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African Journal of Biotechnology

Full Length Research Paper

Advancing adoption of genetically modified crops as food and feed in Africa: The case of Kenya

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Genetically modified organisms (GMOs) and Genetic Engineering (GE) technology has been around since mid 1990s. Numerous successful applications of genetically modified (GM) crops have been recorded in different parts of the world. The technology has been adopted steadily in several countries with acreage under GM crops steadily increasing in many cases. Socio-economic studies show GMO adoption result in improved productivity, reduced cost of labour, and reduced pesticide use. More than 20 years later and in spite of the foregoing, opposition to GMO remains almost the same especially in Kenya. Although the past few months have seen a move toward favourable enabling and political good will, a current report published in the economist magazine indicated that agricultural productivity in Africa and Kenya in particular has remained stagnant for the last 40 years. This points to the vulnerability of Kenya in ensuring food security for its growing population which has actually increased at least 6 folds since 1960s. For food security to be achieved, considerations should be given to traditional as well as modern technologies that can greatly increase productivity, in the shortest time possible, while also taking care devastating effects of pests, diseases, drought, poor soils, and climate change. The genetically engineered crops have been eaten by millions of people from around the world, and have also been fed to millions of animals and poultry all over the world. For Kenya to move forward toward sustainable food security, bold, deliberate actions based on sound science and embedded in the uniqueness of the Kenyan agricultural systems and culture ought to be taken into consideration. This paper reviews the matter of GM foods, their implications for Kenya and all the underlying factors meriting consideration.

Key words: Genetic engineering, Genetically modified organisms, Biotechnology, Biosafety, Public Acceptance, Kenya.

INTRODUCTION

What is the GMO technology: Process or product?

Humans have been improving the quality of domesticated crops for thousands of years and this has mostly been through conventional breeding where important traits are encouraged, picked, and passed down from one generation to the next (Keetch et al., 2014; European Safety, 2019). Genetic engineering however picks over from here and aims to achieve the modification of the crops by selecting novel genes from other crops or

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organisms and incorporating these into the gene of interest of distantly related species (Weebadde and Maredia, 2011). This has proven to be a faster way than the 10 to 15 years of conventional breeding often required to improve a crop for general release. Whereas with conventional breeding, over 1000 to 10,000 genetic material is transferred between species, the genetic engineering aims at a single gene or few well selected novel genes to be moved across species (Baudo et al., 2006). The resulting food crops are referred to as genetically engineered (GE) or genetically modified (GM) foods. To this extent, genetic engineering has been lauded by proponents as faster, more targeted, more precise and efficient way of acquiring intended traits than through conventional breeding (The Royal Society, 2020).

So far, many crops have been modified by genetic engineering technology to provide beneficial traits to farmers (GMO Answers, 2019). Most of the crops have been modified for, herbicide tolerance, insect pest resistance, disease resistance, among other farmer benefiting traits (GMO Answers, 2019). The most widely grown GM crops by acreage have been maize or corn, cotton, soy bean, and canola, while other crops are also being grown such as *brinjal*, papaya, and others (GMO Answers, 2019). In Kenya, several applications have been made for commercialization of GMO crops (National Biosafety Authority, 2019). Top among them being *Bacillus thuringiensis* (Bt) maize, *B. thuringiensis* Cotton, Water Efficient Maize for Africa (WEMA) maize, virus resistant cassava, virus resistant bananas, late blight fungal disease resistant potatoes, among others (National Biosafety Authority, 2019). The WEMA project mainly focused on drought tolerance technology through conventional breeding. Its successor, the TELA is working toward introducing the Bt. gene to WEMA varieties. Yet, none of these applications have ever gone past field trials and unto commercialization, except for *B. thuringiensis* cotton which only recently got the go ahead for commercialization through cabinet approval in December 2019 (Vijida, 2019).

What are the motivators of this technology?

Farmers had been losing money for years from their crops due to attacks from pests, diseases, and weeds while yields were stagnating or diminishing following success with *green revolution* (GMO Answers, 2019). To continue improving farm productivity means finding remedies to the pests and diseases devastating the crops and better ways to reduce farm expenses especially labour; given the increased cost of labour in many parts of the world (Alhassan and Adekunle, 2014). On the other hand, the industry supplying the agro-chemicals have been under pressure regarding the toxic nature of the insecticides and herbicides (especially given that a majority of the insecticides and herbicides residues end in water ways, and polluting the soils since a very small percentage is actually being absorbed by plants). Furthermore, farmers have realized the fact that some of the target insects and weeds have developed resistance to the insecticides and herbicides. Alternatives to both challenges occupied the minds of the industry for a while and by the time the agro-chemical industry revealed that they could actually transform plants with the *B. thuringiensis* gene, so the crop will produce the toxin by itself. This means farmers could stop heavy reliance on chemical applications, this was received as extremely good news. However, control of weeds was still a major challenge especially in large scale farms. Herbicides especially glyphosates were being used in large scale but they could not be used on the crops because they would kill them too since the active ingredient is a broad spectrum and systemic to plants. A herbicide tolerant and insect resistant *B. thuringiensis* maize was the novel answer the industry introduced for the farmers who were yearning for a way to reduce not just cost of pesticides but more so, labour and mechanical costs of controlling weeds.

The glyphosate tolerant and insect resistant *B. thuringiensis* crops were well received by farmers and contributed to ease the need for weed control and insect control by mechanical or by manual and other means. In retrospect, the farmers may have been over motivated by this prospect resulting into planting much *B. thuringiensis* and herbicide tolerant crops and ignoring the other Integrated Pest Management Practices (IPM) that would have helped to prevent or delay the development of resistance for much longer. Despite this challenge, farmers in many countries around the world including Canada, USA, Japan, Argentina, India, and the Philippines have enjoyed the advantages brought about by GMOs for more than two decades (ISAAA, 2019).

Enumerated benefits of adoption of GMO crops in different countries

A study sponsored or conducted by the European Commission to trace the benefits of GM crops for past 19 years from 1996 to 2014 suggested a drastic reduction in use of pesticides by 581 million kg thus reducing environmental footprint associated with GMO by 20% (Brookes and Barfoot, 2017). In USA alone, planting GMOs reduced pesticide use and resulted in reduction of 46.4 million pounds in 2003. The *B. thuringiensis* cotton in China resulted in reduced use of formulated pesticides by 78,000 tonnes in 2001 an equivalent of a quarter of all the pesticides sprayed in China in the mid-1990s (European Commission, 2010). With reduction of pesticide use, comes the reduced exposure and potential poisoning of farmers and farm workers. Insecticide used in control cotton bollworms reduced from as much as 5,748 metric tons of active ingredients in 2001 to as low as 222 metric tons of active ingredients in 2011; a 96%

reduction (Perry et al., 2016). The adoption of GMO technology equally contributed to continued expansion of no-tillage agriculture in the U.S. saving 1 billion tons of soils through herbicide tolerant crops (Perry et al., 2016). *B. thuringiensis* Cotton in US and Australia has been documented to result in improved number and diversity of beneficial insect in the cotton growing fields (Qaim and Klumper, 2014).

Even with all the enumerated benefits of GMOs, Kenyan farmers were not sure how much longer they will have to wait until they grow these crops in their fields. Thankfully, the cabinet approval of *B. thuringiensis* cotton's commercialization was well received; and seen by many as a positive step toward ensuring the much-needed progress. In deed, the country has made good progress since then by flagging off of the planting of GMO cotton in selected farms by the Cabinet Secretary for Agriculture. The pressing question remains: How did a country with such great enthusiasm about the promises of biotechnology turn to one of such skepticism after all the research available for consideration? Kenya was one of the first countries in the world not just in Africa to ratify the Cartagena Protocol on Biosafety to the Convention on Biological Diversity in the Route to Food (Mungai, 2019). The National Biosafety Authority website and documented regulations show that, there is enough preparation to proceed with the aspects of ensuring GMO adoption. In spite of the efforts of investors, scientists and GMO enthusiasts, lack of a favourable environment has led to some of these pro-GMO crusaders developing a cold feet with their efforts in the country. As a matter of fact, Kenya is experiencing loss of opportunity to attract additional investment for the continuous development of the technology as most organizations shift their support to countries with favourable political climate (National Biosafety Authority, 2019).

MATERIALS AND METHODS

The results presented is a culmination of over 3 months of research involving in depth discussions with various stakeholders including farmers, seed industry representatives, academia, biotechnology industry visits, lectures by prominent industry players in GMO technology and participating in international short courses and training offered by the World Technology Access Program (WorldTAP) during the senior authors stay at Michigan State University through the Norman E. Borlaug Fellowship Programme.

RESULTS

From the results of this study, it is clear that there are several reasons that stand in the way for adoption of GMOs in Africa in general and Kenya in particular. The issues range from felt or unfounded fears regarding effect of GMO, the mixed signals from EU about health and safety of GM foods, the potential risk of GMOs to the environment and biodiversity. Other reasons include the fear of possible effect on non-target organisms and potential development of resistance to insect-pests by the GM crops. Lastly, food safety fears of GMOs remain pertinent in some parts of the continent. These cases are presented in detail in the following.

Unearthing the fears of GMO adoption

Kenya already drafted Regulations on Biosafety in 1998 and was poised to be one of the few counties to take advantage of the new technology when it was first released to this part of the world. However, the moratorium placed on GMOs in 2012 by the Ministry of Health, dealt a big blow to continued development, promotion, and adoption of GMO crops in Kenya (Ministry of Sanitation and Public Health (MoSPH), 2012). The ban has stayed on seven years later and with the latest direction from government being that GMO activity in the country will be handled on case by case basis. This is what delivered the cabinet approval for the commercialization of the *B. thuringiensis* cotton in December, 2019 (Vijida, 2019). There is evidence of many promising projects and opportunities to improve on African crops and especially so from the public research institutions especially, Kenya Agricultural and Livestock Research Organization (KARLO), International Livestock Research Institute (ILRI) and International Centre for Insect Physiology and Ecology (ICIPE). However, lack of funds and expertise, have been noted as a bottle neck to unveiling technology due to the highly regulated nature of biosafety (Wambugu and Kamanga, 2014). It is also vital to note that the appropriateness of specific technologies depend on current agricultural systems, practices, and surrounding natural environment especially with regard to environmental safety (Wambugu and Kamanga, 2014). This fact tends to be ignored and instead the opponents of biotechnology prefer to wholesomely dismiss the technology without considering socio-economic benefits, and utility of the technology as an option for safeguarding environmental resources.

The mixed signal: EU, WHO and UN? To follow the science or the politics?

The European Food Safety Authority (EFSA) studies have repeatedly demonstrated that the GMO foods are as safe both to environment and to humans as their conventional counterparts (European Commission, 2010). Yet, the European Union (EU) still has restrictions on growing of GMO crops in Europe. This stance has bewildered many observers (Tagliabue, 2017). The European Commission grants authorizations to place GM food and feed on the European market for a period of ten years, yet they have constantly requested EFSA to publish as much as about 5 new guidelines just in the past 5 years alone and then for some reason repeatedly

ignoring the EFSA opinions that demonstrate that GMOs are just as safe as their conventional counterparts. Whereas the EU may be justified to call for these improvements, to the casual observer, the EU may simply be doing these to avoid the backlash from the technology developers or simply laying more layers of roadblocks through over regulations. This leaves developing countries especially those with limited capacity at a point of confusion as it seems the European Commissions' decisions on GMO crops are rather the result of political than science-based decisions. But this is where developing countries ought to break from the mold and begin to chart their own course because they must realize the priority for Europe and Africa are different. This realization would help developing countries handle the mixed signals not just coming from EU, but also from the WHO and FAO-UN. The FAO-UN is on one hand calling for addressing of malnutrition by the developing nations based on modern biotechnology (FAO, 2013). While on the other hand, is warning of the dangers of GMO to the environment (FAO, 2013).

GMO for Africa: What are the drivers and opposition?

Unintended and adventitious harmful effects of GMOs on the environment are one reason of the fiercest oppositions raised by opponents of GM crops (Wambugu and Kamanga, 2014). Yet, more than 100 independent, U.S. European, and international scientific societies have addressed the relative safety of GMO and their conventional counterparts and arrived at the conclusion that properly regulated GMOs, pose no new risk to the environment and human health as compared to conventional counterparts (The National Academies of Sciences Engineering Medicine, 2016). Studies have also revealed that farming insect resisitant *B. thuringiensis* corn in the Philippines has not demonstrated reduced number and diversity of insects (Pringle, 2013). A 10 year study commissioned by USDA in 2006 demonstrated that there is no increased risk of invasiveness or persistence in wild habitats for GM crops (oilseed rape, potatoes, corn, and sugar beet) and traits (herbicide tolerance, insect protection) (Fernandez-Cornejo and Caswell, 2014). The same conclusions were arrived at on the basis of a study by the European Commission (European Commission, 2010). These studies do not conclude all possibility for crops to form persistent weedy relatives only that the productive GM crops are unlikely to survive out of cultivation conditions. The more reason the studies have always focused on case by case evaluation and recommend need for post release Monitoring and Evaluation for 10 years or more after release (European Commission, 2010).

From a development point of view however, it is critical to place the opposition to GMOs in the context of opposition to other technologies that experienced similar if not even worse opposition. Normally, there are many reasons why societies oppose and block new technology besides the inherent nature of the technology itself. Most of the initial opposition has to do with the creative disruption that new technologies embody across a number of different fields. That is the sole reason why society must not discourage the unchanted voices of our time. They may be the best people we need to leapfrog the current set of challenges and have a quantum leap (Juma, 2016). As Einstein once said, 'Problems cannot be solved at the same level of thinking that created them in the first place'. Innovations and inventions are how we circumvent this closed thinking by employing a different way or approach to solving our current problems. The apparent opposition to new technologies including GMOs may need to be understood in this context. This may be the reason Wambugu and Kamanga (2014), conclude that without serious investment, the support of critical mass at regulation, astuteness on government political affairs in gaining good will, excellent issue management of GMO lobby groups, and well-resourced outreach, GMOs are likely to fail. This list is a true reflection of the matters that are not part of the GMO science yet must be tackled to address the challenges and drive adoption of the technology.

How about direct effects on the non-intended/targeted organisms?

The early warning of *B. thuringiensis* crops possible impact on *Monarch butterfly larvae* caused panic and many people begun to wonder whether there be any possibilities that the GMO crops were actually causing death of the USA's most loved butterfly (Holt-Giménez, 2019). In 2001 a collaborative research by Scientists from Canada and U.S. observed that the possibility was negligible (Sears et al., 2001b). Report by U.S. Environmental Protection Agency (EPA) stated that according to data presented, *B. thuringiensis* did not present any unreasonable adverse effect on the unintended wildlife in the environment (Sears et al., 2001a). Despite this, opponents are still raising the same questions (Sears et al., 2001a).

How about development of insect-pest resistance?

The management of insect resistance is a concern for scientists and governments including regulatory authorities (Purdue University, 2019). The recommendation of biosafety practice is ensuring that there must be a provision for associate refuge of non-GMO crops so the insects grow without selection pressure to insect resistant varieties (Difonzo, 2019). Post release monitoring and evaluation of GM crop and surrounding environment also acts as a tool to control

any resistance. Post release monitoring requires a well-trained and coordinated effort and an information sharing forum all across the country. Recent GMO developments now use a multiple number of genes conferring different types of traits (stacked genes) and these can help discourage the selection pressure burden that would lead to development of resistance. For this to work, county governments in Kenya must be empowered to report any early cases of potential 'exhaustion' of resistance and appropriate action to be taken before it gets out of hand (Difonzo, 2019). The best agronomic practices and integrated pest management (IPM) strategies are vital for resistance management (Difonzo, 2019).

Safety of GMOs

One critical requirement of food and any new products is that it must not just satisfy hunger. It must be safe, nutritious and acceptable by consumers as a legitimate source of food for which the consumers make independent choices and not out of coercion. The GMO foods have not escaped this aspect one bit. The main opposition that has been witnessed as far as GMO foods is concerned has centered around the three major areas: food safety, environmental safety, and socio-cultural aspects (The National Academies of Sciences Engineering Medicine, 2016). Food safety is the most critical of these factors while talking purely from a science-based perspective. Whereas in many cases there are ways those food scientists and safety experts use to test safety of products including chemical, biological and physical testing. Such approaches are best usable where a single ingredient is at stake. This case is not very effective for whole foods and hence the reason why scientists have resorted to other means to arrive at a determination of safety of GMO to plants and feeds. This concept that was embraced and ratified by Cartagena Protocol for Biosafety, is the concept of substantial equivalence. The aim is not to determine the absolute safety of a GMO but to compare its main food nutrition and safety related attributes to the conventional counterpart. Of all the over 10 most commercially grown GMO foods, scientific consensus so far reported indicate that there are no significant harmful effects on health of both food and feed attributable to the consumption of GMOs (The National Academies of Sciences Engineering Medicine, 2016).

DISCUSSION

Decision making parameters for accelerating GMO adoption in Kenya

From the results it is evident that fear presents one of the most prominent reasons for negative view of GM crops in the world and especially in Kenya. The fear may be real or imagined. To address these issues, the proper understanding of potential risks and benefits of GMOs, the nature of the forgone opportunity cost is vital. Furthermore, the potential risks must be stated clearly and the role of politics in enhancing or hindering steps in the GM adoption process. It is only by doing this that the countries can make informed choices as unfounded fear is cleared. Any real fears will then be evaluated by informed decision based on risk assessment and characterization. The discussion dissects the issues and offers information that can be considered in respect to decision making on GMOs in Kenya and Africa.

The benefits of biotechnology

One of the factors that do not get much attention in the GMO debate is the attendant benefits that many countries have enjoyed due to the introduction of biotechnology. A study assessing the global economic and environmental impacts of biotech crops for the first twenty-one years of adoption (1996-2016), showed that biotechnology has reduced pesticide spraying by 671.2 million kg and has reduced environmental footprint associated with pesticide use by 18.4% (Perry et al., 2016). The technology has also significantly reduced the release of greenhouse gas emissions from agriculture equivalent to removing 16.75 million cars from the road (Brookes and Barfoot, 2017). At the same time, a meta-analysis by of the impact of biotechnology (Qaim and Klumper, 2014), reported that GM technology has reduced pesticide use by about 37%. In the USA alone between 1998 and 2011, non-adopters of herbicide resistance corn reduced their herbicide use by 1.2% while adopters of insect tolerant crops reduced insecticide use by 11.2% (Perry et al., 2016). Other studies detailing the impact of GMOs in China, reported that the use of *B. thuringiensis* cotton resulted in reduction in pesticide use of 78,000 tons of formulated pesticides in 2001. This value accounted for about 25% of all the pesticides sprayed in China in the mid-1990s (Tao and Shudong, 2006). In yet another important study by the USDA covering data collected from 1999 to 2012, it was shown that *B. thuringiensis* cotton adoption has caused a significant reduction in pesticides use in India (Fernandez-Cornejo and Caswell, 2014). There are many other benefits that go unmentioned as opponents lure the public to most controversial and sometimes immeasurable issues which appeal to feelings and emotions other than facts.

Opportunity cost of delayed use or adoption of biotechnology

Studies have tempted to address the matter of forgone

benefits of delayed adoption of important food crops improvement by GE technology in Africa. One of such papers reported work done by Wesseler et al. (2017) in which they examined the opportunity cost for delay in adoption of biotechnology in several countries in Africa. Under their estimation, their model projects such a delayed cause of action in implementing GMO technology to be very substantial. For example, they estimated that the cost of one year delay in approval of the pod-borer resistant cowpea in Nigeria would cost the country about USD 33 million to 46 million and result in loss of 100 and 3,000 lives hypothetically. Given that Kenya too had an opportunity to adopt GMO crops after South Africa, Wesseler et al. (2017) estimated the forgone benefit of that delay too to the Kenyan economy. According to report by Insect Resistant Maize Insect Resistant Maize for Africa (IRMA), it was very possible that Kenya would have adopted GMO technology soon after South Africa but this did not happen and hence, up to 4000 lives could theatrically have been saved. However, this must be looked at in the context of complacency in government and where all other factors like improved production systems, irrigation use of improved seeds among other factors are kept constant (Wesseler et al., 2017).

Risk assessment and capacity of adoption of GMO in the Kenya

The Kenya National Biosafety Authority was established by the Biosafety Act No. 2 of 2009 to exercise general supervision and control over the transfer, handling, and use of genetically modified organisms (GMOs) (National Biosafety Authority, 2019). Because of the nature and the complex matrix of food, the purpose of safety tests is to evaluate that the GM crop is just as safe as its widely consumed relative both to humans and to the environment. The safety assessment criterion is based on internationally developed and agreed guidelines, and best practices by UN-FAO, WHO, Codex Alimentarius and other respected global organizations. According to the procedures published by the National Biosafety Authority, once the committee has received an application for evaluation of a request to commercialize a GMO crop (dossier), they must publish it within 14 days to the public. This is where the competence and systems based functioning regulatory authorities come into play. The Biosafety Authority does not only need to understand all the requirements of the dossier, but also must be able to determine based on sound science whether the application has captured sufficient data and material as to allow unbiased assessment of the application. The National Biosafety Authority of Kenya can request for additional data, should they need it to help with a determination about a product. Furthermore, they have access to expert resources not just from the pool of scientists in Kenya but even from the African Union and other Biosafety Networks in Africa who can help with specific expertise necessary for the evaluation (Wangari, 2019).

The NBA will then request for written comments from public, scientists, and other interested parties to be submitted within 21 days of their publishing the dossier. After this, the evaluation is done and the NBA stipulates to communicate back to applicant within at least 90 days of the application and not more than 150 days after the receipt of the complete application (National Biosafety Authority, 2019). There are two important fundamentals of science that allows such a system to work and provide checks and balances along the process of commercialization of GMO used as food and feed. The first, is the concept of good laboratory practice (GLP) that is done to ensure reproducibility of test results. Second, is the principle of direct data entry which stipulates that any resulting data must be entered as to and when an observation is made. Lastly, scientific results go through a rigorous double blinded peer review which often removes bias and allow for work to be examined on the basis of its own scientific merit and not based on subjective means such as consideration of who the authors are.

Whereas the regulatory demands of GMOs have created a scenario where it is very expensive currently to deregulate (commercialize) the crops, a case for joint Risk Assessment body to service the continent and allow countries share the burden of the regulatory process of GMOs such as establishment of East Africa Food Safety Authority (EFSA) seems a plausible idea. Africa may benefit greatly from the creation of an Africa Biosafety Authority body that resembles the EFSA that can at least reduce the burden on the less developed countries to afford commercializing of GMOs. By so doing experiences can be quickly shared and expertise quickly deployed among countries as desired.

Harnessing the political will

Many people from several quarters all over the world and especially the scientific community have relegated the debate and final blockade to utilization of GMO foods in the developing countries to the presence or absence of the ‘political will’. In their book chapter ‘*Does Africa Need political Will to Overcome Impediments to GM crop* (Alhassan and Adekunle, 2014) reported that countries like Brazil, Argentina, India, and the Philippines developed the political will to engage. However, they make this assertion without defining what is the political will, how did these countries develop it, and how the other countries can develop it as well? The matter of relegating the challenge of biotechnology adoption in Africa and Kenya especially to political will needs a careful attention. The reason being that whereas at this stage the political will is needed to help adopt the GM crops, leaving such a decision to political will is

dangerous where the politics takes over and runs amok and announces support for the adoption of a harmful or irrelevant technology. Anchoring anything such as GMO development on politics is one sure way to embed the technology on *quick sand*. The point ought to be the pursuit of political will to promote sound scientific evidence, strong and effective biosafety institutions. Since these are embedded in law, ensuring laws of the nation are respected and upheld irrespective of the political office bearer is a much better guarantee to the kind of investments undertaken to commercialize the technology, than can be offered by any political will.

It is also very important that the political and policy makers look at the alternative scenarios. Failing to adopt GMOs or even to review the ban would mean continued status quo. In an honest evaluation we must realize that we are not just avoiding risk, since nothing is risk free, even continuing the ban exposes us to risk of some kind especially of very limited markets for imports in case of drought. The aim must be risk balancing. The overarching question may need to be where we would be if we continue what we are doing now? And whatever the scenario happens to be the country must then ask, can it afford that position? The answers countries get from these questions should provide the best impetus for driving adoption or continuation of the moratorium on GMO and GE crops.

One of the areas that have been suggested is harnessing of the technology for addressing very pertinent and closer issues to the country in question. For example, the development of the Water Efficient Maize for Africa, Insect Resistant Maize for Africa, and other local crops being modified to provide for more beneficial effects, that can directly address the consumers' needs are more welcome (CYMMIT, 2018). This initiative has the advantage that it is being supported through philanthropic means and National parastatals which are public institutions. The sponsors of this project include Bill and Melinda Gates Foundation, Howard G. Buffett Foundation and the U.S. Agency for International Development (CYMMIT, 2018).

On one hand, one cannot fail to realize that government officials of developing countries are overwhelmed by the weight of all the information either for or against the technology. This can be very disturbing especially for those in leadership. It seems that the scientific community has not done a good job of convincing the decision makers at the political level about the safety and attending benefits of GMO crops for the people and the economy of these nations. At the same time, government officials ought to carry out independent research, practice critical thinking which goes beyond what is said to why it is said, beyond who is offering the report to why the report. At least the relevant GMO technology should be selected for use in Kenya given that some of the foremost Biotech crops being fronted were grown in some countries decades ago and newer and superior GMO offerings are currently available in the developed countries. The question is whether these first-generation GMOs are the appropriate ones for Kenya or by adopting them, will we be repeating the mistakes that led to their being abandoned or up graded for farmers in the developing countries.

In closing, going through the 'Regulations and Guidelines for Biosafety in Biotechnology for Kenya', it is difficult to see where any issues of lack of safety should be raised if the process detailed in the documents were followed. It goes to prove that there is a tendency to bash the GMO crops without necessarily having had a chance to read through the regulations in place. But there is also a possibility that the level of trust that countries have on their governments, and attending institutions, have a direct bearing on their trust about GMO technology irrespective of the facts as outlined in science.

Conclusions

Decades of growing of GM crops have allowed millions of farmers around the world to not only increase productivity, but also have control of some serious insect pests, diseases and weeds in the fields, resulting in reduced use of pesticides, increased productivity, and hence profitability. Kenya has made slow but steady progress with GMO crops' commercialization with *B. thuringiensis* cotton being approved for field trials and later on released to farmers in 2020. There are other beneficial GM crops (Bt. Maize and Bacterial wilt Resistant Cassava) in the pipeline for NBA regulatory process and hopefully release into the environment in the future. In encouraging adoption of GMO crops, capacity building and taking into account the uniqueness of the agro-ecological conditions and farming systems of the country is vital. Kenya's NBA, and relevant authorities, must ensure farmers' ability to afford seeds, and responsive regulation of GMOs which both are critical factors to bear in mind. Perhaps the lifting of the existing ban based on evaluation of available scientific evidence will provide a conducive environment for full exploitation of the biotechnology and encourage rigorous research to deal with any unfolding safety situation. With the bold step the government has taken of commercializing *B. thuringiensis* cotton technology in 2020, there can be no reason why the progressive adoption of the technology in Kenya cannot be realized. This will also allow the country to be better prepared to take advantage of available food grains for importation in the unfortunate incidence of drought resulting in hunger and starvation.

CONFLICT OF INTERESTS

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

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REFERENCES

Alhassan WS, Adekunle AA (2014). Does Africa Need Political Will to Overcome Impediments to GM Crop Biotechnology Applications for Agricultural Economic Growth and Development, as in the Case of Brazil, Argentina, and India? In: Biotechnology in Africa, pp. 257-269. Springer.

Answers G (2019). GMO Basics [Internet document] URL https://gmoanswers.com/gmo-basics. Accessed.

Baudo MM, Lyons R, Powers S, Pastori GM, Edwards KJ, Holdsworth MJ, Shewry PR (2006). Transgenesis has less impact on the transcriptome of wheat grain than conventional breeding. Plant Biotechnology Journal 4(4):369-380. Available at: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1467-7652.2006.00193.x

Brookes G, Barfoot P (2017). GM crops: Global socio-economic and environmental impacts 1996 - 2015. https://www.bezpecnostpotravin.cz/UserFiles/publikace/2017globalimpactstudy.pdf

CYMMIT (2018). Water Efficient Maize for Africa (WEMA) [Internet document].

Difonzo C (2019). Interactions between industry, academics, and government agencies with regard to regulating Bt corn traits. Seminar Proceedings presented on: The seminar is Monday Oct 21, 4pm, in 243 Natural Science Building, Michigan State University.

European Commission (2010). A decade of EU-funded GMO research (2001–2010). Luxembourg, Publications Office of the European.

European Safety Authority (2019). GMO [Internet document] URL https://www.efsa.europa.eu/en/topics/topic/gmo. Accessed.

FAO (2013). The State of Food and Agriculture: Food Systems for better Nutrition. [Internet document].

Fernandez-Cornejo J, Caswell M (2014). The first decade of genetically engineered crops in the United States. Genetically Engineered Crops in America: Analyses, Adoption, Trends, pp. 75-113.

GMO Answers (2019). What GMO crops are currently available on the market? [Internet document] URL https://gmoanswers.com/current-gmo-crops. Accessed.

Holt-Giménez E (2019). Can we feed the world without destroying it by Eric Holt-Gimenez. Cambrige, UK: Polity Press.

ISAAA (2019). Biotech Crops Continue to Help Meet the Challenges of Increased Population and Climate Change [Internet document]. ISAAA Brief 54-2018: Executive Summary URL http://www.isaaa.org/resources/publications/briefs/54/executivesummary/default.asp. Accessed.

Juma C (2016). Innovation and Its Enemies: Why People Resist New Technologies. Oxford: Oxford Scholarship.

Keetch DP, Makinde D, Weebadde CK, Maredia KM (2014). Biosafety in Africa: Experiences and best practices. Available at: https://agris.fao.org/agris-search/search.do?recordID=US2018H01475.

Weebadde C, Maredia KM (2011). Environmental Biosafety Issues Associated with Genetically Engineered Crops. Chapter in the Book titled “Environmental Safety of Genetically Engineered Crops” Edited by Rebecca Grumet, James Hancock, Karim M. Maredia and Cholani Weebadde, Pages 21-30, Published by Michigan State University Press, East Lansing, Michigan, USA.

Ministry of Public Health and Sanitation (MoSPH) (2012). Banning of GMO in Kenya. Accessed on 12th November, 2020 from https://www.youtube.com/watch?v=2qV75NOjsuY

Mungai C (2019). GMOs: The right to food and the right to know in route to food. Available at: https://routetofood.org/gmos-the-right-to-food-and-the-right-to-know/

National Biosafety Authority (2019). National Biosafety Authority-Championing for a Biosafe Nation [Internet document]. National Biosafety Authority.

Perry ED, Ciliberto F, Hennessy DA, Moschini G (2016). Genetically engineered crops and pesticide use in U.S. maize and soybeans. Science Advances 2:e1600850.

Pringle P (2013). Food Inc: Mendel To Monsanto - The Promises and Perils of the Biotech Harvest. 1st edn. Simon & Schuster Trade.

Purdue University (2019). How do GMOs affect insects? Beetles, Butterflies, Bees and More: The Relationship Between Bugs and GMOs [Internet document]. Dr. Christian Krupke URL: https://ag.purdue.edu/GMOs/Pages/GMOsandInsects.aspx. Accessed.

Qaim M, Klumper W (2014). A Meta-Analysis of the Impacts of Genetically Modified Crops. 9. Available at: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0111629

Sears MK, Hellmich RL, Stanley-Horn DE, Oberhauser KS, Pleasants JM, Mattila HR, Siegfried BD, Dively GP (2001b). Impact of Bt corn pollen on monarch butterfly populations: A risk assessment. Proceedings of the National Academy of Sciences 98:119370-11942.

Sears MK, Hellmich RL, Stanley-horn, DE, Oberhauser KS, Pleasants JM, Mattila HR, Siegfried BD, Dively GP (2001a). Impact of Bt corn pollen on monarch butterfly populations: A risk assessment Proceedings of the National Academy of Sciences 98(21):11937-11942

Tagliabue G (2017). The EU legislation on “GMOs” between nonsense and protectionism: An ongoing Schumpeterian chain of public choices. GM Crops and Food 8:57-73.

Tao Z, Shudong Z (2006). The Economic and Social Impact of GMOs in China », China Perspectives [Online], 47 | May-june 2003, Online since 10 November 2006, connection on 12 June 2020. URL: http://journals.openedition.org/chinaperspectives/359. DOI: https://doi.org/10.4000/chinaperspectives.359.

The National Academies of Sciences Engineering Medicine (2016). Report in Brief Genetically Engineered Crops: Experiences and Prospects. Board on Agriculture and Natural Resources. Accessed on 25th July, 2020: Available at: https://www.nap.edu/catalog/23395/genetically-engineered-crops-experiences-and-prospects

The Royal Society (2020). How does GM differ from conventional plant breeding? [Internet document] URL https://royalsociety.org/topics-policy/projects/gm-plants/how-does-gm-differ-from-conventional-plant-breeding/. Accessed.

Vijida P (2019). Cabinet approves commercial farming of GMO cotton. THE STAR. Available at: https://www.the-star.co.ke/news/2019-12-19-cabinet-approves-commercial-farming-of-gmo-cotton/

Wambugu F, Kamanga D (2014). Biotechnology in Africa. Emergence, Initiatives and Future. Nairobi, Kenya: Springer International Publisher Switzerland.

Wangari D (2019). GMO position in Kenya. Personal Communications.

Wesseler J, Smart RD, Thomson J, Zilberman D (2017). Foregone benefits of important food crop improvements in Sub-Saharan Africa. PLoS ONE 12:e0181353.

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Treatment of mangrove and well contaminated water using activated charcoal from coconut shell in Douala, Cameroon

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The main objective of this study was to evaluate the contribution of activated carbon, based on coconut shell, in the treatment of mangrove and well contaminated water from the BONAPRISO district. More precisely, it consisted of preparing the activated carbon, then characterizing the mangrove water and the well water through a manual of sampling procedure; and finally verifying that the activated carbon produced had a good capacity for absorption of bacteria contained in the different sampled waters. Therefore, it emerged from this study that the activated carbon produced from coconut pods carbonized at 561°C, ground and activated at 443°C, had a specific surface of 658 m^2/g and pore sizes of 20 µm. Thereafter the various waters characterized showed a yellowish coloration for well water and whitish for mangrove water, a pH ranging from 6.5 and 6.8, respectively; and the total flora had a total load of 2.18×10^5 CFU/ml for well water and a total load of 3.24×10^5 CFU/ml for mangrove water. Finally, the effectiveness of activated carbon in fixing bacteria, such as total flora, streptococci, and faecal coliforms showed that it was adsorbed in well water: 86.45% total flora; 91.67% streptococci; 100% faecal coliforms, and therefore, acted at 92.7%. Similarly, in mangrove water, there is 51.22% of total flora, 100% streptococci, and 92.4% faecal coliforms were fixed and, therefore, acted at 81.20%. The remaining bacteria, in well water (13.55% total flora; 8.33% streptococci) and in mangrove water (48.78% total flora; 7.6% faecal coliforms), respectively, could not be fixed.

Key words: Coconut shell, faecal coliforms, streptococci, mangrove, activated charcoal.

INTRODUCTION

Water has been an essential resource in human life and its environment for decades. It is incumbent that this is a natural need either for consumption or for current use. So if it is contaminated with pathogens or other toxic elements, it will immediately lead to diseases or the death of certain plants. These contaminating substances can

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come either from the physical environment in which the water has evolved, or from the releases of certain human activities of which the water has become the receptacle (Kanohin et al., 2017). Long-term risks are linked to poor physico-chemical quality (pollution resulting from technological and industrial development), while short-term risks are linked to microbiological characteristics (Kuitcha et al., 2010; Njoku et al., 2014). Water, as a reservoir of germs, constitutes an essential link in the transmission of digestive tract infections (Ndjama et al., 2008). The quality of bathing water was sometimes assessed by researchers through the isolation of coliphages and bacteriophages (Palmateer et al., 1991; Katte et al., 2003; Chun-Han et al., 2011; Caitlin et al., 2014). That of water for human consumption has been studied by other researchers who isolated the bacteria that control faecal contamination (bio-indicators) such as faecal coliforms and faecal streptococci. However, only few studies had been carried out on the characterization of surface water in the equatorial zone where there is an abundance of water. Moreover, in the cities of this climatic zone, especially in poor urban areas where the habitat is spontaneous, the traditional latrines are very shallow and rub shoulders with water points. It should also be noted that some families empty their septic tanks directly into the drains during the heavy rains, especially in cities where there is a significant amount of water (Djuikom et al., 2009; Akoachere et al., 2013). Wells and mangroves, for example, are the most affected because they are the receptacles of this wastewater, which affects them with important pathogens or toxic agents (Jamieson et al., 2004).

The objective of this work was, therefore, to assess the contribution of activated carbon in the treatment of mangrove water and well water from the BONAPRISO district in Douala. More specifically, it was a question of producing the activated carbon based on coconut pods, then doing a bacteriological analysis of the mangrove water and the well water by filtration of the activated carbon produced.

MATERIALS AND METHODS

Sampling site

The sampling site was in the BONAPRISO district in Douala (Cameroon) where KEMIT ECOLOGY was located (Figure 1).

Coal preparation, sampling and experimentation

Sampling, filtration, and analysis of the results were carried out in the structure. Thus, the well and the mangrove sources were the two types most frequently used by the populations of this district.

Preparation of activated carbon

For the utility, 16.2 kg of pods were imprisoned in coconuts in barrels, embers of fire added and the barrels closed with their tight lids. All of the barrels were placed in a closed enclosure protected from oxygen. The heat exchanges inside each barrel transformed the material (coconut pods) into inactive carbon or carbon; this was the carbonization process. The carbonization parameters were variable and measurable (initial temperature and its evolution), and the initial masses were known. All the charred pods were recovered after cooling, crushed, and sifted until carbon powders with a diameter of less than 5 mm were obtained. The coal powders were then subjected to an artisanal physical activation which consisted of using a semi-adiabatic muffle (Njoku et al., 2014).

Sampling

Taking the mangrove water samples, the sampling was done through the wading sampling protocol which describes how the samplers must enter the watercourse, the place from which the sample should be taken, as well as how to collect a sample (Figure 2) while minimizing the risk of contamination (CCME, 2011).

Taking samples of well water, which were assimilated to the protocol for sampling the water column from a bridge, and also describes how the samples should be attached, where the sample should be taken, and how to carry out sampling to minimize the risk of contamination, from the road surface or the bridge structure, were strictly observed (Figure 3) (CCME, 2011).

Preservation and preparation of samples

The manual and general safety protocols relating to the preservation of samples in the field were used to preserve and the samples were prepared. The sample was kept in a cooler with ice cream, and then stored in a refrigerator. Then the sample was treated according to the solutes sought and analysis technique was used. However, a prior filtration step is common and was carried out on a coffee filter in order to remove debris or other impurities (Tita et al., 2009).

Water filtration and bacteriological analysis of sampled water

The water filtration was done through a manufactured prototype (Figure 4) container, among other things: cotton fabric or cotton; activated carbon; fine sand; small pebbles; medium pebbles; large stones; and water sampled.

As for the bacteriological analysis, it will relate to bacteria such as streptococci and faecal and total coliforms; and the total flora, because they are responsible for several diseases in humans. The method consisted of quantifying these microbes present in the water samples taken in the laboratory and it was done as follows: the respective culture media were the BEA for streptococci (quantities: 56.6 g/1 L); and Endo for coliforms (quantities: 23.5 g/1 L) which was dissolved by heating. Then followed the preparation of the physiological waters for the inoculated in the samples (9 ml of physiological water for 1 ml of sampled water); finally, they were put in an oven at respective temperatures of each microbe for 3 days in order to count them.

RESULTS AND DISCUSSION

Preparation of activated carbon

Carbonization

Carbonization is the chemical transformation of dry

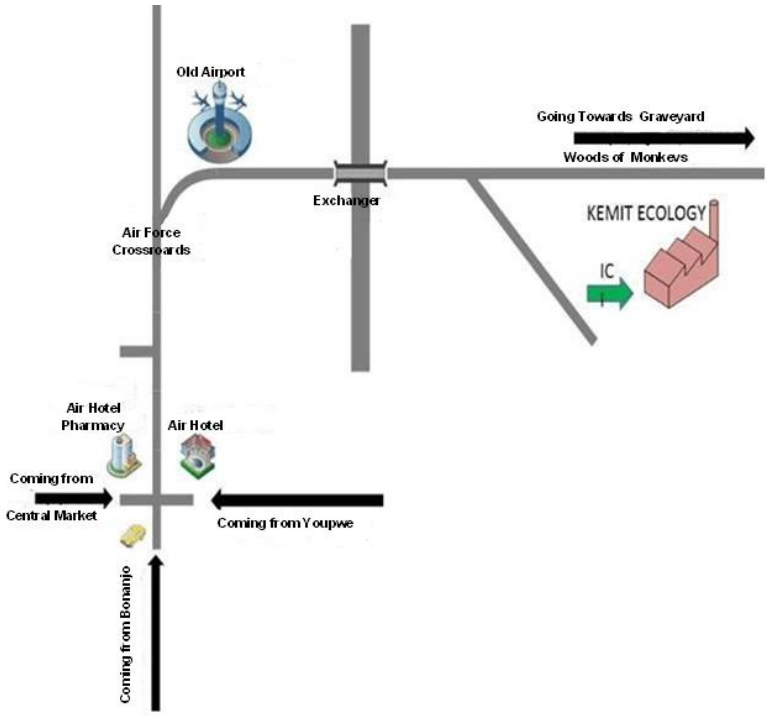

Figure 1. Location map of the study area.

Figure 2. Water sampling procedure.

Figure 3. Sampling from a bridge.

Figure 4. Experimental filter.

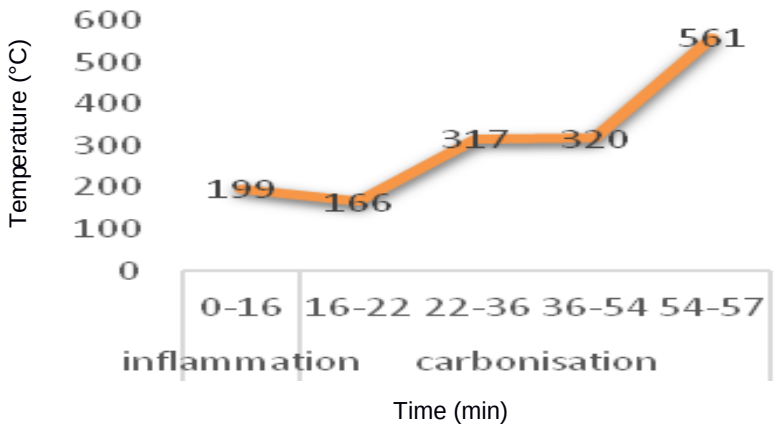

Figure 5. Duration of charring of coconut pods.

vegetable matter under the action of heat and oxygen from the air into carbon black powder. During this process of converting dry matter into carbon black powder, there is a release of greenhouse gases, water vapour, uncondensed oils, and volatile organic matter (Yaning et al., 2019).

$$CHNOS + heat + Oxygen \rightarrow Carbon + Water\ vapor + gaz + oil + xt$$

where xt are the residues of thermolysis only identifiable to X-ray diffraction.

The results show that the carbonization lasted 57 min for temperatures ranging from 166 to 561°C, in the context of the study. It took place in three phases. The ignition corresponded to the ignition of the fire in the pods and its duration was 16 min and its ignition temperature is 199°C. The carbonization proper which was the coal production phase had a duration ranging from 16 to 57 min for carbonization temperatures ranging from 166 to 561°C; jet cooling of 1.3 kg of water cooled the coal where a temperature of 41°C was obtained (Hatem et al., 2014).

Thus, the coal obtained weighed 3.65 kg after carbonization of 16.2 kg of coconut pod, the yield obtained was, therefore, Ro = 22.53%, and subsequently, 90 g of ground material for activation were used in the study (Figure 5).

The coconut pods had undergone carbonization for a duration of 57 min at a final temperature of 561°C; on the other hand, activation had a duration of 3 min for a final activation temperature of 443°C. However, according to the work of Daud and Ali (2004), the activation temperature of coal based on these same pods and having undergone the same principle of carbonization is 800°C for a period 2 h. This variation was due to the fact that:

(1) There was a loss of energy during carbonization, because the metal oven was exposed to air, which increased the rate of ash in the coal, and reduced the temperature and the duration of activation;

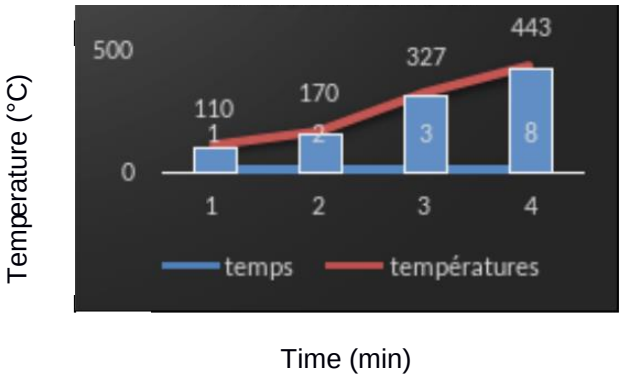

Figure 6. Activation of activated carbon.

(2) The conditions of physical activation were not the same; they activated in a centrifuge devoid of air, and were hermetically closed;
(3) Daud and Ali (2004) used carbon dioxide (CO^2) in their carbonization reaction medium to prevent the calcination of coconut pods after 443°C. These results are, therefore, in contradiction because of the conditions of the reaction medium.
(4) State-of-the-art equipment for optimal activation conditions were deployed in their study: a sterilized pot and a gas plate for heating. But for the researcher's activation, this took place in the laboratory in a thermo-programmable oven.
(5) The duration and the temperature submitted in our study could not be in excess because the activated carbon would be reduced to ash.

In the present research, their temperature was maintained by the presence of CO^2 for a contact time of 2 h which would reduce the O^2 present in the oven (reduction instead of oxidation) so that the carbon did not reduce in ash, which was not our case.

Activation

Activation consisted of increasing the specific surface and increasing the size of the coal pores during a given period and temperature. Figure 6 shows the different temperatures and time recorded during the activation of said carbon.

The results show that the activation lasted 8 min at temperatures between 110 and 443°C, temperatures taken at the centre of the hot plate. And the mass obtained after activation was 41.3 g for an activation rate of 73.75%:

(1) The temperature 110°C corresponded to the temperature of the plate taken directly after the lighting of the fire;
(2) From 110 to 170°C activation was started, corresponding to roasting of coal;
(3) From 170 to 443°C, the actual activation took place, which corresponded to the release of tar pores and other compounds (organic matter, ash);
(4) The temperature 443°C marked the end of the activation by extinguishing the fire.

Specific surface and pore size

Adsorbent used for carrying out tests for the elimination of bacteria is an activated carbon (Riddel-de Haen) with a pore size of 20 µm and a specific surface of 658 m^2/g (Emanuele et al., 2012).

Water treatment and bacteriological analysis of sampled water

The treatment of the water sampled on activated charcoal made it possible to highlight a bacteriological analysis of this water before and after filtration, in order to count the quantity of total flora, total streptococci and coliforms, and faeces present in these waters and to see their evolution. Table 1 shows their presence and their evolution before and after filtration.

Taken under normal conditions of temperature and pressure (CNTP) (P = 1 bar and T = 25°C), the analysis shows that the well water contained 2.14×10^5 CFU/ml of total flora as long as Mangrove water contained a total flora of 3.28×10^5 CFU/ml. The culture media were prepared by agar on haggard (iced sugar) to identify and count streptococci and faecal coliforms. The total load of streptococcus in well water was, therefore, 1.2×10^4 CFU/ml and that of mangrove was 1×10^3 CFU/ml and the faecal coliforms thereof were 1.3×10^4 CFU/ml. For well water, faecal coliforms were not counted because of their abundance in this water. Total coliforms also could not be counted for the two types of water sampled. Otherwise, the analysis of the water after treatment with activated charcoal based on coconut pods, with a particle size of 20 µm and a specific surface of 658 m^2/g (Table 1) revealed that the treated well water contained a total flora of 2.9×10^4 CFU/ml and that mangrove water contained 1.6×10^5 CFU/ml. In the gels, the streptococci contained in the treated well water had a load of 1×10^3 CFU/ml, the total coliforms of 1.16×10^5 CFU/ml and the faecal coliforms were completely absent. However, in the treated mangrove water the count showed that streptococci were rather absent, that there were 2.4×10^4 CFU/ml of total coliforms and 1×10^3 CFU/ml of faecal coliforms.

In the two samples analysed, it was noted in Table 1, a significant or even an uncountable quantity of certain bacteria. This was the case of total coliforms which, exceeding millions of CFU/ml, are considered dangerous in consumption. It is the cause of many diseases such as cholera, ringworm, scabies, and many others (Akoachere

Table 1. Parameters for bacteriological analysis before and after filtration with activated carbon.

Parameter (CFU/ml)	Well water		Mangrove water	
	Not treated	Treated	Not treated	Treated
Total flora	2.14×10^5	2.9×10^4	3.28×10^5	1.6×10^5
Streptococci	1.2×10^4	1×10^3	1×10^3	Absent
Total coliforms	Countless>1×10^2	1.16×10^5	> 1×10^2	2.4×10^4
Faecal coliforms	Countless>1×10^2	Absent	1.3×10^4	1×10^3

et al., 2013). Likewise, there was a strong presence of faecal coliforms in well water, but a total flora which was not abundant. We can deduce that this was due to the fact that the well is a closed vase which does not flow its water naturally like the mangrove, or might even have come from an unhygienic behaviour of the users or from the use of non-clean equipment (buckets, ropes, covers, coping, lack of personal hygiene), or other exogenous contamination which would encourage their multiplication (Benajiba et al., 2013). However, the mangrove with few faecal coliforms has circulating water that is not stagnant. Thanks to its trees, it manufactures organic matter for the food of certain species such as fish. The streptococci in this water are also scarce because they are digested by these species. In well water, on the other hand, there are a large number (1.2×10^4 CFU/ml for well water and 1×10^3 CFU/ml for mangrove water). Their contamination is very harmful for the organism.

There is a link between faecal coliforms and streptococci. Indeed, the two groups of germs are hosts of the digestive tract, with the difference that the faecal coliforms testify to a recent faecal contamination while the faecal streptococci, more resistant in the environment, testify to an old faecal contamination (Trevisan et al., 2002). Their presence in water marks faecal contamination from household waste water, toilets built near used water sources, individual deposits, and/or soil contamination (Gondim et al., 2016; Sepehrnia et al., 2017).

However, activated carbon filtration based on coconut pods, with pore sizes of 20 µm and specific surface of 658 m^2/g of the different sampled waters showed after analysis a significant reduction or even absence of certain groups of bacteria such as streptococci and faecal coliforms. This issue proves the effectiveness of activated carbon in water treatment and the importance of introducing it into a water treatment circuit for any production; but also in improving the living conditions of the populations of BONAPRISO where access to the potable water is difficult. Figure 7 demonstrates this. The adsorption percentages recorded showed the sensitivity of coliforms, streptococci, and total flora to activated carbon. Activated carbon had adsorbed 86.45% of total flora; 91, 67% streptococci and 100% faecal coliforms for well water. Likewise, in water in mangroves, activated carbon fixed 100% streptococci, 92.4% faecal coliforms and 48.78% total flora. Such a case is considerable because, fixed at more than 50%; the efficiency of the activated carbon must be improved either by increasing the specific surface and the size of the pores, or by meeting the conditions for taking samples as much as possible to reach 100%. This activated carbon, therefore, acted on average in well water at 92.70%, a satisfactory percentage for the study; and in mangrove water at 81.20%. The work of Bryant and Tetteh (2015) also shows that after filtration of its waters the activated charcoal adsorbed the bacteria to more than 85%. Activated carbon fixed 100% streptococci, 92.4% faecal coliforms and 48.78% total flora.

To properly conduct the studies by filtering water at 100% adsorption with activated carbon, these precautions were taken beforehand:

(1) Taking samples was under maximum conditions after reading the instructions carefully before taking samples;
(2) The installation of a city equipment in liquid sanitation system;
(3) The ban on uncontrolled discharges of domestic and industrial wastewater was respected;
(4) Regular monitoring and chlorination of groundwater was carried out as it could significantly reduce the degree of bacteriological contamination of the water studied;
(5) The provincial protocol in the event of a flood was followed;
(6) The well that did not comply with current standards was upgraded;
(7) Invested in a treatment system.

It, therefore, appeared that the activated carbon adsorbed 86.45% of total flora for 13.55% of remaining flora, 91.67% of streptococci for 8.33% remaining, and 100% of faecal coliforms for the water of well; and that it fixed 100% of streptococci, 92.4% of faecal coliforms, therefore leaving 7.6% of total flora, and 48.78% for mangrove water. These results show the effectiveness of coconut powder on the treatment or filtration of water for domestic needs.

Conclusion

In general, this study was all about evaluating the

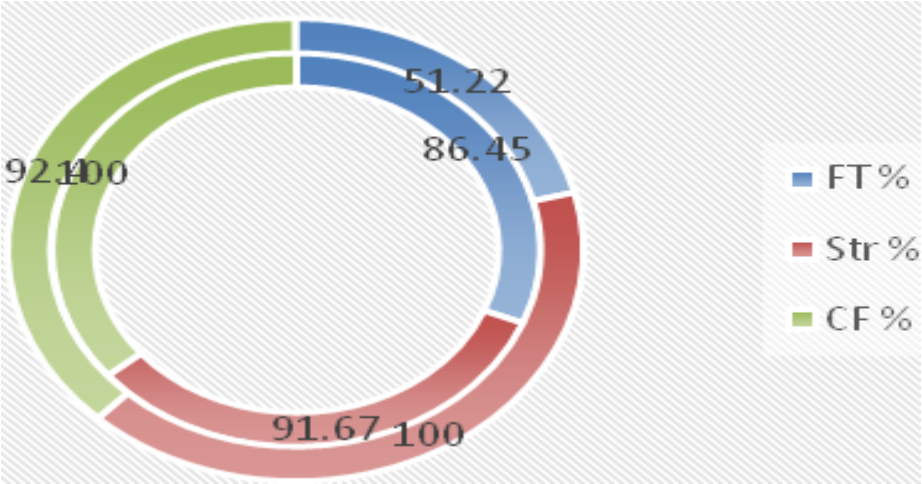

Figure 7. Percentage of adsorption of activated carbon on water.

contribution of coconut powder as activated carbon in the filtration of mangrove water and well water from the BONAPRISO district in Douala. Specifically, it was a question of producing the activated carbon from coconut shells, then carrying out a bacteriological analysis of the mangrove water and the well water by filtration of the activated carbon produced. However, it emerged that after production of an activated carbon of 658 m^2/g of specific surface and pore size of 20 µm introduced into a prototype filter, it was possible to separate the macromolecules from molecules invisible to the naked eye like bacteria. These different bacteria: total flora, coliforms and streptococci made it possible to characterize the waters by their quantity in the two types of samples. After analysis, each type of water contained within it the three types of bacteria. Some were uncountable like total coliforms and faecal coliforms. But after treatment with activated carbon, there was a significant reduction in these bacteria, even absent such as streptococci in the mangrove and faecal coliforms in well water. In this case, the activated carbon produced fixed on average of 92.70% bacteria in sampled well water and 81.20% in sampled mangrove water. Providing an activated carbon treatment system would be a solution to limit water pollution and harmful diseases.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Akoachere J, Omam L, Massalla T (2013). Assesment of the relationship between bacteriological quality of dug-wells, hygiene behaviour and well characteristics in two cholera endemic localities in Douala, Cameroon. BMC Public Health 13:692.

Benajiba M, Saoud Y, Lamribah A, Ahrikat M, Amajoud N, Ouled-Zian O (2013). Evaluation of the microbial quality of water from the Martil water table in Morocco. Journal of Water Science 26(3):223-233.

Caitlin J, Mike R, Dorothy B, Glenda V (2014). Does increased salinity reduce functional depth tolerance of four non-halophytic wetland macrophyte species. Aquatic Botany 116:13-18.

Chun-Han K, Tsai-Ming L, Fang-Chih C, Shu-Ping L (2011). The correlations between system treatment efficiencies and aboveground emergent macrophyte nutrient removal for the Hsin-Hai bridge phase II constructed wetland. Bioresource Technology 102(9):5431-5437.

Djuikom E, Temgoua E, Jugnia L, Nola M, Baane M (2009). Bacteriological pollution of water wells used by populations in the Urban Community of Douala-Cameroon. International Journal of Biological and Chemical Sciences 3(5).

Daud W, Ali W (2004). Comparison on pore development of activated carbon produced from palm shell and coconut shell. Bioresource Technology 93(1):63-90.

Emanuele GP, Jadir NS, Jofran LO, Cassio SM (2012). Sustainable energy : A review of gasification technologies. Renewable and Sustainable Energy Reviews 16:4753-4762.

Gondim-Porto C, Platero L, Nadal I, Navarro-Garcia F (2016). Fate of classical faecal bacteria markers and ampicillin-resistant bacteria in agricultural soils under Mediterranean climate after urban sludge amendement. Sciences Total Environment 565:200-210.

Jamieson R, Gordon R, Joy D, Lee H (2004). Assessing microbial pollution of rural surface waters: a review of current watershed late modeling approaches. Journal of Agriculture and Water Management 70:1-17.

Kanohin F, Otchoumou E, Yapo OB, Dibi B, Bonny AC (2017). Physico-chemical and bacteriological characterization of Bingerville groundwater. International Journal of Biological and Chemical Sciences 11(5):2495-2509.

Kuitcha D, Ndjama J, Tita AM, Lienou G, Kamgang KBV, Ateba BH, Ekodeck GE (2010). Bacterial contamination of water points of the upper Mfoundi watershed, Yaounde, Cameroon. African Journal of Microbiological Research 4(7):568-574.

Katte VY, Fonteh MF, Guemuh GN (2003). Domestic water quality in urban center in Cameroon: a case study of Dschang in the West Province. African Water Journal 10:43-54.

Hatem A, Rosiyah Y, Jamil M, Radzi B (2014). Adsorption of methylene blue on activated carbon fiber prepared from coconut husk: isotherm,

kinetics and thermodynamics studies. Desalination and Water Treatment 52:6720-6732.
Bryant I, Tetteh N (2015). Using slow sand filtration system with activated charcoal layer to treat Salon wastewater in a selected community in Cape Coast, Ghana. Journal of Advanced Chemical Engineering 5(4):1-8.
The Canadian Council of Ministers of the Environment (CCME) (2011). Protocol Manual for Water Quality sampling in Canada. ISBN 978-1-896997-79-7.
Ndjama J, Kamgang KBV, Sigha NL, Ekodeck GE, Awah TM (2008). Water supply, sanitation and health risks in Douala, Cameroon. African Journal of Environmental Sciences and Technology 2(12):422-429.
Njoku VO, Foo KY, Asif M, Hameed BH (2014). Preparation of activated carbons from rambutan (Nephelium lappaceum) peel by microwave-induced KOH activation for acid yellow 17 dye adsorption. Chemical Engineering Journal 250:198-204.
Palmateer GA, Dutka BJ, Janzen EM, Meissner SM, Sakellaris MG (1991). Coliphage and bacteriophage as indicators of recreational water quality. Water Resources 25(3):355-357.
Sepehrnia N, Memarianfard L, Moosavi A, Bachmann J, Guggenberber G, Rezanezhad F (2017). Bacterial mobilization and transport through manure enriched soils: experiment and modeling. Journal of Environmental Management 201:388-396.
Trevisan D, Vansteelant JY, Dorioz JM (2002). Survival and leaching of faecal bacteria after slurry spreading on mountain hay meadows: consequences for the management of water contamination risk. Water Research 36(1):275-283.
Tita MA, Kamgang KBV, Tsala GN (2009). Microbial pollution of surface water and its health impact in the Nkoup River Basin in Foumbot, West Cameroon. Journal of Microbiological Research 8:52-57.
Yaning Z, Yulei C, Paul C, Shiyu L, Nan Z, Kuan D, Liang LF, Peng P, Min M, Yunpu W (2019). Gasification technologies and their energy potentials. In book: Sustainable Resource Recovery and Zero Waste Approaches 193-206.

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Genetic progresses from over four decades of sorghum [*Sorghum bicolor (L.)* Moench, Poaceaea family] breeding in Ethiopia

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Information on genetic progress achieved over time from a breeding program is absolutely essential to develop effective and efficient breeding strategies. Thirty-seven improved lowland sorghum varieties released between 1976 and 2016 and promising advanced lines were evaluated to estimate the genetic progresses made in 40 years of sorghum breeding in Ethiopia. The study was conducted at 2 environments during 2018 cropping seasons in a randomized complete block design with 3 replications. Records taken on grain yield and yield attributes were subjected to statistical analysis. Combined analysis of variance revealed highly significant differences among the genotypes and the test environments for most of the traits, the G×E interaction effects being significant for grain yield. Regression analysis revealed an increase in estimated average annual rate in grain yield potential of 12.2 kg ha^{-1} year^{-1} with annual relative genetic change of 0.60% year^{-1} over the last 40 years of sorghum improvement. Increasing trends along variety release year were also evident for biomass yield, grain yield production per day, biomass production rate and seed growth rate. Stepwise regression analysis revealed that seed growth rate was the most important character, which greatly contributed to the improvement in grain yield. Grain yield was positively correlated with biomass yield, biomass production rate, grain yield production per day, seed growth rate, and thousand seed weight. It is, therefore, strategically advisable that breeding efforts in the future should give due emphasize traits such as seed growth rate.

Key words: Sorghum, genetic improvement, grain yield.

INTRODUCTION

Globally, sorghum [*Sorghum bicolor (L.)* Moench, Poaceaea family] 2n = 20) is the 5th most important cereal crop and is the dietary staple of more than 500 million people in 30 countries (FAO, 2011). It is grown on 40 million hectare in 105 countries of Africa, Asia, Oceania and the Americas. Africa and India account for

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the largest share (>70%) of global sorghum area while USA, India, Mexico, Nigeria, Sudan, and Ethiopia are the major producers (FAO, 2011). Sorghum plays an important role as dietary staple for millions of people, especially in arid and semi-arid countries of Africa and Asia. In Ethiopia, the crop stands third next to tef and maize both in area and total production (CSA, 2015) and it is predominantly cultivated in dry areas where drought causes frequent failures of other crops (Geremew et al., 2004). The current sorghum production in Ethiopia is estimated to be 4.3 million tones on an area of 1.8 million hectare of land giving the national average grain yield of 2.4 tones ha^{-1} (CSA, 2015). Besides being an important food crop, the grain is used for the preparation of local beverages and the stalk is used for construction, fuel and animal feed. Despite the economic importance of sorghum and Ethiopia's position in terms of domestication and diversity, its productivity has been constrained by wide array of biotic and abiotic stresses such as drought, shoofly, stem borer, midge, grain mold and *Striga*.

Since the inception of research on sorghum in Ethiopia, considerable efforts have been made to improve the productivity of sorghum targeting the dry lowland areas of Ethiopia. The researches have been focusing on addressing the major constraints mainly developing early maturing or drought tolerant varieties and varieties resistant to *Striga*, the major parasitic weed affecting sorghum production in the targeted environment (Mekbib, 2006). A total of 26 varieties were released for the dry land areas (Asfaw, 2007). In addition, 6 varieties and 5 hybrids were identified as potential and, in the process, to be verified and released for production. Even though, information on genetic progress achieved over time from a breeding program is absolutely essential to develop effective and efficient breeding strategies by assessing the efficiency of past improvement works in genetic yield potential and give suggestion on future selection direction to facilitate further genetic improvement (Slafer, 2003). Likewise, the importance of this study may be used as the base line for yield potential experiments for several years.

In Ethiopia, apart from some comparative observations in variety trials by breeders, results of field demonstrations and popularization programs by different stakeholders, where a few varieties might be tested together under common environments. Among a few studies in Ethiopia include studies on wheat (*Triticum aestivum* L.) (Amsal et al., 1995a, b), haricot bean (*Phaseolu vulgaris* L.) (Kebere et al., 2006), on barley (*Hordeum vulgare* L.) (Wondimu, 2010), on sorghum [*Sorghum bicolor (L.)* Moench] (Mihret et al., 2015) and on Teff [Eragrostis tef (Zucc.) Trotter] (Yifru and Hailu, 2005). However, there has been limited information generated on the genetic gain and morphological attributes for increased productivity, if there is any, attained through breeding on sorghum. Therefore, the current study was designed:

1. To determine the amount of genetic gain made over time in yield potential of lowland sorghum in Ethiopia
2. To identify the magnitude and direction of change in morphological and agronomic characters associated with genetic improvement in grain yield.
3. To investigate the correlations among yield and yield related traits of sorghum in Ethiopia.

MATERIALS AND METHODS

Description of the experimental sites

The trial was conducted at 2 sites, namely Sheraro (Tigray regional state) and Miesso (Harari regional state). Sheraro is suited at an altitude of 1028 m, above sea level. The area receives an average annual rainfall of 677 mm, with the average monthly maximum and minimum temperature of 32.9 and 18.8°C, respectively. The climate is typical of major sorghum producing regions of Ethiopia representing the dry lowlands. The second test site was Miesso, which is a substation of Melkassa Research Center located at an altitude of 1400 m located at a latitude of 9°23´N and longitudes 40°77´E. The area has a bimodal rainfall pattern and receiving an average annual rainfall of 763 mm, with the average monthly maximum and minimum temperature of 30.5 and 15.2°C, respectively. Generally, the two experimental sites do not differ considerably in their edaphic and climatic conditions.

Experimental materials

A total of 37 released varieties since 1976 by the national and regional research system and pipeline sorghum varieties and hybrids were used for this study. Sorghum genotypes (varieties) used for this study are presented in Table 1.

Experimental procedures and crop management

The test genotypes were laid using a randomized complete block design (RCBD) with 3 replications. Genotypes were randomly assigned to each of the experimental units for each of the testing site. Each plot has 11.25 m^2 areas which contained 3 rows of 5 m length. Spacing of 75 cm between rows and 15 cm between plants was used. The spacing between plots and blocks was 0.75 and 1.0 m, respectively. The experimental materials were planted in July 2016 at both locations. Phosphorus and nitrogen fertilizers were applied at the recommended rates of 46 kg P2O5 ha^{-1} and 50 kg nitrogen ha^{-1} in the form of diammonium phosphate and urea, respectively. DAP (diammonium phosphate) fertilizer was applied at time of planting, whereas urea was applied in split (half at planting and the other half at knee height). The plots were weeded as frequently as needed. Data were recorded on plot and plant basis on the train under consideration.

Statistical analysis

All measured parameters were subjected to analysis of variance (ANOVA) using PROCANOVA of SAS software version 9.0 (SAS institute, 2004) to assess the difference among the tested varieties in each location. The homogeneity of error variance between the two locations was tested by Bartlett 's test and combined analyses of variance was performed for the traits whose error mean squares

Table 1. List of genotypes used for genetic gain study in the lowland environment.

S/N	Variety	Pedigree	Year of variety release	Breeder/seed source
1	Gambella 1107	Gambella 1107	1976	MARC
2	76T1# 23	76T1# 23	1976	MARC
3	Seredo	Seredo	1986	MARC
4	Dink mash	Dink mash	1986	MARC
5	Meko	M-36121	1998	MARC
6	Abshir	P-9403	2000	MARC
7	Gobiye	P-9401	2000	SARC
8	Teshale	3443-2-op	2002	MARC
9	Yeju	Icsv-111 Inc	2002	SARC
10	Birhan	Key#8566	2002	SARC
11	Abuare	ICSV-1x (TSx135/4/2/3/1)	2003	SARC
12	Hormat	ICSV-1112 BF	2005	SARC
13	Macia	Macia	2007	MARC
14	Red Swazi	Red Swazi	2007	MARC
15	Raya	PGRC/EX 222878xKAT-369-1	2007	SARC
16	Miskir	PGRC/E 69441x KAT-369-1	2007	SARC
17	Girana-1	CR; 35XDJ1195X KAT-369-1	2007	SARC
18	Gedo-1	Gambella 1107x KAT-369-1	2007	SARC
19	Melkam	WSV-387	2009	MARC
20	ESH-1	P-9501AxICSR14	2009	MARC
21	ESH-2	ICSA21A xICSR50	2009	MARC
22	Mesay	MekoxGobye-2	2011	SARC
23	Chare	PGRC/E#222880	2011	DBARC
24	Dekeba	ICSR 24004	2012	MARC
25	Melkamash-79	Melkamash-79	2013	MARC
26	ESH-3	ICSA-15Xm-5568	2014	MARC
27	2005MI5064 (Argeti)	WSV387/P9404	2016	MARC
28	2005MI5065	WSV387/P9405	2016	MARC
29	PU209A/PRL021071	PU209A/PRL021071	2016	MARC
30	PU209A/PU304(ESH4)	PU209A/PU304	2016	MARC
31	ICSA15/AWN87	ICSA15/AWN87	2016	MARC
32	P9534A/Gambella1107	P9534A/Gambella1107	2016	MARC
33	Kari Metama-1	Kari Metama-1	2016	MARC
34	IESV23007DL	IESV23007DL	2016	MARC
35	P9511A/PRL020817	P9511A/PRL020817	2016	MARC
36	ETSC 300001	Teshale/B35//Teshale	2016	MARC
37	ETSC 300002	Teshale/E361//Teshale	2016	MARC

MARC= Melkassa Agricultural Research Center; SARC= Sirinka Agricultural Research Center, DBARC= Debrebirhan Agricultural Research Center.

were homogenous using PROC ANOVA procedure of SAS. Mean separation was carried out using Duncan's Multiple Range Test (DMRT) at 5% of significance. Locations and replications within locations were considered random while the varieties were considered fixed effects. The following model was used for combined ANOVA:

$$Yijk = \mu + Gi + Lj + GLij + Bk(j) + Eijk$$

Where: Yijk = observed value of variety i in block k of location j, μ = grand mean, Gi = effect of variety I, Lj = effect of location j, Bk (j) = effect of block k in location j, GLij = the interaction effect of variety i with location j and Eijk = error (residual) effect of variety i in block k of location j. Linear regression analysis was used to calculate the genetic gain for each trait measured. The breeding effect was estimated as a genetic gain for grain yield and associated traits in sorghum improvement by regressing mean of each character for each variety against the year of release of that variety using PROC REG procedure.

The coefficient of linear regression gives the estimate of genetic gain in kg ha^{-1} $year^{-1}$ or in % per year (Evans and Fisher, 1999). For this study, the year of release was expressed as the number of years since 1976 for the varieties; the year when the first Sorghum variety was released. The relative annual gain achieved in the last 40 years (1976-2016) was determined as a ratio of genetic gain to the corresponding mean value of oldest variety and

Table 2. The Average yields (kg/ha) of these 4 groups when tested at Sheraro and at Miesso and when averaged over the 2 locations.

Group of adaptation	Test		
	Sheraro	Miesso	Over Loc
Adapted to Sheraro	2848.0	1848.2	2348.1
Adapted to Miesso	2374.8	2587.3	2481.1
Widely adapted	2882.4	2695.1	2748.6
Un-adapted	2272.5	1870.2	2071.4

expressed as percentage.

RESULTS AND DISCUSSION

Performance of the genotypes

The difference between the 37 sorghum genotypes in the combined analysis was statistically significant for DTE, HWT, GFP, PW, GYHA, HI, GYPDAY and SGR (Table 4). The difference between the genotypes was significant at both locations for these traits except for DTE (Table 3). The average grain yield of all sorghum varieties was 2448.03 kg ha^{-1}, which ranged from 1861.2 kg ha^{-1} for 76T1#23 to 3190.3 kg $ha^{-1}$for P9534A/Gambella 1107 (elite genotype in NVT 2016). The recently advanced elite genotype P9534A/Gambella 1107 was the first best yielder (Table 8) among the 37 varieties. The superiority of the higher yielder variety, P9534A/Gambella 1107 represents 1152.7 kg ha^{-1} or 36.1% increment (Table 5) over the average of the first two older varieties (Gambella 1107 and 76T1#23) released in 1976.

The G x E interaction was highly significant for all traits except for DTF, DTM, FLW, NLPP and GL, indicating the inconsistency of performance of the genotypes over the 2 locations for most of the traits (Table 4). Due to the low G x E Mean square against which they were tested, the difference between the genotypes for DTF, DTM, FLW, NLPP and GL was significant. These five traits also had the lowest variance due to rank change (from 13 to 62 with mean variance of 45.4) (results not presented). For PHT, NTPP, FLL, PE, PL, and NSPP, the G x E interaction was significant, but did not mask the difference between the genotypes; mean square of genotypes was also significant, indicating the difference between the 37 genotypes. The variance of rank changes for these traits was also not very high and ranged between 39 and 86 with a mean of 64.7. Most of the interaction seems to be not that due to lack of correlation but due heterogeneity of variances and the performance over locations for PHT, NTPP, FLL, PE, PL, and NSPP is also relatively consistent. For the majority of the remaining traits, DTE, HWT, GFP, PW, TSW, GYHA, BYHA, HI, GYPDAY, BPR and SGR, where the G x E interaction was highly significant while the difference between genotypes was non-significant. For example, for HWA, GFP, GYHA, HI, and SGR, where the G x E was highly significant. The variance of ranks was high for these traits and ranged between 81 and 127 with mean of 101.5. There was large rank change of the varieties over the two locations for these traits. This means that some varieties performed best at Sheraro only while others performed best only at Miesso for this trait. For example, the 37 genotypes can be classified into four categories according to their adaptation (i) widely adapted genotypes with above-average grain yield at both locations (9, 16, 19, 21, 22, 27, 28,31, 32, 34 and 37), (ii) genotypes adapted to Sheraro (3, 4, 5, 7, 13, 14, 25, 26, 29 and 35), (iii) genotypes adapted to Miesso (8, 11, 12, 20, 23, 24, 33 and 36) and (iv) genotypes not adapted to any of the locations [(1, 2, 6, 10, 15, 17, 18 and 30) (Figure 1).

All the 11 highest yielding and superior genotypes were of 2000 and 2010s release (4^{th} and 5^{th} decades) (6 are advanced genotypes in the pipeline in 2016 and 5 were among those released in the 2000s). If we divide the 37 sorghum genotypes into 5 decades of release, that is, 1970s, 1980s, 1990s, 2000s and 2010s, mean grain yield of the 5 decades was 1872, 1846, 2095, 2190 and 2451 kg ha^{-1} (Table 5). Genotypes of the recent decade had a yield advantage of 607.1 kg ha^{-1} or 23.0% (Table 5). It can, therefore, be concluded that grain yield has been improved over the 40 years of the national sorghum improvement for the dry lowlands of Ethiopia. It is also interesting to note that the three varieties released in the second and third decades (1986 and 1998) still give high grain yield at Sheraro (Table 6). The following is the average yield of these four groups when tested at Sheraro, at Miesso and when averaged over the 2 locations

The 11 genotypes with narrow adaptation to Sheraro had a yield advantage of 473.2 Kg/ha (19.9%) over the mean of the 8 genotypes adapted to Miesso, when tested at Sheraro (2848.0 vs. 2374.8 Kg/ha, respectively). The 11 genotypes had a disadvantage of 739.1 Kg/ha (28.6%) as compared to the mean of the 8 genotypes specifically adapted to Miesso, when tested at Miesso (1848.2 vs. 2587.3 Kg/ha), respectively (Table 2). The widely adapted genotypes gave the highest yields at both locations and over both locations; however, their

Table 3. Mean squares, CV and R^2 for separate analysis of variance for seed yield and yield related traits in variety evaluated at Sheraro and Miesso in 2016 cropping season.

Trait[#]	Sheraro				Miesso			
	Variety (36)[$]	Mean	CV (%)	R^2	Variety (36)[2]	Mean	CV (%)	R^2
DTE	2.00***	6.60	9.47	0.72	0.59^{ns}	6.06	10.41	0.43
DTF	66.64***	57.42	3.60	0.89	50.41**	71.56	6.65	0.53
DTM	88.99***	97.05	3.56	0.80	73.53***	107.64	3.50	0.73
PHT	3368.62***	182.86	10.54	0.82	73.53***	156.44	10.55	0.78
HWT	950.4***	5.51	7.56	0.74	526.65***	3.44	7.97	0.78
GFP	29.42***	39.63	8.97	0.54	41.43***	42.11	6.56	0.78
NTPP	0.08***	1.13	12.4	0.66	0 .04^{ns}	0.89	18.04	0.44
NLPP	3.17 ***	10.29	9.45	0.63	1.92***	9.51	9.29	0.55
FLL	38.11***	40.13	3.80	0.89	34.20***	39.01	8.43	0.62
FLW	0.69^{ns}	6.97	9.54	0.44	1.05***	6.15	10.13	0.59
PE	15.49***	5.41	15.98	0.91	5.29***	4.47	21.88	0.73
PL	33.21***	26.38	7.69	0.80	23.82***	25.95	7.99	0.74
PW	1.01**	8.01	9.82	0.45	4.13***	9.70	12.18	0.60
NSPP	1355769.2***	2718.99	7.39	0.94	1507233.28***	3186.00	19.28	0.67
TSW	34.52***	32.08	4.54	0.89	9.63***	24.26	5.24	0.75
GL	0.02***	2.61	2.99	0.66	0.03***	2.13	4.82	0.62
GYPH	324768.99***	2631.51	9.64	0.72	683676.66***	2264.55	11.33	0.84
BYPH	1578104.68**	8700.73	11.11	0.47	117.40***	38.43	14.63	0.65
HI	10.19^{ns}	30.25	10.29	0.54	96.23**	55.23	15.30	0.41
GYPDAY	42.20***	27.21	10.22	0.73	57.29***	21.06	11.63	0.83
BPR	221.58***	89.53	11.87	0.50	518.14***	54.69	13.67	0.83
SGR	40.20***	66.73	16.43	0.45	57.29***	21.06	11.63	0.83

[$]= Numbers in parenthesis represent degrees of freedom, **, *, ns= Significant at P ≤ 0.01, significant at P ≤ 0.05, and non-significant respectively; CV= coefficient variance, R^2= determination coefficient, DTE= days to emergence, DTF= days to flowering, DTM= days to physiological maturity, PHT=plant height in cm, HWT=head weight tons per hectare, GFP= grain filling period (days), NTPP=number of productive tillers per plant, NLPP=number of leaves per plant, FLL=flag leaf length in cm, FLW=flag leaf width in cm, PE=panicle exertion in cm, PL=panicle length in cm, PW=panicle width in cm, NSPP=number of seeds per panicle, TSW=thousand seed weight in gram, GL=grain size in mm, GYPH= grain yield Kg per hectare, BYPH= above ground biomass yield Kg per hectare, HI=harvest index (%), GYPDAY= grain yield production per day (Kg ha-1 y-1day-1), BPR=biomass production rate (Kg ha-1y-1), and SGR=seed growth rate (Kg $ha^{-1}y^{-1}$).

advantage over both the Sheraro-adapted and Miesso-adapted genotypes is the highest when tested over both locations. They were superior to Sheraro-adapted genotypes by 1.2% at Sheraro, to Miesso- adapted genotypes by 4.2% at Miesso and by 17.1 and 10.8% to the two groups when tested over locations. This indicates the importance of testing the varieties across locations and over years to check their stability for use as reliable genetic materials for crop improvement in a specific location.

In this study, the result of significant interaction of variety by location for grain yield is in contrary to the finding of Hailu et al. (2009) who reported no variety x environment interaction for grain yield.

Genetic improvement in sorghum grain yield

Mean grain yields of varieties released in the years such as 1986, 1998, 2000, 2002, 2003, 2005, 2007, 2009, 2011,2012, 2013, 2014 and 2016 exceeded that of the average of the first released older varieties. Moreover, Seredo, Dinkmash by 300.2 kg ha^{-1}(12.84%), Meko by 587.0 kg ha^{-1}(22.36%), Abshir, Gobiye by 217.5 kg ha^{-1}(9.65%), Teshale, Yeju and Birhan by 408.8 kg ha^{-1}(16.71%), Abuare by 338.85 kg ha^{-1} (14.26%), Hormat by 457.2 $kgha^{-1}$ (18.33%), Macia, Red swazi and Raya by 194.23 $kgha^{-1}$ (8.70%) (Table 5). The least and highest increases were 194.3 kg ha^{-1} (8.71%) and 648.7 $kgha^{-1}$(24.15%), respectively, over varieties released in 1976 (Table 5). These indicated that there was a gradual increase in grain yield across years of release although this increment was not consistent over the years. For example; varieties 6. Abshir, released in 2000, 10. Birhan released in 2002, 15. Raya released in 2007 and 18. Gedo-1 released in 2007 was among the lowest yielding varieties. Among the advanced genotypes that were in the pipeline in 2016, genotype 30. PU209A/PU304 (ESH-4) was very low yielding (Table 5). This emphasizes the care that should

Table 4. Mean squares, CV and R^2 from combined analysis of variance for seed yield and other traits in varieties evaluated over two locations (Sheraro and Miesso) in 2016 cropping season.

Trait	Source of variation					
	Location (1) ++	Variety (36)	Location variety (36)	Mean	CV %	R^2
DTE	16.22***	1.53***	1.06***	6.33	9.85	0.66
DTF	11089.01***	101.52***	15.53^{ns}	64.49	5.71	0.89
DTM	6219.03***	150.86***	11.66^{ns}	102.35	3.51	0.87
PHT	38749.95***	3523.75***	1828.29***	169.65	10.65	0.83
HWT	237.83***	672.30***	804.70***	4.47	7.96	0.94
GFP	340.63***	40.63***	30.22***	40.87	8.34	0.65
NTPP	3.16***	0.08***	0.04**	1.01	14.90	0.69
NLPP	33.32***	3.88***	1.20^{ns}	9.90	9.39	0.63
FLL	69.82***	58.97***	13.35***	39.57	6.45	0.74
FLW	37.30***	1.30***	0.44^{ns}	6.56	9.88	0.62
PE	49.66***	14.92***	5.86***	4.94	18.92	0.87
PL	9.95 ^{ns}	41.77***	15.26***	26.17	7.79	0.77
PW	159.21***	2.98***	2.15***	8.86	11.26	0.70
NSPP	12104406.5***	2074565.45***	788437.15***	2952.50	1 5.38	0.79
TSW	3399.28***	22.70***	21.44***	28.17	4.82	0.95
GL	13.04***	0.05***	0.01^{ns}	2.37	3.84	0.93
GYPH	7473507.36***	559930.66***	448514.98***	2448.03	10.41	0.82
BYPH	427538713.9***	1269197.2**	12202251.7^{ns}	7312.98	12.38	0.81
HI	3544.01***	83.04***	63.00***	34.43	13.42	0.74
GYPDAY	2102.53***	55.48***	42.01***	24.14	10.88	0.85
BPR	66425.08***	176.50***	128.52^{ns}	72.23	12.73	0.86
SGR	8720.69***	481.12***	341.68***	60.96	14.18	0.78

++ = Number in parenthesis is degree of freedom, **, *** = Mean square of characters was significant at 0.05 and 0.01 respectively. CV= coefficient variance, R^2= determination coefficient, DTE=days to emergence, DTF = days to flowering, DTM=days to maturity, PHT=plant height(cm), HWT=head weight tons per hectare, GFP=gra in filling period, NTPP= number of productive tillers per plant, NLPP=number of leaves per plant(main stem), FLL=flag leaf length (cm), FLW=flag leaf width(cm), PE=panicle exertion(cm), PL=panicle length(cm), PW=panicle width(cm), NSPP=number of seeds per panicle, TSW=thousand seed weight (gram), GL=grain length(mm), GYPH=grain yield kg per hectare, BYPH= Above ground biomass yield per hectare kg per hectare, HI=harvest index(%), GYPDAY=grain yield production kg per hectare per day, BPR= biomass production rate (kg per hectare per year), and SGR=seed growth rate (kg per hectare per year).

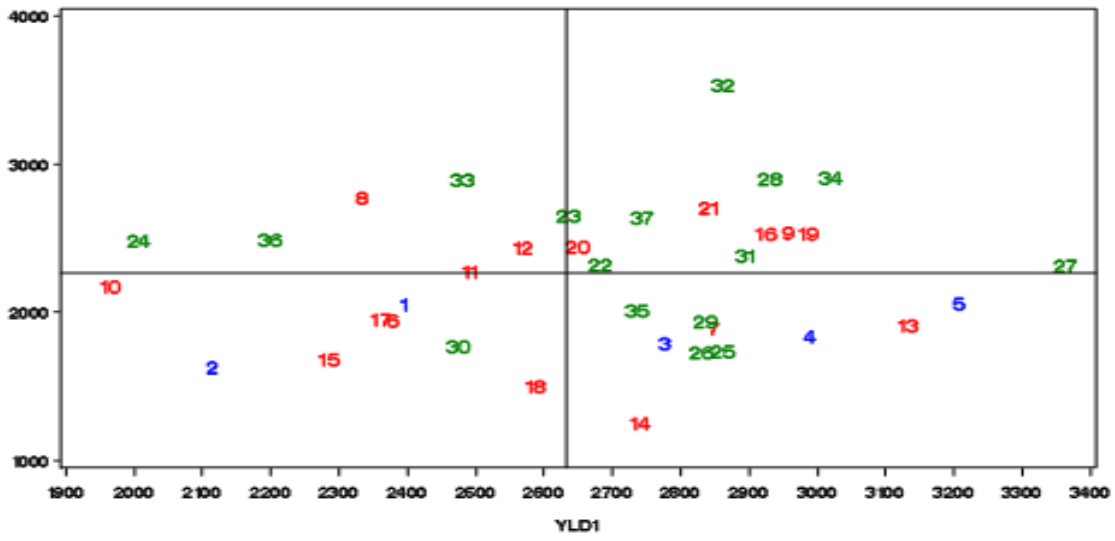

Figure 1. The yield (Yld1) at Sheraro (X) and Miesso (Y).

Table 5. Trends in genetic progress in grain yield for sorghum varieties over the average of the 1st older varieties (Gambella 1107 and 76t1#23) released in 1976.

Variety	Year	Mean yield	Increment over average of the 1st older varieties (1976s)	
		Kg ha^{-1}	Kg ha^{-1}	%
Gambella 1107	1976	2037.6	-	-
76T1#23	1976			
Seredo	1986	2337.8	300.2	12.84
Dinkmash	1986			
Meko	1998	2625.2	587.6	22.38
Abshir	2000			
Gobiye	2000	2255.01	217.41	9.64
Teshale	2002			
Yeju	2002	2446.5	408.9	16.71
Birhan	2002			14.25
Abuare	2003	2376.3	338.7	
Hormat	2005	2494.8	457.2	18.33
Macia	2007			
Red Swazi	2007			
Raya	2007	2233.65	194.23	8.70
Miski	2007			
Girana-1	2007			
Gedo-1	2007			
Melkam	2009			
ESH -1	2009	2688.26	648.67	24.15
ESH-2	2009			
Mesay	2011			
Chare	2011	2567.25	529.65	20.63
Dekeba	2012	2237.8	200.2	8.95
Melkamash-79	2013	2291.6	254.0	11.08
ESH-3	2014	2274.5	236.9	30.9
2005 MI5064	2016			
2005MI5065	2016			
PU209A/PRL021071	2016			
PU209A/PU304	2016			
ICSA15/AWN87	2016	2644.7	607.1	**23.0**
P9534A/Gambella 1107	2016		(1152.7)	(36.1) alone
Kari Metama 1	2016			
IEsV23007DL	2016			
P9511A/PRL020817	2016			
ETSC300001, TSC300002, ETSC300002E	2016			
ETSC300002	2016			
Total yield increment	1986--2016	2471.48	433.9	17.56

be taken in the process of variety release. The candidate advanced genotypes should be subjected to rigorous multi-environment and multi-season testing before release. When the candidates are tested only in one season over very few sites, the best ones (widely adapted high yielding genotypes) may be missed. For example, among the 11 elite advanced genotypes tested in 2016, only two (27) 2005MI5064 (Argeti) and (30) PU209A/PU304 (ESH-4)) were released, while genotypes 34. IESV23007DL and 36. ETSC 300001, that gave higher yield than varieties 27 and 30 (Figure 1) were missed from release and are testing in national variety trial (NVT). The average rate of increase in yield potential per year of release over the last 40 years period from the slope of linear regression was 12.2**kg ha^{-1} year^{-1} (Figure 2). Although there was

Table 6. Mean performance of characters from separate analysis of variance for sorghum varieties grown in the yield potential trial at Sheraro.

S/N	VAR	DTE	DTF	DTM	PHT	HWT	GFP	NTPP	NLPP	FLL	FLW	PE
1	Gambella 1107	5.33^{fg}	59.33^{bg}	101.33^{bc}	130.33^{k}	4.56^{lk}	39.67^{bf}	1.13^{ad}	10.83^{ac}	40.67^{ci}	6.33^{b}	1.33^{o}
2	76T1#23	5.33^{fg}	62.67^{b}	91.00^{jk}	141.67^{hk}	3.64^{m}	38.67^{bg}	0.93^{df}	8.50^{f}	41.57^{bh}	6.67^{ab}	5.33^{hk}
3	Seredo	5.67^{eg}	62.67^{b}	91.33^{ik}	132.33^{k}	5.07^{fl}	40.00^{bf}	1.23^{a}	8.67^{ef}	39.20^{el}	7.33^{ab}	5.00^{hl}
4	Dinkmash	6.67^{be}	49.67^{m}	99.67^{bf}	179.33^{dh}	6.22^{ac}	38.33^{cg}	0.93^{df}	10.50^{ac}	37.63^{im}	7.33^{ab}	2.67^{mo}
5	Meko	5.67^{eg}	62.00^{bc}	92.00^{hk}	222.33^{ac}	5.81^{ag}	39.33^{bf}	0.93^{df}	9.83^{ce}	38.75^{fl}	7.33^{ab}	3.33^{ln}
6	Abshir	5.00^{g}	61.67^{bc}	92.00^{hk}	227.00^{ab}	4.89^{hl}	36.33^{dg}	0.93^{df}	8.67^{ef}	44.60^{ab}	7.67^{ab}	6.67^{dh}
7	Gobiye	5.33^{fg}	61.67^{bc}	93.33^{fk}	201.67^{ae}	6.28^{ab}	38.67^{bg}	1.00cf	8.50^{f}	41.67^{bh}	7.00^{ab}	3.33^{ln}
8	Teshale	7.00^{ad}	61.67^{bc}	93.67^{ek}	135.33^{jk}	4.98^{gl}	36.67^{dg}	1.00cf	10.00^{bd}	33.33^{no}	7.00^{ab}	8.67^{b}
9	Yeju	6.67^{be}	61.33^{bd}	92.67^{gk}	137.33^{ik}	5.22^{el}	40.67^{bf}	0.93^{gf}	9.83^{ce}	36.97^{jm}	7.00^{ab}	6.00^{fi}
10	Birhan	8.00^{a}	61.33^{bd}	93.33^{fk}	188.33^{cf}	4.53^{lk}	40.00^{bf}	1.27^{a}	8.83^{df}	37.27^{im}	6.33^{b}	5.67^{gj}
11	Abuare	8.00^{a}	60.67^{be}	101.67^{b}	179.33^{dh}	5.81^{ag}	42.67^{ae}	1.23^{ab}	10.00^{bd}	40.40^{ci}	6.67^{ab}	3.00^{mn}
12	Hormat	6.00^{dg}	60.00^{bf}	94.33^{dk}	204.67^{ad}	5.54^{bi}	41.00^{bf}	1.03^{be}	10.50^{ac}	38.30^{hm}	7.00^{ab}	3.00^{mn}
13	Macia	7.00^{ad}	60.00^{bf}	96.33^{bk}	225.00^{ac}	5.66^{bi}	40.00^{bf}	1.03^{be}	9.67^{cf}	40.23^{dj}	8.00^{a}	4.33^{im}
14	Red Swazi	7.67^{ab}	59.67^{bg}	90.33^{k}	205.33^{ad}	4.62^{jl}	0.67^{bf}	1.00^{cf}	8.67^{ef}	36.10^{kn}	6.33^{b}	6.00^{fi}
15	Raya	5.00^{g}	74.00^{a}	123.00^{a}	218.33^{ac}	5.72^{ah}	49.00^{a}	1.17^{ac}	11.17^{ab}	42.75^{ae}	7.67^{ab}	7.33^{cf}
16	Miskir	6.67^{be}	59.00^{bg}	99.33^{bg}	133.67^{k}	5.04^{gl}	36.67^{dg}	0.97^{cf}	9.67^{cf}	38.97^{fl}	6.67^{ab}	5.00^{hl}
17	Girana -1	6.33^{cf}	58.33^{ch}	92.00^{hk}	218.33^{ac}	5.25^{el}	32.00^{g}	1.00^{cf}	10.67^{ac}	39.07^{fl}	7.33^{ab}	11.00^{a}
18	Gedo -11	6.67^{be}	58.00^{ch}	95.00^{bk}	172.00^{dj}	5.78^{ag}	34.33^{eg}	1.10^{ae}	10.67^{ac}	43.63^{ad}	7.33^{ab}	4.00^{jm}
19	Melkam	7.00^{ad}	57.33^{di}	100.33^{be}	202.00^{ae}	6.52^{a}	38.33^{cg}	1.03^{be}	10.17^{ac}	39.33^{el}	7.00^{ab}	2.00^{no}
20	ESH -1	7.00^{ad}	57.33^{di}	96.33^{bk}	143.67^{gk}	5.19^{el}	39.00^{bg}	1.00^{cf}	10.50^{ac}	40.87^{ci}	6.67^{ab}	8.00^{bd}
21	ESH-2	7.33^{ac}	57.33^{di}	94.33^{dk}	222.67^{ac}	5.19^{el}	40.33^{bf}	0.97^{cf}	9.67^{cf}	38.13^{hm}	6.67^{ab}	7.00^{cg}
22	Mesay	7.00^{ad}	57.00^{ei}	98.67^{bh}	166.67^{ek}	5.10^{el}	40.67^{bf}	1.03^{be}	10.83^{ac}	38.20^{hm}	7.67^{ab}	5.00^{hl}
23	Chare	7.00^{ad}	56.33^{fj}	100.33^{be}	238.00^{a}	6.07^{ad}	40.33^{bf}	1.03^{be}	9.83^{ce}	38.47^{gl}	6.67^{ab}	4.33^{im}
24	Dekeba	7.00^{ad}	55.67^{gk}	97.67^{bj}	150.67^{gk}	5.45^{ci}	42.00^{be}	1.03^{be}	9.50^{cf}	37.83^{im}	6.33^{b}	4.00^{jm}
25	Melkamash-79	6.00^{dg}	51.33^{lm}	99.00^{bg}	205.33^{ad}	5.39^{dj}	41.67^{be}	1.00^{cf}	9.50^{cf}	42.03^{ag}	6.67^{ab}	5.00^{hl}
26	ESH-3	7.67^{ab}	55.67^{gk}	94.67^{ck}	204.00^{ae}	5.57^{bi}	41.67^{be}	1.00^{cf}	9.50^{cf}	44.87^{ab}	7.33^{ab}	7.67^{be}
27	2005 MI5064	6.67^{be}	54.67^{hl}	98.33^{bh}	148.33^{gk}	5.04^{gl}	37.00^{cg}	0.81^{fg}	9.83^{ce}	41.67^{bh}	6.67^{ab}	2.67^{mo}
28	205MI5065	7.00^{ad}	54.00^{il}	96.33^{bk}	202.33^{ae}	5.90^{af}	36.67^{dg}	1.17^{ac}	9.50^{cf}	39.53^{ek}	6.67^{ab}	4.00^{jm}
29	PU209A/PRL021071	7.00^{ad}	53.67^{il}	99.33^{bg}	147.00^{gk}	6.31^{ab}	45.667^{ab}	1.07^{be}	8.50^{f}	45.27^{a}	7.33^{ab}	8.33^{bc}
30	PU209A/PU304	6.33^{cf}	53.33^{im}	96.33^{bk}	180.00^{dg}	5.93^{ae}	44.00^{ac}	1.00^{cf}	9.50^{cf}	44.03^{ac}	6.33^{b}	6.33^{eh}
31	ICSA15/AWN87	7.33^{ac}	63.33^{b}	102.33^{bg}	218.67^{ac}	5.30^{dk}	36.67^{dg}	1.00cf	10.83^{ac}	39.28^{el}	7.33^{ab}	6.33^{eh}
32	P9534A/Gambell1107	5.67^{eg}	53.00^{jm}	96.67^{bk}	174.67^{di}	5.33^{dk}	38.33^{cg}	0.97^{cf}	10.17^{ac}	39.27^{el}	6.67^{ab}	8.33^{bc}
33	Kari Mtama 1	6.67^{be}	52.67^{jm}	99.33^{bg}	197.67^{be}	4.83^{jl}	42.00^{be}	0.67^{g}	10.33^{ac}	40.27^{dj}	6.33^{b}	5.00^{hl}
34	IESV23007DL	6.33^{cf}	52.33^{jm}	101.00^{bd}	149.33^{gk}	6.31^{ab}	39.33^{bf}	0.90^{ef}	11.33^{a}	34.93^{mn}	7.67^{ab}	4.33im
35	P9511A/PRL020817	7.67^{ab}	52.33^{jm}	95.00^{bk}	159.33^{fk}	6.34^{ab}	43.33^{ad}	0.967^{ef}	10.33^{ac}	42.23^{af}	7.00^{ab}	10.67^{a}
36	ETSC300001	7.33^{ac}	52.00^{km}	97.67^{bj}	172.00^{dj}	4.44^{l}	36.00$_{eg}$	0.97^{cf}	11.17^{ab}	30.73^{o}	6.33^{b}	6.00^{fi}

Table 6. Contd.

37	ETSC300002	6.33^{cf}	51.67^{km}	98.00^{bi}	231.33^{ab}	5.42^{cj}	38.67^{bg}	0.90^{ef}	10.17^{ac}	35.93^{ln}	7.67^{ab}	3.67^{km}
Grand mean		6.60	57.42	97.05	182.86	5.51	39.63	1.13	10.29	40.13	6.97	5.41
C.V (%)		9.47	3.60	3.56	10.54	7.56	8.97	12.42	9.45	3.80	9.54	5.98
R^2		0.72	0.89	0.79	0.82	0.74	0.54	0.66	0.63	0.89	0.44	0.91
S/N	**VAR**	**PL**	**PW**	**NSPP**	**TSW**	**GL**	**GYPH**	**BYPH**	**HI**	**GYPDAY**	**BPR**	**SGR**
1	Gambella 1107	24.33^{fj}	7.33^{bc}	2860.0^{gi}	32.83^{fk}	2.53^{fg}	2343.0^{gn}	8038.7^{cg}	30.97^{ad}	23.13^{gk}	80.23^{cf}	63.80^{ae}
2	76T1#23	24.00^{gj}	8.00^{ac}	1696.7^{r}	33.10^{ej}	2.50^{gh}	2062.7^{ln}	7371.2^{eg}	30.90^{ad}	22.70^{gk}	78.37^{df}	8.27^{ce}
3	Seredo	24.33^{fj}	9.33^{a}	2078.3^{nq}	36.10^{ad}	2.70^{bd}	2723.0^{bj}	9048.9^{ag}	30.47^{ad}	29.90^{ad}	101.40^{ac}	73.07^{ae}
4	Dinkmash	19.67^{k}	7.67^{bc}	3059.0^{eh}	36.27^{ad}	2.77^{ab}	2936.3^{af}	8897.8^{ag}	31.43^{ad}	72.23ae	29.50^{ae}	90.43^{af}
5	Meko	28.66^{be}	8.67^{ab}	2971.7^{fi}	33.10^{ej}	2.73^{ac}	3155.6^{ab}	10134.3^{a}	33.33^{a}	34.20^{a}	105.93^{a}	85.37^{a}
6	Abshir	28.00^{bg}	8.33^{ac}	3730.3^{b}	30.63^{km}	2.60^{dg}	2325.9^{hn}	7353.1^{fg}	30.20^{ad}	25.33^{dj}	77.90^{df}	59.93^{be}
7	Gobiye	28.33^{bf}	7.33^{bc}	2466.7^{jm}	33.43^{ei}	2.63^{cf}	2797.1^{bh}	8506.7^{ag}	29.80^{ad}	29.90^{ad}	90.13^{af}	63.77^{ae}
8	Teshale	26.67^{di}	8.33^{ac}	3049.7^{eh}	33.93^{dh}	2.63^{cf}	2281.5^{in}	8107.7^{bg}	27.83^{ad}	24.40^{dj}	84.07^{bf}	58.87^{be}
9	Yeju	25.00^{ej}	8.33^{ac}	2123.3^{mq}	28.50^{mn}	2.63^{cf}	2903.7^{af}	9089.4^{af}	29.80^{ad}	31.37^{ac}	98.76^{ad}	72.13^{ae}
10	Birhan	25.33^{ej}	7.00c	1955.3^{pr}	27.73^{no}	2.67^{be}	1920.0^{n}	8143.2^{bg}	28.57^{ad}	20.60^{ik}	83.83^{cf}	58.67^{be}
11	Abuare	25.33^{ej}	7.00^{c}	3394.3^{ce}	36.93^{ab}	2.70^{bd}	2447.4^{em}	7129.9^{g}	32.97^{ab}	24.03^{ej}	70.80^{ef}	57.27^{ce}
12	Hormat	25.67^{ej}	7.67^{bc}	2800.7^{gj}	30.87^{jm}	2.50^{gh}	2524.4^{dl}	9943.7^{ac}	26.03^{dc}	26.77^{ch}	103.73^{ab}	65.33^{ae}
13	Macia	27.00^{dh}	8.67^{ab}	3492.3^{bd}	32.10^{fk}	2.63^{cf}	3087.4^{ac}	8697.3^{ag}	33.37^{a}	32.13^{ac}	89.93^{af}	70.70^{ae}
14	Red Swazi	26.33^{di}	7.33^{bc}	2021.3^{or}	26.10^{op}	2.57^{eh}	2696.3^{bj}	9158.5^{af}	29.50^{ad}	29.90^{ad}	97.17^{ad}	66.20^{ae}
15	Raya	24.67^{ej}	8.33^{ac}	1857.0^{qr}	33.43^{ei}	2.57^{eh}	2240.0^{jn}	8000.0^{cg}	29.93^{ad}	18.17^{k}	70.03^{f}	52.63^{de}
16	Miskir	31.00^{ac}	7.33^{bc}	1815.7^{qr}	36.63^{ac}	2.53^{fh}	2880.0^{af}	9317.5^{ae}	29.43^{ad}	29.07^{ae}	94.17^{ad}	68.53^{ae}
17	Girana -1	27.67^{cg}	8.67^{ab}	1874.0^{qr}	30.70^{km}	2.63^{cf}	2317.0^{hn}	9403.5^{ad}	27.80^{ad}	25.10^{dj}	98.10^{ad}	75.00^{ac}
18	Gedo -11	28.33^{bf}	7.00^{c}	3412.0^{ce}	37.07^{ab}	2.67^{be}	2542.2^{dl}	8930.4^{ag}	29.13^{ad}	26.77^{ch}	92.47^{ad}	68.23^{ae}
19	Melkam	25.33^{ej}	8.67^{ab}	3119.7^{dh}	31.67^{hk}	2.50^{gh}	2942.2^{ae}	10017.8^{ab}	30.00^{ad}	29.40^{ae}	102.77^{ab}	80.93^{ab}
20	ESH -1	30.00^{ad}	8.00^{ac}	2647.3^{il}	24.80^{p}	2.47^{h}	2604.5^{ck}	8442.5^{ag}	31.00^{ad}	27.10^{ch}	86.30^{af}	63.20^{be}
21	ESH-2	24.67^{ej}	8.33^{ac}	2370.0^{ko}	35.27^{be}	2.57^{eh}	2797.0^{bh}	8423.7^{ag}	31.10^{ad}	29.70^{ad}	89.10	68.67^{ae}
22	Mesay	33.67^{a}	8.33^{ac}	2410.0^{kn}	37.77^{a}	2.60^{dg}	2637.0^{ck}	8369.4^{ag}	32.30^{ac}	26.77^{ch}	85.86^{af}	73.63^{af}
23	Chare	33.33^{a}	8.33^{ac}	2279.0^{lp}	28.70^{ln}	2.53^{fh}	2589.6^{ck}	9242.5^{af}	30.80^{ad}	73.53^{ad}	25.77^{di}	94.97^{ad}
24	Dekeba	31.67^{ab}	7.00^{c}	1983.3^{pr}	32.63^{fk}	2.70^{bd}	1961.5^{mn}	7499.3^{dg}	28.33^{ad}	50.83^{e}	20.07^{jk}	78.17^{df}
25	Melkamash-79	26.00^{ei}	8.67^{ab}	2941.0^{fi}	32.53^{fk}	2.53^{fg}	2817.8^{bh}	9221.^{7af}	30.57^{ad}	67.50^{ae}	28.47^{bf}	95.37^{ad}
26	ESH-3	30.00^{ad}	8.33^{ac}	2173.0^{mq}	31.67^{hk}	2.63^{cf}	2785.2^{bi}	9019.2^{ag}	30.70^{ad}	67.20^{ae}	29.60^{ae}	91.90^{ae}
27	2005 MI5064	28.00^{bg}	8.00^{ac}	2732.7^{hk}	32.00^{gk}	2.53^{fh}	3318.5^{a}	9677.0^{ac}	32.77^{ab}	85.53^{a}	33.73^{ab}	97.00^{ad}
28	205MI5065	23.33^{hk}	8.00^{ac}	3129.7^{dg}	32.13^{fk}	2.57^{eh}	2885.9^{af}	9080.5^{ag}	31.07^{ad}	78.73^{ac}	29.93^{ad}	95.17^{ad}
29	PU209A/PRL021071	25.00^{ej}	8.33^{ac}	3425.7^{ce}	32.17^{fk}	2.60^{dg}	2791.1^{bh}	8564.0^{ag}	31.90^{ad}	64.03^{ae}	28.33^{bf}	88.07^{af}
30	PU209A/PU304	23.33^{k}	7.67^{bc}	4874.3^{a}	33.20^{ej}	2.63^{cf}	2429.6^{fm}	8712.1^{ag}	29.43^{ad}	59.57^{be}	25.23^{dj}	89.93^{af}
31	ICSA15/AWN87	23.00^{hk}	7.67^{bc}	2735.0^{hk}	34.40^{cf}	2.60^{dg}	2850.4^{ag}	8522.1^{ag}	30.60^{ad}	67.13^{ae}	28.70^{ae}	80.47^{cf}

Table 6. Contd.

32	P9534A/Gambell1107	31.00^{ac}	8.33^{ac}	2902.0^{fi}	32.83^{fk}	2.77^{ab}	2817.8^{bh}	8190.6^{ag}	32.20^{ad}	64.30^{ae}	29.17^{ae}	84.33^{bf}
33	Kari Mtama 1	22.67^{ik}	8.00^{ac}	3250.7^{cf}	31.23^{ik}	2.63^{cf}	2435.6^{em}	9449.9^{ad}	25.93^{d}	57.37^{ce}	24.53^{dj}	95.47^{ad}
34	IESV23007DL	22.67^{ik}	7.67^{bc}	2812.7^{gj}	24.37^{p}	2.53^{fg}	2974.8^{ad}	8458.1^{ag}	32.03^{ad}	70.03^{ae}	29.47^{ae}	86.33^{af}
35	P9511A/PRL020817	21.67^{jk}	8.67^{ab}	3558.3^{bc}	30.93^{jl}	2.53^{fh}	2690.4^{bj}	8448.4^{ag}	30.53^{ag}	61.97^{be}	28.30^{bf}	87.77^{af}
36	ETSC300001	28.67^{be}	7.67^{bc}	2200.0^{mq}	34.17^{dg}	2.83^{a}	2154.0^{kn}	8744.7^{ag}	26.83^{bd}	61.07^{be}	22.07^{hk}	90.23^{af}
37	ETSC300002	21.67^{jk}	8.33^{ac}	2400.0^{ko}	25.17^{p}	2.77^{ab}	2699.3^{bj}	8571.9^{ag}	29.83^{ad}	63.90^{ae}	27.57^{cg}	85.87^{af}
Grand mean		26.38	8.01	2718.99	32.08	2.61	2631.51	8700.73	30.25	27.21	89.53	66.73
C.V (%)		5.41	9.82	7.3	4.54	9.64	9.64	11.11	10.29	16.43	10.22	11.87
R^2		0.80	0.45	0.94	0.89	0.72	0.72	0.47	0.35	0.45	0.73	0.50

†First and last letter associated with a variety. All letters between these two letters are also associated with the variety.

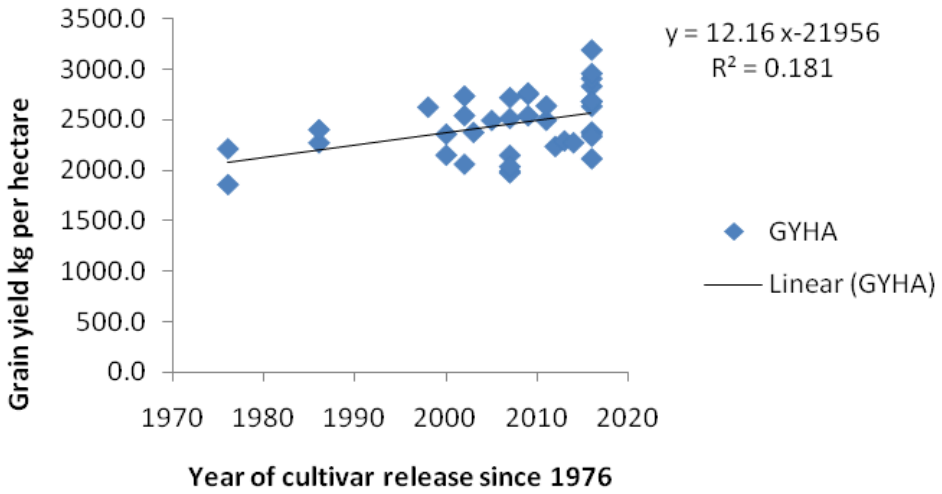

Figure 2. Relationship between year of cultivar release and grain yield over 2 locations.

significant G x E interaction, the direction of yield improvement was positive at both locations; 7.02 kg ha^{-1} year^{-1} at Sheraro and 17.3* kg ha^{-1} year^{-1} at Miesso.

Overall increase in grain yield over the older varieties was estimated to be 433.9 kgha^{-1} (17.56%) considering all varieties in the trial, whereas 1152.7 kgha^{-1} (36.1%) was obtained from variety P9534A/Gambella 1107 (Table 5). Hence, grain yield was found to increase substantially with the release of improved varieties (Figure 2). This agrees with the findings of Karmakar and Bhatnagar (1996) which reported a significant increase in grain yield of new soybean [*Glycine max* (L.) Merrill] cultivars over the older ones. Likewise, Mihret et al. (2015) reported that a significant increase in grain yield of new sorghum [Sorghum bicolor (L.) Moench] varieties over the early

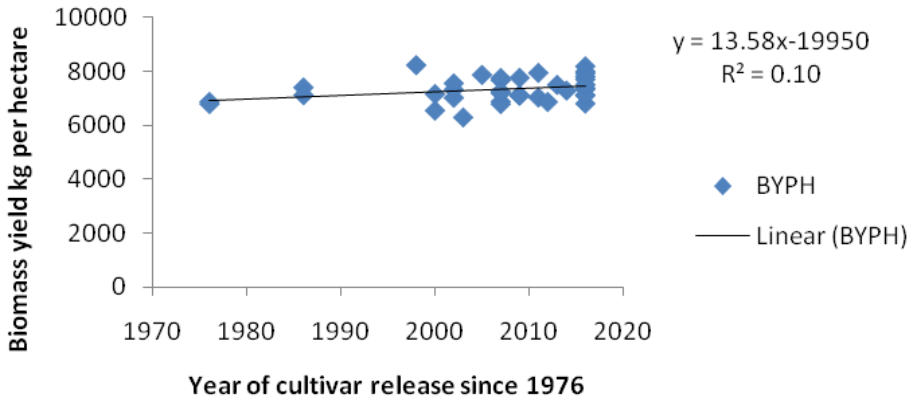

Figure 3. Relationship between the year of cultivar release and biomass yield at Sheraro and Miesso.

released cultivars. Similarly, Yifru and Hailu (2005) in tef [Eragrostis tef (Zucc.) Trotter], Tibebu (2011) in Chickpea (*Cicer arietinum* L.)., Kebere et al. (2006) in haricot bean (*Phaseolu vulgaris* L.) and Wondimu (2010) in food barley (*Hordeum vulgare* L.) who reported a substantial increment in grain yield of modern cultivars over the older ones.

There was no indication of a yield potential plateau in sorghum over the period studied indicating that the opportunity for breeders to further improve yields exists, and that continued progress towards that end may be expected. The average relative annual gain in grain yield of varieties since 1976 was 0.60% per year, or about 0.20% for the whole period of 40 years (Table 10). Present results indicated that plant breeders have made substantial progress for over the past 40 years in improving the yields of sorghum varieties in Ethiopia although; a yield fluctuation was occurring during the release of some of the varieties, Red Swazi (1991.1kg ha^{-1}) and Raya (1977.1kg ha^{-1}) released in (2007) showed yield reduction while, others showed a yield increment (Table 5).

Genetic improvement in biomass yield, harvest index and plant height

Mean biomass yields of varieties released in the years such as 1986, 1998, 2000, 2002, 2005, 2007, 2009, 2011, 2013, and 2016 exceeded that of the average of the first released older varieties. Moreover, Dinkmash by 431.8 kg ha-1(5.96%), Meko by 1400.1 kg ha-1(17.05%), Abshir, Gobiye by 20.3kg ha-1(0.30%), Teshale, Yeju and Birhan by 742.7kg ha-1(10.20%), Hormat by 1034.9 kgha-1 (13.18%), Macia, Red swazi and Raya by 430.8 kgha-1 (5.95%), Gedo, Melkam, ESH-1 &2 by 497.5 kgha-1 (6.81%), Messay, Chare by 6.58 kgha-1 (8.81%),Melkamash-79 by 669.6 kg ha^{-1} (8.95%), 2005MI504, 2005MI205 and ETSC300002 by 705.1 kg ha^{-1} (9.38%) (Table 9). The least and highest increases were 20.3kg ha-1(0.30%) and 1034.9 kgha-1 (13.18%, respectively, over varieties released in 1976 (Table 5). These indicated that there was a gradual increase in biomass yield across years of release although this increment was not consistent over years. The regression of the mean biomass yields of variety on the year of release indicated that there was 13.59^{**} kg ha^{-1} $year^{-1}$ average annual rate of increase (Figure 3). The increasing rate was 15.5 and 11.64^{***} kg ha^{-1} $year^{-1}$ at Sheraro and at Miesso, respectively. There was positive significant trend of improvement in biomass over the last 40 years of sorghum improvement. The relative annual biomass yield increment in sorghum varieties was estimated to be 0.20% per year for the last 40 years (Table 10). The present result agrees with the findings of Daniel and Parzies (2011) in the study of genetic improvement of Sesame (*Sesamum indicum* L.) in Ethiopia. The authors reported higher biomass yield in recently developed varieties than in older ones. Similarly, Hailu et al. (2009) indicated that fodder yield of early-maturing soybean varieties can show a positive trend although not significant, the linear regression of fodder yield of variety means on year of release showed an increasing trend (22.81 kg ha^{-1} $year^{-1}$) during a 16 years period. Fano et al. (2016) also indicated that biomass yield in tef was greater in newer varieties and linearly related to variety age which positively and significantly correlated to grain yield. Contrary to these findings, Sinha et al. (1981) reported that breeding had failed to raise the biomass of wheat and the grain yield improvement was solely due to the result of higher harvest index and similarly, Wondimu (2010) reported a non-significant trend in biomass yield in food barley breeding program.

Although the difference between sorghum genotypes was statistically non-significant in the analysis due to the high G x E interaction, this difference was significant at Miesso. At this location, varieties released in the fourth and fifth decades had the highest harvest index (Table 7).

Table 7. Mean performance of characters from separate analysis of variance for sorghum varieties grown in the yield potential trial at Miesso.

S/N	VAR	DTE	DTF	DTM	PHT	HWT	GFP	NTPP	NLPP	FLL	FLW	PE
1	Gambella 1107	6.3^{ac}	70.7^{bc}	108.0^{ck}	137.0^{gk}	3.44^{cj}	40.0gm	1.00ac	10.3^{ad}	41.93^{ac}	5.0^{e}	5.7^{ac}
2	76T1#23	6.0^{ad}	69.7^{bc}	104.3^{fl}	125.7^{jk}	3.11^{gk}	48.0^{ad}	0.80^{be}	8.7^{df}	40.3^{af}	7.0^{ab}	3.3^{eh}
3	Seredo	6.3^{ac}	66.3^{cd}	101.3^{kl}	124.3^{jk}	3.02^{ik}	50.3^{ab}	1.00^{ac}	8.7^{df}	43.0^{a}	5.3^{de}	5.3^{ad}
4	Dinkmash	6.0^{ad}	70.0^{bc}	112.3^{be}	131.7^{hk}	3.35^{ck}	38.7^{jm}	0.73^{ce}	9.3 ^{bf}	26.8^{g}	6.3^{ad}	2.7^{gh}
5	Meko	6.0^{ad}	66.7^{cd}	102.0^{jl}	148.33^{fj}	3.05^{hk}	49.7^{ac}	0.87^{ae}	9.3^{bf}	40.9^{ae}	5.7^{ce}	2.0^{h}
6	Abshir	5.7^{bd}	70.7^{bc}	102.0^{jl}	139.7^{gk}	3.62^{bg}	41.7^{el}	0.93^{ad}	8.3^{ef}	39.4^{af}	6.3^{ad}	5.0^{ae}
7	Gobiye	6.7^{ab}	71.0^{bc}	103.3^{hl}	113.3^{k}	3.26^{ek}	45.3^{bg}	0.80^{be}	8.0^{f}	40.5^{af}	6.3^{ad}	4.3^{cg}
8	Teshale	5.0^{d}	68.7^{bc}	103.7^{gl}	162.0^{ei}	4.12^{b}	42.3^{el}	0.93^{ad}	9.3^{bf}	41.6^{ad}	5.3^{de}	5.7^{ac}
9	Yeju	6.0^{ad}	59.3^{d}	102.7^{il}	136.7^{gk}	3.56^{ci}	51.0^{a}	0.87^{ae}	9.7^{bf}	38.9^{af}	5.7^{ce}	4.3^{cg}
10	Birhan	6.7^{ab}	71.7^{bc}	106.3^{dl}	145.0^{fk}	3.23^{ek}	46.7^{af}	0.93^{ad}	8.3^{ef}	42.3^{ab}	6.0^{be}	3.7^{dh}
11	Abuare	6.7^{ab}	77.3^{ab}	111.7^{bf}	161.3^{ei}	3.08^{gk}	41.7^{el}	1.13^{a}	9.0^{cf}	39.9^{af}	6.7^{ac}	5.7^{ac}
12	Hormat	6.0^{ad}	67.3^{cd}	104.3^{fl}	153.3^{fj}	3.26^{ek}	42.7^{ek}	0.93^{ad}	9.3^{bf}	39.1^{af}	6.0^{be}	5.0^{ae}
13	Macia	6.0^{ad}	72.3^{bc}	106.3^{dl}	141.7^{gk}	2.90^{jk}	41.3^{fl}	0.80^{be}	8.7^{df}	38.6^{af}	7.3^{a}	4.3^{cg}
14	Red Swazi	6.0^{ad}	67.0^{cd}	100.3^{l}	114.3^{k}	2.85^{k}	47.0^{ae}	0.87^{ae}	8.3^{ef}	39.5^{af}	5.7^{ce}	3.3^{eh}
15	Raya	6.3^{ac}	84.7^{a}	125.0^{a}	190.3^{ae}	3.89^{bc}	35.3^{m}	1.00^{ac}	11.7^{a}	38.5^{af}	6.7^{ac}	6.0^{ac}
16	Miskir	5.7^{bd}	69.7^{bc}	109.3^{cj}	151.0^{fj}	3.26^{ek}	40.3^{gm}	0.73^{ce}	9.7^{bf}	43.7^{a}	6.3^{ad}	2.3^{h}
17	Girana -1	5.7^{bd}	76.0^{bc}	102.0^{jl}	190.3^{ae}	3.35^{ck}	37.7^{km}	0.87^{ae}	10.0^{ae}	38.5^{af}	6.7^{ac}	6.7^{a}
18	Gedo -11	5.7^{bd}	70.7^{bc}	105.0^{el}	169.7^{bg}	3.23^{ek}	37.0^{lm}	1.07ab	10.7^{ac}	43.0^{a}	6.3^{ad}	6.0^{ac}
19	Melkam	6.3^{ac}	74.3^{bc}	110.3^{ch}	156.3^{fj}	3.59^{ch}	40.0^{gm}	0.93^{ad}	9.7^{bf}	38.1^{af}	6.3^{ad}	3.3^{eh}
20	ESH -1	6.3^{ac}	71.7^{bc}	114.7^{bc}	146.0^{fk}	3.47^{ci}	40.0^{gm}	0.87^{ae}	9.3^{bf}	39.4^{af}	6.0^{be}	3.7^{dh}
21	ESH-2	6.0^{ad}	72.7^{bc}	104.3^{fl}	190.0^{ae}	3.76^{be}	40.0^{gm}	0.87^{ae}	9.0^{cf}	38.1^{af}	5.7^{ce}	6.3^{ab}
22	Mesay	6.7^{ab}	73.0^{bc}	109.0^{cj}	144.0^{fk}	3.35^{ck}	41.3^{fl}	0.93^{ad}	9.7^{bf}	43.7^{a}	6.7^{ac}	3.3^{eh}
23	Chare	5.7^{bd}	76.0^{bc}	113.3^{bd}	167.3^{cg}	3.62^{bg}	39.0^{im}	1.13^{a}	9.7^{bf}	37.9^{af}	6.7^{ac}	3.3^{eh}
24	Dekeba	5.7^{bd}	70.7^{bc}	109.0 ^{cj}	141.7^{gk}	3.17^{gk}	39.7^{hm}	0.80^{be}	9.7^{bf}	43.6^{a}	5.3^{de}	3.3^{eh}
25	Melkamash-79	6.3^{ac}	72.0^{bc}	109.0^{cj}	154.0^{fj}	3.88^{bc}	40.7^{gm}	0.87^{ae}	9.7^{bf}	34.1^{f}	6.7^{ac}	5.0^{ae}
26	ESH-3	6.7^{ab}	68.0^{bd}	104.7^{fl}	139.0^{gk}	3.05^{hk}	44.3^{di}	0.93^{ad}	9.0^{cf}	37.9^{af}	6.7^{ac}	5.0^{ae}
27	2005 MI5064	5.7^{bd}	74.7^{bc}	108.3^{ck}	169.3^{bg}	3.82^{bd}	41.7^{el}	0.67^{de}	9.7^{bf}	37.8^{af}	6.3^{ad}	3.0^{fh}
28	205MI5065	6.3^{ac}	74.7^{bc}	106.3^{dl}	212.3^{a}	3.73^{bf}	37.7^{km}	1.00^{ac}	9.7^{bf}	39.0^{af}	5.3^{de}	4.7^{bf}
29	PU209A/PRL021071	6.0^{ad}	73.7^{bc}	109.3^{cj}	131.0^{hk}	3.20^{fk}	44.0^{dj}	1.00ac	8.7^{df}	37.7^{af}	5.7^{ce}	5.0^{ae}
30	PU209A/PU304	6.0^{ad}	71.3^{bc}	107.0^{dl}	129.0^{ik}	3.32^{dk}	45.0^{ch}	0.87^{ae}	9.7^{bf}	39.2^{af}	6.3^{ad}	5.7^{ac}
31	ICSA15/AWN87	6.0^{ad}	77.3^{bc}	118.3^{b}	195.0^{ad}	3.44^{cj}	41.3^{fl}	0.87^{ae}	10.3^{ad}	36.3^{bf}	7.0^{ab}	5.3^{ad}
32	P9534A/Gambell1107	5.7^{bd}	71.3^{bc}	106.7^{dl}	198.7^{ac}	5.16^{a}	42.0^{el}	0.87^{ae}	10.0^{ae}	35.4^{cf}	5.3^{de}	6.0^{ac}
33	Kari Mtama 1	5.3^{cd}	73.3^{bc}	109.3^{cj}	175.3^{bf}	3.85^{bd}	40.0^{gm}	0.60^{e}	10.0^{ae}	43.4^{a}	6.0^{be}	2.3^{h}
34	IESV23007DL	6.0^{ad}	73.0^{bc}	111.0^{cg}	163.7^{dh}	3.59^{ch}	39.7^{hm}	0.87^{ae}	11.0^{ab}	35.0^{df}	6.7^{ac}	2.7^{gh}
35	P9511A/PRL020817	7.0^{a}	66.3^{cd}	104.3^{fl}	149.3^{fj}	3.02^{ik}	44.3^{di}	0.93^{ad}	9.3^{bf}	34.7^{ef}	6.0^{be}	6.3^{ab}
36	ETSC300001	6.7^{ab}	75.3^{bc}	107.7^{cl}	201.0^{ab}	3.35^{ck}	40.7^{gm}	0.87^{ae}	11.0^{ab}	34.6^{ef}	5.3^{de}	6.0^{ac}

Table 7. Contd.

37	ETSC300002	5.3^{cd}	72.7^{bc}	110.0^{ci}	189.7^{ae}	3.35^{ck}	40.0^{gm}	0.80^{bee}	9.7^{bf}	40.7^{af}	7.0^{ab}	3.7^{dh}
	Grand mean	6.06	71.56	101.69	156.44	5.51	42.11	0.89	9.51	39.01	6.15	4.47
	C.V (%)	10.41	6.65	3.5	10.55	7.97	6.56	18.04	9.29	8.43	10.13	21.88
	R^2	0.43	0.53	0.73	0.78	0.78	0.78	0.44	0.55	0.62	0.59	0.73
S/N	**VAR**	PL	PW	NSPP	TSW	GL	GYPH	BYPH	HI	GYPD	BPR	SGR
1	Gambella 1107	25.0^{dk}	9.3^{cf}	2675.0^{eh}	23.5^{el}	2.10^{ch}	2085.3^{em}	5656.8^{b}	38.17^{af}	19.30^{gm}	52.73^{b}	53.64^{ac}
2	76T1#23	26.7^{bh}	10.03^{ce}	2954.3^{df}	25.8^{af}	2.07^{dh}	1659.3^{mo}	6179.3^{ab}	35.57^{bf}	15.93^{lo}	57.83^{ab}	50.79^{bc}
3	Seredo	24.7^{ek}	7.3^{f}	1891.0^{fh}	22.3^{im}	2.03^{ei}	1822.2^{kn}	5721.8b	32.33^{df}	18.07^{in}	57.57^{ab}	37.26^{c}
4	Dinkmash	30.3^{ab}	9.7^{cf}	3552.3^{ae}	25.0^{bh}	2.23^{ae}	1869.6^{jn}	5304.7^{b}	38.67^{ae}	16.67^{ko}	47.23^{b}	53.80^{ac}
5	Meko	24.0^{gl}	9.3^{cf}	2711.0^{eg}	27.8^{a}	2.27^{ad}	2094.8^{em}	6288.9^{ab}	34.77^{bf}	20.60^{el}	59.47^{ab}	47.12^{c}
6	Abshir	28.7^{ae}	9.0^{cf}	3389.0^{be}	22.8^{gm}	2.33^{ab}	1977.4^{hn}	5706.7^{b}	38.93^{ae}	19.40^{gl}	52.80^{b}	54.28^{a}
7	Gobiye	28.3^{af}	8.7^{df}	4233.3^{ab}	23.5^{el}	2.20^{af}	1920.1^{in}	5776.6^{b}	35.67^{bf}	18.57^{hm}	54.97^{b}	45.24^{c}
8	Teshale	21.7^{km}	10.0^{ce}	3041.0^{bf}	25.5^{af}	2.13^{bh}	2803.0^{bc}	6507.1^{ab}	40.83^{ad}	27.10^{b}	61.03^{ab}	63.62^{ac}
9	Yeju	24.0^{gl}	9.0^{cf}	1568.3^{h}	24.2^{ck}	2.27^{ad}	2566.8^{be}	5993.4^{b}	43.73^{ab}	25.00^{be}	58.67^{ab}	54.97^{ac}
10	Birhan	26.0^{cj}	7.7^{ef}	3555.3^{ae}	25.2^{bg}	2.07^{dh}	2204.4^{dl}	5864.2^{b}	37.30^{af}	20.70^{el}	54.93^{b}	48.38^{bc}
11	Abuare	24.7^{ek}	9.0^{cf}	3211.7^{be}	22.7^{gm}	2.13^{bh}	2305.2^{ck}	5424.0^{b}	41.30^{ad}	20.67^{el}	49.50^{b}	54.19^{ac}
12	Hormat	24.3^{fl}	9.3^{cf}	2896.3^{df}	24.5^{ck}	2.20^{af}	2465.2^{bh}	5757.0^{b}	42.23^{ac}	23.60^{bg}	54.23^{b}	57.80^{ac}
13	Macia	27.7^{bg}	9.3^{cf}	3618.3^{ae}	25.2^{bg}	1.93^{hi}	1942.5^{in}	5029.1^{b}	39.67^{ad}	18.27^{in}	47.23^{b}	47.02^{c}
14	Red Swazi	25.3^{dk}	8.0^{ef}	2801.0^{eg}	21.2^{ln}	2.13^{bh}	1285.9^{o}	6154.4^{ab}	29.50^{ef}	12.80^{o}	58.50^{ab}	41.99^{c}
15	Raya	22.3^{im}	10.7^{bd}	1879.7^{fh}	27.2^{ab}	2.00^{fi}	1714.3^{lo}	5542.7^{b}	33.03^{cf}	13.73^{no}	47.43^{b}	48.06^{bc}
16	Miskir	26.3^{bi}	8.0^{ef}	2826.3^{eg}	25.0^{bh}	2.20^{af}	2560.1^{be}	6162.2^{ab}	41.07^{ad}	23.43^{bg}	56.33^{b}	62.62^{ac}
17	Girana -1	24.3^{fl}	11.3^{ac}	2721.7^{eg}	20.5^{mn}	2.20^{af}	1982.0^{gn}	4953.6^{b}	38.20^{af}	19.47^{fl}	46.37^{b}	50.50^{bc}
18	Gedo -11	26.7^{bh}	10.7^{bd}	3484.0^{be}	22.0^{kn}	2.13^{bh}	1534.8^{no}	5557.7^{b}	35.93^{bf}	14.63^{mo}	52.03^{b}	52.78^{ac}
19	Melkam	28.0^{ag}	12.3^{ab}	3086.7^{bf}	24.7^{cj}	2.20^{af}	2560.0^{be}	5468.4^{b}	42.40^{ac}	23.20^{bh}	50.70^{b}	58.79^{ac}
20	ESH -1	32.0^{a}	11.0^{bd}	3733.3^{ae}	25.8^{af}	2.07^{dh}	2477.6^{bg}	5724.6^{b}	41.93^{ad}	21.60^{dj}	50.63^{b}	60.20^{ac}
21	ESH-2	26.7^{bh}	9.7^{cf}	3920.3^{ae}	26.3^{ad}	1.87^{i}	2736.3^{bc}	5780.5^{b}	42.06^{ac}	26.30^{bd}	55.17^{b}	63.94^{ac}
22	Mesay	26.3^{bi}	9.3^{cf}	3798.0^{ae}	25.2^{bg}	2.10^{ch}	2355.6^{cj}	5659.5^{b}	39.73^{ad}	21.70^{dj}	52.73^{b}	60.30^{ac}
23	Chare	20.3^{lm}	10.7^{bd}	3043.7^{bf}	25.7^{af}	1.97^{gi}	2686.7^{bd}	6608.3^{ab}	39.33^{ad}	23.70^{bg}	59.73^{ab}	64.00^{ac}
24	Dekeba	29.7^{ac}	10.0^{ce}	3345.0^{be}	25.0^{bh}	2.17^{ag}	2514.1^{bf}	6201.0^{ab}	36.67^{af}	21.27^{ek}	55.13^{b}	56.33^{ac}
25	Melkamash-79	27.3^{bg}	9.7^{cf}	3610.3^{ae}	24.2^{ck}	2.37^{a}	1765.3^{ln}	5740.4^{b}	29.00^{f}	16.20^{lo}	53.60^{b}	40.62^{c}
26	ESH-3	29.0^{ad}	9.0^{cf}	3430.7^{be}	22.2^{jm}	2.10^{ch}	1763.8^{ln}	5524.0^{b}	39.13^{ad}	16.87^{jo}	50.83^{b}	53.82^{ac}
27	2005 MI5064	24.0^{gl}	9.7^{cf}	3766.3^{ae}	26.7^{ac}	2.10^{ch}	2345.7^{cj}	6657.9^{ab}	38.47^{af}	21.63^{dj}	60.13^{ab}	64.68^{ac}
28	205MI5065	23.0^{hm}	9.3^{cf}	2905.0^{df}	24.8^{bi}	2.03^{ei}	2930.4^{b}	6501.8^{ab}	46.13^{a}	27.63^{b}	61.77^{ab}	79.91^{a}
29	PU209A/PRL021071	28.3^{af}	9.0^{cf}	4205.3^{ac}	22.5^{hm}	2.17^{ag}	1970.3^{hn}	5013.3^{b}	37.33^{af}	18.07^{in}	46.67^{b}	44.27^{c}
30	PU209A/PU304	29.0ad	9.7^{cf}	4129.7^{ad}	24.8^{bi}	2.07^{dh}	1804.4^{kn}	5491.1^{b}	37.20^{af}	16.93^{jo}	51.60^{b}	51.11^{bc}
31	ICSA15/AWN87	27.7^{bg}	10.7^{bd}	2985.0^{cf}	26.0^{ae}	2.07^{dh}	2417.6^{ci}	6194.3^{ab}	35.23^{bf}	22.13^{ci}	54.43^{b}	57.91^{ac}

Table 7. Contd.

32	P9534A/Gambell1107	29.0^{ad}	10.7^{bd}	2761.7^{eg}	25.0^{bh}	2.17^{ag}	3562.9^{a}	7754.2^{a}	39.27^{ad}	33.37^{a}	72.23^{a}	75.62^{ab}
33	Kari Mtama 1	25.0^{dk}	10.0^{ce}	3376.3^{be}	23.8^{dk}	2.17^{ag}	2925.4^{b}	6373.2^{ab}	38.97^{ae}	26.73^{bc}	58.53^{ab}	63.70^{ac}
34	IESV23007DL	22.0^{jm}	9.3^{cf}	4739.7^{a}	24.3^{ck}	2.30^{ac}	2942.2^{b}	6186.2^{ab}	40.70^{ad}	26.50^{bc}	56.73^{b}	63.58^{ac}
35	P9511A/PRL020817	28.0af	9.7^{cf}	3625.3^{ae}	23.3^{fl}	2.07^{dh}	2047.4^{fm}	5745.7^{b}	36.30^{bf}	19.67^{fl}	54.10^{b}	51.04^{bc}
36	ETSC300001	19.7^{m}	9.7^{cf}	1648.0^{gh}	19.8^{n}	2.10^{ch}	2521.5^{bf}	6629.9^{ab}	38.83^{ae}	23.43^{bg}	61.70^{ab}	59.15^{ac}
37	ETSC300002	24.3^{fl}	13.3^{a}	2762.0^{eg}	23.5^{el}	2.10^{ch}	2668.4^{bd}	6399.0^{ab}	40.63^{ad}	24.27^{bf}	57.20^{b}	65.57^{ac}
Grand mean		25.95	9.70	3186.00	24.26	2.13	2264.55	5925.23	38.28	21.06	54.93	55.64
C.V (%)		7.99	12.18	19.28	5.24	4.82	11.33	14.29	12.48	21.06	13.76	25.09
R^2		0.74	0.6	0.67	0.75	0.62	0.84	0.39	0.46	0.83	0.42	0.40

First and last letter associated with a Variety. All letters between these two letters are also associated with the variety. DTE=days to emergence, DTF=days to flowering, DTM=days to physiological maturity, PHT= plant height (cm), HWT=head weight tons per hectare), GFP=grain filling period, NTPP= number of productive tillers, NLPP= Number of leaves per plant (main stem), FLL=flag leaf length(cm) FLW=flag leaf width(cm), PE= panicle exertion(cm), PL=panicle length(cm), PW=panicle width(cm), NSPP=Number of seeds per panicle, TSW=Thousand seed weight(gram), GL= grain length(mm), GYH = grain yield kg per hectare, BYH= biological yield kg per hectare, HI= harvest index in percent.

In combined analysis, the highest harvest index was recorded for varieties, 34(IESV23007DL) released in 2016 (42.0%), P9534A/Gambella 1107(41.6%) released in 2016, 21(ESH-2) (40.8%) released in 2009, and 19(Melkam) (38.6%) released in 2009 (Table 8).

Genotypes 1(Gambella1107) and 2(76T1# 23), which were released in 1976 had harvest index of 33.2 and 28.6% (Table 8), respectively; at Miesso higher harvest index is associated with higher grain yield. Harvest index was increased by 0.12% year^{-1} in the combined analysis (Figure 4). It was increased by 0.25%* at Sheraro, but declined by 0.059% at Miesso (Table 10); this is the consequence of the highly significant G x E interaction. The present finding is similar to the findings of Tafesse et al. (2011) that reported newer sesame varieties had high harvest index; harvest index was improved at an annual rate of 0.97% year^{-1} over a period of 47 years. Similarly, Wondimu (2010) showed that newer food barley varieties developed in Ethiopia had higher harvest index and the regression slope of the trait over years of release was 0.004. Jin et al. (2010) reported that harvest index increased significantly with year of release, averaging 0.40% per year, rising from 0.31 to 0.38 for soybean cultivars released from 1950 to 2006 in Northeast China. In the same way, yield potential improvement in bread wheat produced marked positive change in harvest index (0.42% year^{-1}) in Ethiopia (Amsal, 1994). In contrary, Fano et al. (2016) in tef, Kebere, et al. (2006) in haricot bean and Tamene (2008) in faba bean who have reported that harvest index was not steadily changed with the year of release of the varieties in the respective crops they investigated. Likewise, Era et al. (2009) also reported that soybean varieties did not show significant differences for harvest index over the period of the genetic improvement. Besides the increment in biomass, there was also a consistent gradual increment in plant height from the older to the newer varieties.

G*enetic improvement in growth parameters*

PHT was increased by 1.05*** cm year^{-1} (Figure 5). The same tendency was observed at Sheraro (0.90 cm year^{-1}) and at Miesso (1.20 cm year^{-1}). NTPP was reduced by 0.003* tillers plant^{-1} year^{-1} (Table 10). It was reduced by 0.005* and by 0.001 tillers plant^{-1} year^{-1} at Sheraro and at Miesso, respectively. PE was extended (increased) by 0.04* cm year^{-1}. This increment was 0.07* cm and 0.01 cm year^{-1} at Sheraro and at Miesso, respectively. PW was improved by 0.02* cm year^{-1} over the two locations. This improvement was 0.003 cm year^{-1} and 0.03* cm year^{-1} at Sheraro and at Miesso, respectively. There was a tendency for number of leaves plant^{-1} to increase over time. It increased by 0.01, 0.02* and 0.02 leaves plant^{-1} year^{-1}, at Sheraro, Miesso and over locations (Table 10).

PL and FLW had non-significant positive slope; there was a tendency to increase over time, but this increment was statistically non-significant. FLL remained unchanged over time, although there was a tendency of decrease in FLL at Sheraro and in the combined analysis.

A combined analysis averaged over both locations indicated that there was highly significant

Table 8. Mean values of different traits from combined analysis of variance for sorghum varieties in the yield potential trials at Sheraro and Miesso, 2016 cropping season.

S/N	Variety	Trait									
		DTE	DTF	DTM	PHT	HWT	GFP	NTPP	NLPP	FLL	FLW
1	Gambella 1107	5.8^{eg}	66.17^{bf}	104.7^{bf}	133.67^{lm}	4.98^{ac}	40.2^{di}	1.1^{ad}	10.8^{ac}	40.7^{ci}	5.7^{g}
2	76T1#23	5.7^{fg}	61 .00^{fk}	97.7^{jl}	133.67^{lm}	4.21^{jn}	43.8^{af}	0.9^{df}	8.5^{f}	41.6^{ah}	6.8^{ae}
3	Seredo	6.0^{dg}	58.83^{jl}	96.3^{kl}	128.33^{m}	4.04^{lo}	45.8^{ab}	1.2^{ab}	8.7^{ef}	39.2^{el}	6.3^{cg}
4	Dinkmash	6.3^{bf}	65.7^{bg}	107.3^{be}	155.50^{fl}	4.79^{ah}	38.5^{fj}	0.9^{df}	10.5^{ac}	36.8^{im}	6.5^{ae}
5	Meko	5.8^{eg}	59.67^{il}	97.0^{jl}	185.33^{bd}	4.43^{el}	44.8^{ad}	0.9^{df}	9.8^{ce}	38.8^{fl}	6.5^{bg}
6	Abshir	5.3^{g}	63.17^{ck}	97.0^{jl}	183.33^{be}	4.25^{in}	39.2^{ej}	0.9^{df}	8.7^{ef}	44.6^{ab}	7.0^{ad}
7	Gobiye	6.0^{dg}	62.83^{dk}	98.3^{il}	157.50^{fl}	4.77^{ah}	42.2^{ah}	1.0^{cf}	8.5^{f}	41.7^{bh}	6.7^{bf}
8	Teshale	6.0^{dg}	62.83^{dk}	98.7^{hl}	148.67^{hm}	4.55^{gl}	39.7^{ej}	1.0^{cf}	10.0^{bd}	33.3^{no}	6.2^{dg}
9	Yeju	6.3^{bf}	55.67^{l}	97.7^{jk}	137.00^{km}	4.39^{em}	43.3^{a}	0.9^{df}	9.8^{ce}	37.0^{jm}	6.3^{cg}
10	Birhan	7.3^{a}	62.50^{ek}	99.8^{fl}	166.67^{dj}	3.88^{no}	42.2^{af}	1.3^{a}	8.8^{df}	37.3^{im}	6.2^{dg}
11	Abuare	7.3^{a}	68.17^{bc}	106.7^{bd}	170.33^{di}	4.44^{el}	42.0^{ag}	1.2^{ab}	10.0^{bd}	40.4^{dj}	6.7^{bf}
12	Hormat	6.0^{dg}	60.33^{hl}	99.3^{gl}	179.00^{cg}	4.40^{el}	40.8^{ah}	1.0^{be}	10.5^{ac}	38.3^{hm}	6.5^{bg}
13	Macia	6.5^{af}	64.33^{bi}	101.3^{ek}	183.33^{be}	4.28^{hn}	43.8^{bh}	1.0^{be}	9.7^{cf}	40.2^{dj}	7.7^{a}
14	Red Swazi	6.8^{ad}	58.33^{kl}	95.3^{l}	159.83^{ek}	3.73^{o}	43.0ae	1.0^{cf}	8.7^{ef}	36.1^{kn}	6.0^{eg}
15	Raya	5.7^{fg}	79.33^{a}	124.0^{a}	204.33^{ab}	4.80^{ag}	38.5^{ag}	1.2^{ac}	11.2^{ab}	42.8^{ae}	7.2^{ac}
16	Miskir	6.2^{cg}	66.17^{bf}	104.3^{bg}	142.33^{jm}	4.15^{ko}	35.7^{fj}	1.0^{cf}	9.7^{cf}	39.0^{fl}	6.5^{bg}
17	Girana -1	6.0^{dg}	68.00^{bd}	97.0^{jl}	204.33^{ab}	4.23^{gn}	37.^{17j}	1.0^{cf}	10.7^{ac}	39.1^{fl}	7.0^{ad}
18	Gedo -11	6.2^{cg}	65.67^{bf}	100.0^{fl}	170.83^{dh}	4.50^{cl}	39.3^{ij}	1.1^{ae}	10.7^{ac}	43.6^{ad}	6.8^{ae}
19	Melkam	6.7ae	68.17^{bj}	105.3^{be}	179.17^{cf}	5.05^{ab}	39.5^{ej}	1.0^{be}	10.2^{ac}	39.3^{el}	6.7^{bf}
20	ESH -1	6.7^{ae}	64.50^{bi}	105.5^{be}	144.83^{jm}	4.33^{fn}	40.2^{ej}	1.0^{cf}	10.5^{ac}	40.9^{ci}	6.3^{cg}
21	ESH-2	6.7^{ae}	63.33^{bj}	99.3^{gl}	206.33^{ab}	4.47^{dl}	41.2^{ci}	1.0^{cf}	9.7^{cf}	38.1^{hm}	6.2^{dg}
22	Mesay	6.8^{ad}	65.50^{bg}	103.8^{bg}	155.33^{fl}	4222.2^{jn}	39.8^{bh}	1.0^{be}	10.8^{ac}	38.2^{hm}	7.2^{ac}
23	Chare	6.3^{bf}	68.00^{bf}	106.8^{bc}	202.6^{ac}	4.85^{ad}	39.7^{ei}	1.0^{be}	9.8^{ce}	38.5^{gl}	6.8^{f}
24	Dekeba	6.3^{bf}	63.17^{ck}	107.8^{b}	146.17^{im}	4.31^{fn}	43.3^{bh}	1.0^{be}	9.5^{cf}	37.8^{im}	5.8^{fg}
25	Melkamash-79	6.2cg	64.67^{bi}	104.0^{bg}	179.67^{cf}	4.64^{bk}	39.0^{ah}	1.0^{cf}	9.5^{df}	42.0^{ag}	6.7^{bf}
26	ESH-3	7.2^{ab}	60.50^{gk}	99.7^{fl}	171.50^{dh}	4.31^{fn}	39.5^{ag}	1.0^{cf}	9.5^{cf}	44.9^{ab}	7.0^{ad}
27	2005 MI5064	6.2cg	68.00^{bd}	103.3^{bh}	158.83^{fk}	4.43^{el}	38.3^{ej}	0.8^{fg}	9.8^{ce}	41.7^{bh}	6.5^{bg}
28	205MI5065	6.7^{ae}	67.17^{be}	101.3^{ek}	207.33^{ab}	4.81^{ae}	45.2^{hj}	1.2^{ac}	9.5^{cf}	39.5^{ek}	6.0^{eg}
29	PU209A/PRL021071	6.5^{af}	63.67^{bj}	104.3^{bg}	139.00^{km}	4.76^{bi}	44.5^{ac}	1.1^{be}	8.5^{f}	45.3^{a}	6.5^{bg}
30	PU209A/PU304	6.2^{cg}	61.83^{fk}	101.7^{dj}	154.50^{gl}	4.62^{bk}	39.0^{ad}	1.0^{cf}	9.5^{cf}	44.0^{ac}	6.3^{cg}
31	ICSA15/AWN87	6.7^{ae}	68.00^{bd}	104.3^{bg}	206.83^{ab}	4.37^{en}	40.7^{ej}	1.0^{cf}	10.8^{ac}	39.3^{el}	7.2^{ac}
32	P9534A/Gambell1107	5.7^{fg}	64.83^{bh}	101.7^{dj}	186.67^{ad}	5.24^{a}	41.0^{ci}	1.0^{cf}	10.2^{ac}	39.3^{el}	6.0^{eg}
33	Kari Mtama 1	6.0^{dg}	65.33^{bh}	104.3^{bg}	186.50^{ad}	4.34^{en}	39.5^{bh}	0.7^{g}	10.3^{ac}	40.3^{dj}	6.2^{dg}
34	IESV23007DL	6.2^{cg}	67.33^{be}	106.0^{be}	156.50^{fl}	4.95^{ad}	44.5^{ej}	0.9^{ef}	11.3^{a}	34.9^{mn}	7.2^{ac}
35	P9511A/PRL020817	7.3^{a}	59.00^{jl}	99.7^{fl}	154.33^{hl}	4.68^{bj}	38.5^{ae}	1.0^{cf}	10.3^{ac}	42.2^{af}	6.5^{bg}

Table 8. Contd.

36	ETSC300001	7.0^{ac}	68.50^{b}	102.7^{ci}	186.50^{ad}	3.90^{mo}	39.3^{gj}	1.0^{cf}	11.2^{ab}	30.7^{o}	5.8^{fg}
37	ETSC300002	5.8^{eg}	66.00^{bf}	104.0^{bg}	210.50^{a}	4.39^{em}	41.8^{ej}	0.9^{ef}	10.2^{ac}	35.9^{ln}	7.3^{ab}
	Grand mean	6.33	64.49	102.35	169.65	4.43	40.87	1.01	9.90	39.57	6.65
	CV (%)	9.85	5.71	3.51	10.54	8.04	8.34	14.90	9.39	6.45	9.88
	R^2	0.66	0.89	0.87	0.83	0.94	0.65	0.69	0.63	0.74	0.62

S/N	Variety	PE	PL	PW	NSPP	TSW	GL	GYPH	BYPH	HI	GYPDAY	BYPR	SGR
1	Gambella 1107	3.50^{ik}	24.7^{ik}	8.3^{dg}	2767.5^{ei}	28.2^{em}	2.3^{fh}	2214.2^{in}	6847.8^{ce}	33.17^{ch}	21.22^{hj}	65.88^{dg}	55.90^{gl}
2	76T1#23	4.33^{gi}	25.3^{hk}	9.0^{cf}	2325.5^{ij}	29.5^{ce}	2.3^{gi}	1861.0^{o}	6775.2^{ce}	28.58^{hi}	19.32^{j}	70.75^{bf}	44.06^{l}
3	Seredo	5.17^{eg}	24.5^{jk}	8.3^{dg}	1984.7^{j}	29.2^{cf}	2.4^{eg}	2272.6^{hn}	7385.4^{ae}	30.90^{fi}	23.98^{ei}	77.95^{ae}	52.72^{il}
4	Dinkmash	2.67^{k}	28.2^{ag}	8.7^{cg}	3305.7^{be}	25.1^{o}	2.5^{ad}	2403.0^{dk}	7101.2^{ae}	34.25^{ch}	23.08^{fi}	68.22^{bf}	62.68^{di}
5	Meko	2.67^{k}	26.3^{dk}	9.0^{cf}	2841.3^{di}	32.1^{a}	2.5^{ac}	2625.2^{bh}	8211.6^{a}	32.15^{ch}	27.40^{be}	86.02^{a}	61.09^{ei}
6	Abshir	5.83^{cf}	28.3^{af}	8.7^{cg}	3559.7^{bc}	28.0^{em}	2.5^{ab}	2151.6^{jo}	6529.9^{de}	33.47^{ch}	22.37^{gj}	68.07^{bf}	56.09^{gk}
7	Gobiye	3.83^{hk}	28.3^{af}	8.0^{eg}	3350.0^{bd}	27.1^{jn}	2.4^{cg}	2358.6^{fl}	7141.6^{ae}	33.67^{ch}	24.23^{di}	73.37^{ae}	57.32^{fk}
8	Teshale	7.17^{b}	21.7^{mo}	9.2^{be}	3045.3^{cg}	29.5^{ce}	2.4^{dg}	2542.2^{ci}	7307.4^{ae}	35.70^{ag}	25.75^{bg}	74.88^{ae}	64.71^{ci}
9	Yeju	5.17^{eg}	25.3^{hk}	8.7^{cg}	1845.8j	29.1^{ci}	2.5^{be}	2735.2^{bd}	7541.4^{ae}	37.55^{ae}	28.18^{ac}	78.28^{ae}	61.30^{ei}
10	Birhan	4.67^{fi}	25.5^{gk}	7.3^{cg}	2755.3^{ei}	26.8^{ko}	2.4^{eg}	2062.2^{ko}	7003.7^{ae}	31.25^{ei}	20.65^{ij}	71.50^{bf}	48.07^{kl}
11	Abuare	4.33^{gi}	25.0^{ik}	8.0^{eg}	3303.0^{be}	25.2^{o}	2.4^{cg}	2376.3^{el}	6277.0^{e}	38.42^{ad}	22.35^{gj}	59.37^{fg}	56.45^{gk}
12	Hormat	4.00^{gj}	24.8^{ik}	8.5^{eg}	2848.5^{ei}	30.7ac	2.5^{be}	2494.8^{cj}	7850.4^{ac}	34.13^{ch}	25.18^{ch}	80.27^{ab}	59.69^{ej}
13	Macia	4.33^{gi}	26.7^{dj}	9.0^{dg}	3555.3^{bc}	28.0^{em}	2.2^{hi}	2514.9^{ci}	6863.2^{be}	37.12^{af}	25.20^{bg}	68.95^{bf}	62.50^{di}
14	Red Swazi	4.67^{fi}	23.5^{kn}	7.7^{fg}	2411.2^{hj}	26.6^{lo}	2.4^{dg}	1991.1^{mo}	7656.5^{ad}	25.45^{i}	21.35^{hj}	81.57^{ab}	46.91^{kl}
15	Raya	6.67^{bc}	24.7^{ik}	9.5^{bd}	1868.3^{j}	26.7^{lo}	2.3^{gi}	1977.1^{no}	6771.4^{ce}	29.47^{gi}	15.95^{k}	54.67^{g}	47.13^{kl}
16	Miskir	3.67^{ik}	26.3^{dk}	7.7^{fg}	2321.0^{ij}	29.2^{cf}	2.4^{dg}	2720.0^{be}	7739.9^{ad}	36.37^{af}	26.25^{bf}	75.35^{ae}	71.75^{ae}
17	Girana -1	8.83^{a}	23.8^{jm}	10.0^{ac}	2297.8^{ij}	28.6^{dk}	2.4^{eg}	2149.5^{jo}	7178.5^{ae}	32.28^{dh}	22.28^{gj}	75.43^{ae}	62.50^{di}
18	Gedo -11	5.00^{eh}	25.7^{fk}	8.6^{cf}	3448.0^{bd}	26.4^{mo}	2.4^{df}	2038.5^{lo}	7244.1^{ae}	28.78^{hi}	20.70^{ij}	73.47^{ae}	57.82^{fk}
19	Melkam	2.67^{k}	29.5^{ac}	10.5^{ab}	3103.2^{cg}	30.9^{ac}	2.4^{bf}	2751.1^{bd}	7743.1^{ad}	38.55^{ac}	26.30^{bf}	75.03^{ae}	71.03^{ae}
20	ESH -1	5.83^{cf}	29.8^{ac}	9.5^{bd}	3190.3^{bg}	28.8^{dj}	2.3^{gi}	2541.0^{ci}	7083.5^{ae}	37.05^{af}	24.35^{di}	68.85^{bf}	64.80^{ci}
21	ESH-2	6.67^{bc}	27.5^{ci}	9.0^{cf}	3145.2^{cg}	25.6no	2.2^{i}i	2766.7^{bc}	7102.1^{ae}	40.75^{ab}	28.00^{ac}	72.40^{af}	69.12^{af}
22	Mesay	4.17^{gi}	25.8^{ek}	8.8^{cf}	3104.0^{cg}	30.2^{bd}	2.3^{eh}	2496.3^{cj}	7014.4^{ae}	36.47^{af}	24.23^{di}	68.38^{bf}	62.65^{di}
23	Chare	3.83^{hk}	21.8^{lo}	9.5^{bd}	2661.3^{fi}	31.7^{ab}	2.3^{gi}	2638.1^{bg}	7925.4^{ac}	34.38^{bh}	24.73^{ch}	75.22^{ae}	67.02^{cg}
24	Dekeba	3.67^{ik}	26.2^{dk}	8.5^{dg}	2664.2^{fi}	26.9^{ko}	2.4^{eg}	2237.8^{in}	6850.1^{ce}	33.25^{ch}	20.67^{ij}	64.57^{eg}	54.67^{hl}
25	Melkamash-79	5.00^{eh}	28.0^{bh}	9.2^{be}	3275.7^{bf}	29.2^{cg}	2.6^{a}	2291.6^{gn}	7481.1^{ae}	30.67^{fi}	22.33^{gj}	73.00^{ae}	55.86^{gl}
26	ESH-3	6.33^{bd}	29.5^{ac}	8.7^{cg}	2801.8^{ei}	27.4^{fn}	2.4^{cg}	2274.5^{hn}	7271.6^{ae}	31.88^{dh}	23.23^{fi}	74.30^{ae}	54.13^{hl}
27	2005 MI5064	2.83^{jk}	24.3^{jl}	8.8^{cg}	3249.5^{bf}	29.6^{ce}	2.3^{fh}	2832.1^{bc}	8167.5^{ab}	34.87^{bh}	27.68^{bc}	79.92^{ac}	53.67^{ad}
28	205MI5065	4.33^{gi}	28.3^{af}	8.7^{cg}	3017.3^{ch}	28.3^{el}	2.3^{eg}	2908.2^{ab}	7791.1^{ad}	38.43^{ad}	28.78^{ab}	77.77^{ae}	78.45^{ab}
29	PU209A/PRL021071	6.67^{bc}	30.8^{a}	8.7^{cg}	3815.5^{b}	27.3^{hn}	2.4^{eg}	2380.7^{el}	6788.6^{ce}	35.95^{ag}	23.20^{fi}	66.33^{cg}	53.67^{hl}
30	PU209A/PU304	6.00^{be}	30.3^{ab}	8.7^{cg}	4502.0^{a}	28.5^{dl}	2.3^{fh}	2117.0^{ko}	7101.6^{ae}	30.63^{fi}	21.08^{hj}	70.88^{bf}	48.47^{jl}

Table 8. Contd.

31	ICSA15/AWN87	5.83^{cf}	28.8^{ad}	9.2^{be}	2860.0^{di}	29.1^{ci}	2.3^{eh}	2634.0^{bg}	7358.2^{ae}	36.33^{af}	25.42^{bg}	71.20^{bf}	68.59^{af}
32	P9534A/Gambell1107	7.17^{b}	28.5^{ae}	9.5^{bd}	2831.8^{di}	29.1^{ci}	2.4^{cg}	3190.3^{a}	7972.4^{ac}	41.58^{a}	31.27^{a}	78.65^{ad}	79.79^{a}
33	Kari Mtama 1	3.67^{ik}	25.0^{ik}	9.0^{cf}	3313.5^{be}	29.1^{ch}	2.4^{dg}	2680.5^{bf}	7911.6^{ac}	36.05^{af}	25.63^{bg}	76.75^{ae}	65.77^{ch}
34	IESV23007DL	3.50^{ik}	20.8^{o}	8.5dg	3776.2^{b}	28.6^{dk}	2.5^{ab}	2958.5^{ab}	7322.1^{ae}	42.03^{a}	27.98^{ac}	69.75^{bf}	75.23^{ac}
35	P9511A/PRL020817	8.50^{a}	29.5^{ac}	9.2^{be}	3591.8^{bc}	27.3^{gn}	2.4^{eg}	2368.9^{el}	7097.0^{ae}	33.80^{ch}	23.98^{ei}	71.98^{bf}	54.30^{hl}
36	ETSC300001	6.00^{be}	21.17^{no}	8.7^{cg}	1924.0^{j}	22.1^{p}	2.3^{fh}	2337.8^{fm}	7687.3^{ad}	31.60^{ei}	22.75^{fj}	75.60^{ae}	61.23^{ei}
37	ETSC300002	3.67^{ik}	23.7^{kn}	10.8^{a}	2581.0^{gi}	27.2^{in}	2.3^{fh}	2683.8^{bf}	7485.5^{ae}	36.98^{af}	25.92^{bg}	72.72^{af}	68.54^{af}
Grand mean		4.94	26.17	8.86	2952.5	28.17	2.4	2448.03	7310.0	34.43	24.14	72.63	60.96
CV (%)		18.92	7.79	11.26	15.37	4.82	3.84	10.41	12.38	13.42	10.88	13.25	14.18
R^2		0.82	0.77	0.70	0.79	0.95	0.93	0.82	0.81	0.74	0.85	0.86	0.78

DTE=days to emergence, DTF=days to flowering, DTM=days to physiological maturity, PHT= plant height (cm), HWT=head weight tons per hectare), GFP=grain filling period, NTPP= number of productive tillers, NLPP= Number of leaves per plant (main stem), FLL=flag leaf length(cm) ,FLW=flag leaf width(cm), PE= panicle exertion(cm), PL=panicle length(cm), PW=panicle width(cm), NSPP=Number of seeds per panicle, TSW=Thousand seed weight(gram), GL= grain length(mm), GYH = grain yield kg per hectare, BYH= biological yield kg per hectare, HI= harvest index in percent.

($p \leq 0.01$) and significant difference among locations, varieties and location x variety interaction effect in plant height (Table 4). These highly significant differences observed among varieties for plant height agrees with different authors (Saleem et al., 2002; Fikru, 2004; Melese, 2005). Among those recently released varieties, minimum plant height was observed in genotypes "Seredo (128.0 cm), while, ETSC300002" advanced in 2016 (210.5 cm) exhibited maximum plant height. As it was estimated from regression of variety means against year of release, the annual rate of gain, 0.79 cm ha^{-1} year^{-1} was different from zero (Table 12). This indicated that yield potential improvement program had markedly affected plant height. Similarly, Yifru and Hailu (2005) reported that plant height was higher for the modern tef varieties than the older ones, even though the relative genetic gain over the past 35 years of breeding, was low (0.4285 cm per year) and was not significantly ($p<0.05$) different from zero. Similarly, Amsal (1994) indicated that the newest varieties were significantly taller than the older ones but, it did not show relation with year of variety release. On the contrary, Donmez et al. (2001) reported that modern varieties showed significantly decreased plant height and reduced lodging in winter wheat varieties. Similar reports were presented by different researchers in different crops (Wondimu, 2010 in barley; Mihret et al., 2015 in sorghum, Kebere et al., 2006 in haricot bean).

Yield attributes

HWHA increased by 2.47 tones ha^{-1} year^{-1}. At Sheraro, it decreased by 5.06 tones ha^{-1} year^{-1} while, at Miesso it increased by 10.0* tones ha^{-1} year^{-1}. NSPP increased significantly over the last 40 years by 13.7, 17.4* and 15.5* seeds panicle^{-1} year^{-1} at Sheraro, at Miesso and over locations, respectively. However, TSW was reduced by 0.01 g per thousand weight year^{-1} at both locations and over locations, although this reduction was statistically not significant. Seed length (size) also reduced by 0.001, 0.0001 and 0.001 mm year^{-1} at Sheraro, at Miesso and over locations (Table 10).

Location mean squares from combined analysis of variance were significant ($p \leq 0.05$) for panicle width, number of productive tillers per plant, number of seeds per panicle, grain length and thousand seed weight (Table 4). Likewise, Abebe (1985), Fikru (2004), Melese (2005) and Temesgen (2007), found significant difference in the above yield components traits among tested genotypes in different crops. The mean number of grain (seed) per panicle, panicle width, number of productive tillers per plant and grain length increased significantly over the 40 years period from 2546.50 to 2975.69 (an increase of 14.39%), from 8.67 to 8.84, from 1.03 to 1.04 and from 2.30 to 2.32, respectively. However, over the same period, thousand seed weight decreased from 28.82 to 28.13 cm (by 2.39%).

Generally, older varieties had lower number of grains per panicle and productive tillers than the newer and high yielding varieties. Similar trend was reported by Amasal (1994) who reported 0.438% grains gain in the number of grains per panicle. This difference is reflected in the linear regression coefficient that showed a significant

Table 9. Trends in genetic progress in biomass yield for varieties released in 1976, 1986,1998, 2001, 2002, 2003, 2005, 2007, 2009, 2011, 2012, 2013,2014 and 2016 over the average of the 1st older varieties (Gambella 1107& 76t1#23) released in 1976.

Variety	Year	Mean biomass yield (kg/ha)	Increment over older varieties	
			kg/ha	%
Gambella 1107 76T1#23	1976	6811.5	-	-
Dinkmash	1986	7243.3	431.8	5.96
Meko	1998	8211.6	1400.1	17.05
Abshir	2000			
Gobiye	2000	683.0	20.3	0.30
Teshale	2002			
Yeju	2002	7284.2	742.7	10.20
Birhan	2002			
Abuare	2003	6277.0	-534.5	-8.52
Hormat	2005	7850.4	1034.9	13.18
Macia	2007			
Red Swazi	2007			
Raya	2007			
Miskir	2007			
Girana -1	2007	7242.3	430.8	5.95
Gedo -1	2009			
Melkam	2009			
ESH -1	2009			
ESH -2	2009	7309.0	497.5	6.81
Mesay	2011			
Chare	2011	7469.9	658.4	8.81
Dekeba	2012	6850.1	38.6	0.56
Melkamash-79	2013	7481.1	669.6	8.95
ESH-3	2014	7271.6	460.1	6.33
2005MI504	2016			
2005MI505	2016			
PU209A/PRL021071	2016			
PU209A/PU304	2016			
ICSA15/AWN87	2016	7516.6	705.1	9.38
P9534A/Gambella 1107	2016			
Kari Metama-1	2016			
IEsV23007DL	2016			
P9511A/PRL020817	2016			
ETSC300001	2016			
ETSC300002	2016			

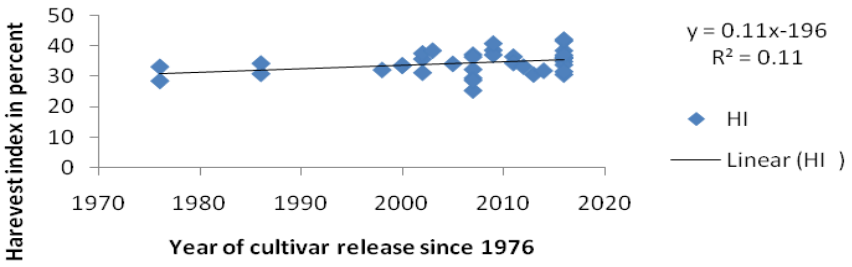

Figure 4. Relationship between the year of cultivar release and harvest index over 2 locations.

Table 10. Estimation of mean values, coefficient of determination (r^2), regression coefficient (b) and intercept for various traits from linear regression of the mean value of each trait for each variety against the year of variety release since 1976.

Trait	Regression analysis for data of individual locations								Regression analysis for data combined over two locations			
	SHERARO				MEISO							
	Mean	RSQ	INTERC	B	Mean	RSQ	INTERC	B	Mean	RSQ	INTERC	B
DTE	6.6	0.31	-61.3*	0.03*	6.1	0.02	11.9	-0.002	6.33	0.22	-24.7*	0.02*
DTF	57.4	0.08	-61.8	0.06	71.6	0.21	-153.1**	0.11**	64.49	0.15	-107.5**	0.09**
DTM	97.1	0.17	-86.6	0.09**	107.6	0.14	-94.8	0.10	102.35	0.17	-90.7**	0.10**
PHT	182.9	0.20	-1618.0**	0.90**	156.4	0.64	-2247***	1.20***	169.65	0.60	-1932.3***	1.05***
HWHA	5509.5	0.06	15665.0	-5.06	3439.0	0.24	-16619.0**	10.0*	4474.47	0.04	-477.0	2.47
GFP	396.0	0.10	-24.8	0.03	42.1	0.25	270.4*	-0.11*	40.87	0.12	122.8	-0.04
NTPP	1.13	0.28	10.2*	-0.005*	0.89	0.02	2.71	-0.001	1.01	0.23	6.47*	-0.003*
NLPP	10.3	0.07	-17.5	0.01	9.5	0.25	-33.8**	0.02*	9.90	0.20	-25.6	0.02**
FLL	40.1	0.01	68.5	-0.01	39.0	0.001	31.8	0.004	39.57	0.00	50.11	-0.01
FLW	7.0	0.00	6.2	0.00	6.2	0.05	-8.4	0.01	6.56	0.03	-1.11	0.004
PE	5.4	0.34	-126.6*	0.07*	4.5	0.02	-12.9	0.01	4.94	0.27	-69.7*	0.04*
PL	26.4	0.18	-125.0**	0.08**	26.0	0.004	45.8	-0.01	26.17	0.05	-39.5	0.03
PW	8.0	0.01	2.4	0.003	9.7	0.28	-53.1**	0.03*	8.86	0.22	-25.4	0.02*
NSPP	2719.0	0.16	-24650.0**	13.68**	3186.0	0.30	-31683.0*	17.4*	2952.50	0.30	-28167.0*	15.5*
TSW	32.1	0.003	50.8	-0.01	24.3	0.01	41.5	-0.01	28.17	0.006	46.2	-0.01
GL	2.6	0.01	3.9	-0.001	2.1	0.004	3.0	-0.00	2.37	0.01	3.42	-0.001
GYHA	2632.0	0.12	-11470.0	7.03	2265.0	0.29	-32442.0*	17.3*	2448.03	0.41	-21956.0**	12.2**
BYHA	8701.0	0.08	-22476.0	15.54	5891.0	0.57	-86351.0**	11.6***	7312.98	0.46	-54413.0**	13.59**
HI	30.13	0.02	60.4	0.25	37.7	0.23	-268.0**	0.06*	34.27	0.20	-103.8**	0.07**
GYPDAY	27.2	0.05	-66.5	0.05	21.1	0.24	-261.2*	0.14	24.2	0.27	-164.0**	0.09*
BYPR	90.0	0.01	-49.8	0.07	54.8	0.48	-694.5**	0.37**	72 .4	0.23	-372.1	0.22*
SGR	65.1	0.06	55.15	0.005	51.7	0.22	-731.2**	0.39**	58.39	0.21	-338.0**	0.20**

**Red colored are significant at 15%.

($p \leq 0.05$) increase in number of grains per panicle with annual rate of gain of 429.19 or by 0.61% $year^{-1}$ as compared to the older variety for the last 40 years in Sorghum varieties improvement program (Table 12). Similarly, Demissew (2010) reported a linear regression of mean which is highly significant increment with a relative genetic gain of 0.61% $year^{-1}$ grain per panicle. Panicle length showed an increasing trend with years of variety release, which indicated that newer varieties had longer panicle length, higher number of productive tillers per plant and higher number of grains per panicle than the older ones. Linear regression of variety means against year of variety release showed significant ($p \leq 0.05$) increment trend in panicle length with relative annual genetic increment of 0.12% (Table 12). Similar reports were published for progress in seed length from soybean breeding in the USA during the period between 1902 and 1977 (Specht and Williams, 1984), in durum wheat improvement (Tafese, 2011) and chickpea breeding in Ethiopia (Tibebu, 2011).

Analysis of variance revealed highly significant

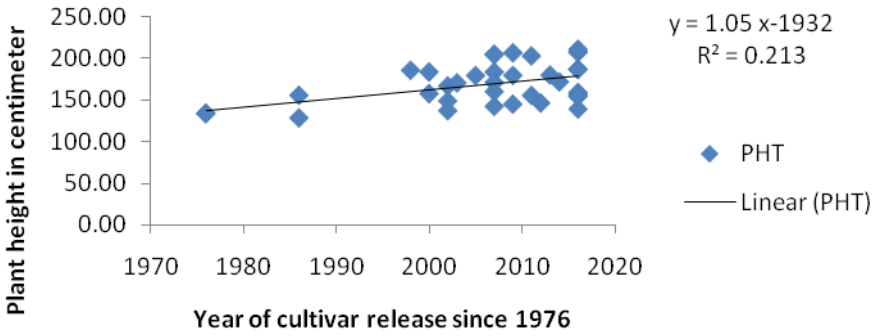

Figure 5. Relationship between the year of cultivar release and plant height over 2 locations.

differences among genotypes for 1000 seed weight. The values for 1000 seed weight ranged from 28.8 to 32.1 g with a mean value of 30.5 g. Accordingly, Meko (released in 1998) and mean of Gambella 1107 and 76T1#23 (oldest varieties) exhibited maximum and minimum 1000 seed weight of 32.1 and 8.82 g, respectively. In line with this, Tamene (2008) reported annual rate of genetic progress of 8 g thousand seed weight year^{-1} with relative genetic gain of 1.06 % in fababean. In the same way, Mihret et al. (2015) reported a significant increase in thousand seed weighs with a relative annual rate of gain was 0.94% year^{-1} over 39 year. However, a highly significant (P ≤ 0.01) decrease in thousand seed weight with a relative annual reduction of 0.96% was obtained in soybean varieties (Demissew, 2010). But Kebere et al. (2006) reported non-significant change in hundred seed weight of haricot bean varieties released between 1972 and 1998.

Results of the present study of the studied genotypes for Panicle width, which ranged from7.33 to10.83cm with mean value of 9.1 and coefficient of variations of 5.71. Highest Panicle width was depicted by genotype ETSC300002 (10.83 cm), while the lowest exhibited by Birhan (7.33 cm). Linear regression coefficient showed increment in Panicle width with annual rate of gain of 0.02*cm year^{-1} or by 0.004% year^{-1} relative increase as compared to the older varieties for the last 40 years in sorghum varieties improvement program (Table 12).

Phonological development traits (flowering and maturity)

The analysis of variance of the phonological traits at individual location revealed that there was significant difference (P ≤ 0.05) among varieties in days to flowering. DTE was increased by 0.03* and 0.02* days year^{-1} at Sheraro and over locations but was reduced by 0.002 days year^{-1} at Miesso. Days to flowering were increased by 0.06, 0.11 and 0.09 days year^{-1}, at Sheraro, Miesso and over locations. These changes were significant at 10% probability level. Days to maturity was also increased by 0.09, 0.10- and 0.10-days year^{-1} at Sheraro, Miesso and over locations and these changes were significant at 10% probability level. However, the grain filling period was decreased by 0.11* and 0.04 at Miesso and over locations, but was increased by 0.03 days year^{-1} at Sheraro (Table 10).

The regression analysis of days to flowering against the year of release indicated a significant annual genetic gain of 0.06 days y^{-1} at Sheraro and a 0.11 days year^{-1} gain at Miesso. In addition, days to physiological maturity showed a significant positive trend with the year of variety release in both locations (Table 10). The relative annual genetic gain since 1976 was found to be 0.11% (Sheraro) and 0.16% (Miesso) for days to flowering and 0.09% (Sheraro) and 0.09% (Miesso) for physiological maturity (Table 11).

A combined analysis of variance across the two test locations showed significant (p ≤ 0.01) differences among locations, among varieties and location by variety interaction for days to maturity (Table 4). In line with these results, Tigist (2003) and Ketema (2007) reported the presence of significant difference among genotypes for days to flowering. Most of the recently released varieties were the earliest in flowering and physiological maturity. Variety Raya (released in 2007) is the variety that took longer period (79 to 125) to flower and mature and the highest yielder "P9534A/Gambella1107 " is among the varieties that flower and mature early. This shift towards early maturity by decreasing the flowering and maturation time without significantly reducing the grain filling period is important to escape from terminal moisture stress in mid and low altitude areas. In the same way, Wondimu (2010) in barley, Tafese (2011) in sesame, Mihret et al. (2015) in sorghum and Tibebu (2011) in Desi type chickpea in Ethiopia reported a decrease in days to flowering. However, Hailu et al. (2009) observed insignificant yield increment with delayed flowering and maturity in soybean genotypes. Similarly, in a study on haricot bean and durum wheat in Ethiopia, Kebere et al. (2006) and Yifru and Hailu (2005) also reported a non-significant increase in days to maturity.

Table 11. Relative genetic gain (RGG) and correlation coefficients (corr.coe) for grain yield and different attributes in different sorghum varieties (in each location) during 2016 cropping season.

Trait	Sheraro		Miesso	
	Relative genetic gain (per year)	Correlation coefficient	Relative genetic gain (per year)	Correlation coefficient
Days to 50% emergence	0.57	-	-0.03	-
Days to flowering	0.11	0.12	0.16	0.31
Days to Maturity	0.09	0.21	0.09	0.6
Plant height (cm)	0.66	0.47	0.91	0.51
Head weight	-123.72	-0.13	0.31	0.17
Number of tillers per plant	-0.42	-0.51	-0.11	0.06
Panicle length	0.33	0.4	-0.04	-0.03
Panicle width	0.04	0.41	0.31	-0.41
Number of seeds per panicle	0.6	-0.07	0.62	0.48
Thousand seed weight (g)	-0.03	0.41	-0.04	-0.29
Grain length (mm)	-0.04	0.09	0	-0.3
Grain yield per hectare	0.32	0.31	0.92	0.57
Biomass yield per hectare	0.2	0.37	0.78	-0.25
Harvest index	-0.07	-0.02	0.47	0.29
Grain production per day	0.69	-0.05	0.8	0.01
Biomass production rate	0.06	0.26	0.66	-0.58**
Seed growth rate	0.01	0.26	0.88	0.4

Biomass production rate, seed growth rates and grain production per day

Biomass production rate, seed growth rate, and grain yield production per day showed significant ($p \leq 0.05$) difference among varieties in both locations. At Sheraro genotypes observed to produce the highest biomass production rate, seed growth rate and grain yield production per day of 90.0; 65.1, 27.2 kg $ha^{-1}day^{-1}$ at Sheraro and 54.8; 54.8, 21.1 kg $ha^{-1}day^{-1}$ at Miesso, respectively (Table 10). Low seed growth rate might be due to early termination of rain which caused lower biomass yield and grain yield. Most of the older varieties produced a higher biomass production rate, seed growth rate and grain yield production per day than the recent varieties at both locations. The relative annual gain of 0.13% per year for biomass production rate, 0.44% per year for seed growth rate and 1.80% for grain production per day (Table 12) was high, indicating that these characters were effectively and significantly improved as a result of the 40 years period of grain yield potential improvement. This agrees with the investigation of Kebere et al. (2006) on haricot bean and Fano et al. (2016) on tef. This data indicated that, biomass production rate; seed growth rate and grain production per day from the five decades of plant breeding and selection was increased by 11.61, 18.86 and 34.17%. The annual genetic gain as estimated from the regression coefficient was 0.22, 0.20 and 0.09 kg ha^{-1} day^{-1} $year^{-1}$ for biomass production rate, seed growth rate and grain yield production per day respectively (Table 10).

According to Kusmenoglu and Muehlbauer (1998), increased seed yield has been obtained through development of cultivars with shorter vegetative and generative growth periods, and greater rates of crop and seed growth. In agreement with the finding of the present study, Pedersen et al. (1998) and Demissew (2010) reported significant increase in seed growth rate in soybean. Similarly, Kebere et al. (2006), Tamene (2008) and Tibebu (2011) reported significant increase in seed growth rate and biomass production rates of haricot bean, fababean and chickpea varieties released in Ethiopia, respectively.

However, Wondimu (2010) showed that there was a significant increase only in seed growth rate in barley. In contrast, Yifru and Hailu (2005) observed non-significant increases in both seed growth rate and biomass yields of tef genotypes over 35 years of breeding and selection. From this, it can be concluded that substantial improvement was apparent in the rate of biomass production, seed growth rate and grain production per day due to grain yield improvement.

BYPR increased by 0.07, 0.37**, and 0.22 kg ha^{-1} day^{-1} at Sheraro, Miesso and over locations, for the last 40 years. GYPDAY also increased by 0.05, 0.14* and 0.09* kg ha^{-1} day^{-1} at Sheraro, Miesso and over locations. SGR increased by 0.14, 0.55** and 0.34 kg ha^{-1} day^{-1} at Sheraro, Miesso and over locations, respectively (Table 10). The highest improvements were achieved at Miesso.

Table 12. Relative genetic gain and correlation coefficients for grain yield and different attributes in different sorghum varieties (over locations) during 2016 cropping season.

Character	Relative genetic gain (% per year)	Correlation coefficients
Days to flowering	0.14	-
Days to maturity	0.1	0.81
Plant height	0.79	0.48
Head weight	0.67	0.32
Number of tillers per plant	-0.03	0.13
Panicle length	0.12	-0.19
Panicle width	0.23	0.32
Number of seeds per panicle	0.61	-0.07
Thousand seed weight	-0.03	-0.17
Grain length (size)	-0.04	-0.21
Grain yield per hectare	0.6	0.08
Biomass yield per hectare	0.45	-0.09
Harvest index	0.23	0.15
Grain production per day	1.8	-0.2
Biomass production rate	0.13	-0.52

Correlation analysis

Biomass yield, harvest index and plant height

The correlation coefficients of grain yield, thousand seed weight and biomass yield with all the traits studied are presented in Table 12. The results of correlation analysis indicated that grain yield showed a highly significant ($p \leq 0.01$) and positive association with biomass yield (r= 0.57***), harvest index (r=0.86***), grain yield production per day(r=0.94**), biomass production rate (r=0.37***), and seed growth rate(r=0.92***). Moreover, biomass yield showed significant positive correlation with biomass production rate (r=0.84***), grain yield production per day (r=0.61***) and seed growth rate(r=0.55***), but non-significant association with all other traits (Table 14) In agreement with the present study, Singh et al. (1990) on chickpea found that biological yield and harvest index had significant positive association with grain yield and therefore simultaneous selection for these two traits would lead to high seed yield. Conversely, Yifru and Hailu (2005) on tef, (Kebere et al., 2006) on haricot bean, Tamene (2008) on faba bean, Hailu et al. (2009 and Demissew (2010) on soybean found a highly significant positive correlations between grain yield and biomass yield, but no significant correlation between grain yield and harvest index. However, Amsal (1994) on bread wheat and Wondimu (2010) on food barley reported a significant and positive association between harvest index and grain yield and a non-significant association between biomass and grain yield. The association between grain yield and plant height was positive (r=0.24). Different authors (Wondimu, 2010 on food barley and Jin et al., 2010 on soybean) found a significant correlation of grain yield and plant height. In contrary, Yifru and Hailu (2005), Kebere et al. (2006), Tamene (2008), Hailu et al. (2009) observed no relation between grain yield and plant height in tef, haricot bean, faba bean, and soybean respectively. In general, grain yield in the modern varieties appears to be associated more with a higher partitioning efficiency to the grain sink than the production of a higher biomass. This indicated that partitioning efficiency may serve as an index for identifying varieties with higher seed yield.

Yield components

Significant and negative correlation was observed between grain yield and number of tillers per plant, grain yield and panicle length, while the association of grain yields with panicle width, head weight and number of seeds per panicle were positive (Table 14). This indicates that these characters are important traits used as indirect selection criteria in breeding for improving grain yield in sorghum. Similar results were also reported by Saleem et al. (2002) in chickpea found that there was significant and negative association of grain yield with spike length. Likewise, Majumder et al., (2008); Degewione and Alamerew (2013) reported that positive and non-significant correlation of grain yield with number of kernel per spike and 1000 seed weight in chickpea.

Phonological traits

Days to flowering and days to maturity showed a non-significant and positive association (r = 0.08) and (r=0.03) with grain yield respectively (Table 14). This is in agreement with the investigation of Tibebu (2011) who

reported a non-significant association of days to flowering and days to maturity with grain yield in chickpea. However, Singh et al. (1990) in chickpea, Hailu et al. (2009) and Demissew (2010) in soybean reported strong positive correlations of grain yield with days to flowering and days to maturity. In contrast, Fikru (2004), reported a negative association between days to flowering and days to maturity with grain yield in wheat. According to Amsal (1994) in wheat and Fano et al. (2016) in tef, days to flowering and days to maturity were association poorly with grain yield. Kebere et al. (2006) in haricot bean reported lack of correlation between grain yield and these phonological traits.

Productivity traits

Biomass production rate (r=0.37**), grain yield production per day (r=0.94**) and seed growth rate (r=0.92**) showed a highly significant (P ≤ 0.01) positive relation with grain yield (Table 14). This clearly showed that improvement in these traits was markedly concurrent with the yield improvement achieved in the past and can further be exploited in future breeding program. Similar results were reported by Kebere et al. (2006) in haricot bean and Demissew (2010) in soybean. Likewise, DeBruin and Pedersen (2009) found positive relation between grain yield and crop growth rate during seed set, seed growth rate, grain yield production per day. Moreover, positive correlation between grain yield with grain yield production per day and biomass production rate (Yifru and Hailu, 2005) on Tef were reported.

Yield components

Non-significant and negative correlation was observed between grain yield and plant height and grain filling period, while the association of grain yields with panicle length and panicle width was positive. This indicates that these characters are important traits used as indirect selection criteria in breeding for improving grain yield in sorghum. Similar results were also reported by Saleem et al. (2002) in chickpea found that there was significant and negative association of grain yield with spike length. Likewise, Majumder et al., (2008); Degewione and Alamerew (2013) reported that positive and non-significant correlation of grain yield with number of kernel per spike and 1000 seed weight in chickpea.

Phonological traits

Days to flowering and days to maturity showed a non-significant and positive association (r = 0.20) and (r=0.13) with grain yield respectively (Table 14). This is in agreement with the investigation of Fikru (2004), reported a negative association between days to flowering and days to maturity with grain yield in wheat, Tibebu (2011) who reported a non-significant association of days to flowering and days to maturity with grain yield in chickpea. However, Singh et al. (1990) in chickpea, Hailu et al. (2009) and Demissew (2010) in soybean reported strong positive correlations of grain yield with days to flowering and days to maturity. According to Amsal (1994) in wheat and Fano et al. (2016) in tef, days to flowering and days to maturity were association poorly with grain yield. Kebere et al. (2006) in haricot bean reported lack of correlation between grain yield and these phonological traits.

Productivity traits

Biomass production rate(r = 0.22NS) revealed a non-significant and negative association with grain yield, while grain yield production per day (r=0.92***) and seed growth rate (r =0.95***) showed a highly significant (P ≤ 0.01) and positive relation with grain yield (Table 14). This clearly showed that improvement in these traits was markedly concurrent with the yield improvement achieved in the past and can further be exploited in future breeding program. Similar results were reported by Kebere et al. (2006) in haricot bean and Demissew (2010) in soybean. Likewise, DeBruin and Pedersen (2009) found positive relation between grain yield and crop growth rate during seed set, seed growth rate, grain yield production per day. Moreover, positive correlation between grain yield with grain yield production per day and biomass production rate (Yifru and Hailu, 2005) on Tef were reported.

Stepwise regression analyses using grain yield as dependant variable and other traits independent variables indicated that, seed growth rate, harvest index, grain production per day and biomass yield production rate are traits which contributed to gain in grain yield. Particularly, 49% of the variation in grain yield was explained by seed growth rate and was the single most important trait that contributed most to the variation in grain yield among others, where as 27, 23 and 20% variation in grain yield were contributed by grain yield production per day, biomass production rate and harvest index respectively (Table 13). This illustrates that the improvement in grain yield was achieved by combination of different factors.

According to Wondimu (2010) results of a stepwise regression analysis of grain yield on selected yield components revealed that harvest index, biomass yield and seed yield per day altogether accounted for 46, 73 and 74% of the variation in grain production respectively. Amsal, (1994) also reported number of grains per meter square alone accounted for most of the variation (>68%) in grain yield while number of gains per meter square, 1000-seed weight, plant height, biomass yield collectively contributed for more than 93% variation in wheat grain yield. About 96% of the variation in

Table 13. Selection from stepwise regression analysis of mean grain yield as dependent variable and the other traits as independent variable.

Independent variable	Regression coefficient (b)	R^2 (%)
Harvest index	0.07**	0.20
Grain yield production per day	0.09*	0.27
Biomass yield production rate	0.22*	0.23
Seed growth rate	0.34**	0.49

**, All regression coefficients are significant at P≤0.015; *: All regression coefficients are significant at P≤0.05.

fababean grain yield was explained by economic (seed) growth rate, whereas economic growth rate, number of pods per plant, harvest index and biomass together accounted for 99% of the variation in grain yield (Tamene, 2008).

Conclusion

Regardless of considerable effort and devotion of resources, the magnitude of genetic progress from sorghum improvement made since its early inception and the associated traits of genetic improvement achieved so far from the same efforts from different years in a common environment have not been studied. Therefore, one set of yield potential experiment comprising 37 sorghum varieties were conducted in randomized complete block design with three replications at Sheraro and Miesso to determine the amount of genetic gain made over time in yield potential of sorghum varieties and to identify changes in morphological characters associated with genetic improvement in grain yield potential of sorghum varieties in Ethiopia. The analysis of variance for each location revealed wider variability ($p<0.01$) for all traits, except for days to emergence and number of productive tillers per plant, indicating wider possibility of selection for these traits. The combined analysis of variance across the two locations revealed that there were significant differences among the sorghum varieties due to genotype, location and genotype x location interaction for most of the traits.

The results from linear regression analysis showed that breeding has made significant improvement in grain yield potential of sorghum through consecutive release of new varieties over the past 40 years. The average grain yield of all sorghum varieties, averaged over the two locations was 2448.03 kg ha^{-1}, which ranged from 1861.0 to 3190.3 kg ha^{-1}. The superiority of the highest yielding variety, P9534A/Gambella1107 represents 1152.7 kg ha^{-1} or 36.13% increment over the average of the first two older varieties (Gambella1107 and 76T1#23) followed by IESV 230007DL (2958.5 $kgha^{-1}$), 2005 MI5065(2908.2 $kgha^{-1}$) and 2005MI5064 (Argeti) (2832.1 $kgha^{-1}$). Varieties derived from indigenous germplasm lines and from introduced (ICRISAT) advanced breeding lines yielded an average grain yield of 2644.7kg ha^{-1}, and exceeding the grain yield of older varieties by 607.1 kg ha^{-1} (30.9%). The average rate of increase in grain yield of sorghum varieties per year of release was 12.2 kg ha^{-1} (.60%). Generally, grain yield showed an increase from old to new varieties during the last five decades of sorghum breeding in Ethiopia. This implies that the grain yield potential of sorghum has not attained plateau in Ethiopia.

For the last 40 years of sorghum improvement, biomass yield increased significantly by 30.8 kg ha^{-1} (0.45%) $year^{-1}$. As the rate of biomass yield has been similar to that of yield gain, harvest index was also steadily modified with the year of release of a variety and there was no consistent gradual reduction in plant height from the older to the newer varieties. Linear regression analysis revealed that there was a significant improvement in most of yield attributes; PHT, NLPP, PE and PW which showed a significant increment and NTPP showed a reduction trend across the years of release. Like that of grain yield and harvest index, all productivity traits showed a highly significant increasing trend for the last 40 years of sorghum improvement program. On the contrary, corresponding to the decrease in flag leaf length, thousand seed weight, and grain length (size) showed a negative trend but not significantly different from zero. Unlike other crops, early maturing genotypes produce a higher seed yield than the late ones in most situations, because when days to maturity increases the phonology of the crops enters to the dry spell, which in turn leads to loss in yield.

Examination of yield components by a series of simple correlation indicated that grain yield was positively and significantly associated with biomass yield, head weight, thousand seed weight, grain production per day, biomass production rate and seed growth rate, whereas all other measured yield components showed non-significant association with grain yield.

Results of stepwise regression analysis indicated that harvest index, grain production per day biomass production rate and seed growth rate contribute most of the variation in grain yield of sorghum. Seed growth rate was by far contributing a lot, which accounted for 49% of the variation in grain yield. In the case of sorghum, the most recently released varieties showed higher grain yield.

Table 14. Correlation coefficients of mean values of yield and yield related traits of varieties represented in the study.

	YVR	DTF	DTM	PHt	HW	NTPP	PL	PW	NSPP	TSW	GL	GYPH	BYPH	HI	BPR	SGR	GYPD
YVR	1																
DTF	0.22^{ns}	1															
DTM	0.20^{ns}	0.81***	1														
PHt	0.46***	0.48***	0.20^{ns}	1													
HW	0.45***	0.32^{ns}	0.36**	0.31^{ns}	1												
NTPP	-0.24^{ns}	0.13^{ns}	0.14^{ns}	-0.07^{ns}	-0.05^{ns}	1											
PL	0.13^{ns}	-0.19^{ns}	-0.04^{ns}	-0.06^{ns}	0.29^{ns}	0.05^{ns}	1										
PW	0.27^{ns}	0.32^{ns}	0.22^{ns}	0.49***	0.39**	-0.31^{ns}	0.07^{ns}	1									
NSPP	0.8^{ns}	-0.07^{ns}	-0.06^{ns}	-0.06^{ns}	0.42**	-0.17^{ns}	0.47***	-0.02^{ns}	1								
TSW	-0.05^{ns}	-0.17^{ns}	-0.13^{ns}	-0.04^{ns}	0.16^{ns}	-0.19^{ns}	0.03^{ns}	0.26^{ns}	0.04^{ns}	1							
GL	-0.07^{ns}	-0.21^{ns}	-0.17^{ns}	-0.12^{ns}	0.24^{ns}	-0.17^{ns}	0.03^{ns}	-0.12^{ns}	0.13^{ns}	0.19^{ns}	1						
GYPH	0.43**	0.08^{ns}	0.03^{ns}	0.24^{ns}	0.56***	-0.36**	0.01^{ns}	0.27^{ns}	0.12^{ns}	0.33**	0.06^{ns}	1					
BYPH	0.32^{ns}	-0.09^{ns}	-0.19^{ns}	0.25^{ns}	0.21^{ns}	-0.42***	-0.20^{ns}	0.18^{ns}	-0.21^{ns}	0.45***	0.18^{ns}	0.57***	1				
HI	0.33^{ns}	0.15^{ns}	0.13^{ns}	0.17^{ns}	0.54^{ns}	-0.19^{ns}	0.11^{ns}	0.25^{ns}	0.25^{ns}	0.14^{ns}	-0.04^{ns}	0.86^{ns}	0.11^{ns}	1			
BPR	0.10^{ns}	-0.52***	-0.68***	0.07^{ns}	-0.06^{ns}	-0.37**	-0.13^{ns}	0.01^{ns}	-0.16^{ns}	0.41**	0.23^{ns}	0.37**	0.84***	-0.04^{ns}	1		
SGR	0.41**	0.27^{ns}	0.05^{ns}	0.37**	0.52***	-0.35**	-0.06^{ns}	0.35**	0.09^{ns}	0.25^{ns}	0.04^{ns}	0.92***	0.55***	0.80***	0.33^{ns}	1	
GYPD	0.33**	-0.20^{ns}	-0.30^{ns}	0.17^{ns}	0.42***	-0.38**	0.04^{ns}	0.19^{ns}	0.12^{ns}	0.36***	0.12^{ns}	0.94**	0.61***	0.77***	0.58**	0.85***	1

This clearly indicated that grain yield was improved consistently as year of release considered.

Ethiopia is known for wide genetic base of sorghum which is a potential for developing improved varieties targeting high yield, disease resistance and other quality traits. However, this huge potential is not yet exploited due to lack of strong breeding program that enable collection, characterization, evaluation and identification of desirable traits for genetic improvement. The Ethiopian sorghum national breeding program was dependent mostly on material introduction from other countries such as Zimbabwe (SAFGRAD) and India (ICRISAT). Moreover, the existing conventional breeding scheme is time taking, laborious and the desirable traits are masked by environmental effect. Hence, the use of modern tools aid to know genetic makeup of different varieties that can be used effectively for breeding and conservation program.

Finally, it should be emphasized that data generated from an experiment conducted for one season may not be sufficient enough to measure the average improvement over the last 40 years. Therefore, similar experiments conducted over many years and over many locations are preferred to make reliable recommendations. But data collected herein from two locations and one season may be used as the base line for yield potential experiments for several years.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Abebe T (1985). Correlation and variation studies on yield and some plant character in some indigenous and exotic chickpea varieties. An M.Sc. Thesis submitted to the School of Graduate Studies, Addis Ababa University.

Amsal T (1994). Yield potential of rain fed wheat in the central high lands of Ethiopia. M.Sc. Thesis Presented to the School of Graduate Studies of Alemaya University.

Amsal T, Turner DG, Getinet G (1995a). Improvement in yield of bread wheat cultivars released in Ethiopia from 1949 to 1987. African Crop Science Journal 3(1):41-49.

Amsal T, Turner DG, Tesfaye T, Getinet G (1995b). Improvement in durum wheat yield and associated effects on morpho-physiological characters. African Crop Science Journal 3(4):433-442.

Asfaw A (2007), The role of introduced sorghum and Millets in Ethiopian Agriculture. Journal of Semi-Arid Tropical Agricultural Research 3(1):1-4.

Daniel EG, Parzies HK (2011). Genetic variability among landraces of sesame in Ethiopia. African Crop Science Journal 19(1):1-13.
CSA (Central Statistical Authority) (2014/15). Agricultural Sample Survey. Area and production of crops. Statistical Bulletin 505, Volume I. Addis Ababa.20p.
Debruin J, Pedersen P (2009). Growth, Yield, and Yield Component Changes among Old and New Soybean Cultivars. Agronomy Journal 101. 10.2134/agronj2008.0187.
Degewione A, Alamerew S (2013). Genetic Diversity in Bread Wheat (Triticum aestivum L.) Genotypes. Pakistan Journal of Biological Sciences: PJBS. 16:1330-1335.
Demissew T (2010). Genetic gain in grain yield and associated traits of early and medium maturing varieties of soybean [*Glycine max* (L.) Merrill]. MSc. Thesis, Presented to the School of Graduate Studies of Haramaya University, Haamaya, Ethiopia.
Donmez E, Sears RG, Shroyer JP, Paulsen GM (2001). Genetic gain in yield attributes of winter wheat in the Great Plains. Crop Science 41:1412-1419.
Era H, Kamara AY, Asafo-Adjei B, Dashiell KE (2009). Improvement in grain and fodder yields of early-maturing promiscuous soybean varieties in the Guinea Savanna of Nigeria. Crop Science 49:2037-2042.
Evans LT, Fischer RA (1999). Yield Potential: Its Definition, Measurement, and Significance. Crop Science 39:1544-1551.
Fano D, Mekbib F, Assefa K, Jigjiga Uinversity (2016). Genetic Gain in Grain Yield Potential and Associated Traits of Tef [Eragrostistef (Zucc.)Trotter] in Ethiopia. Global Journal of Science Frontier Research 16(6).
Fikru M (2004). Genetic variability and inter- relationship of agronomic characters affecting seed yield in Desi type Chickpea (*Cicer arietinum* L.). M.Sc. Thesis Submitted to the School of Graduate Studies, Addis Ababa University.
Mekbib F (2006). Farmer and formal breeding of sorghum [*Sorghum bicolor* (L.) Moench] and the implications for integrated plant breeding. Euphytica 152:163-176.
Food and Agricultural Organization of the United (2011). FAO STAT Prod STAT, Production Crops. http://faostat.fao.org/site/567/default.aspx#ancor (accessed June 23, 2011)
Geremew G, Asfaw A, Taye T, Tesfaye T, Ketema B, Hilemichael H (2004). Development of sorghum varieties and hybrids for dryland areas of Ethiopia. Uganda Journal of Agricultural Science 9:594-605.
Hailu T, Kamara A, Asafo-Adjei B, Dashell K (2009). Improvement in grain and fodder yields of early-maturing promiscuous soybean genotypes in the guinea savanna of Nigeria. Crop Science 49:2037-2042.
Jin J, Liu X, Wang G, Mi L, Shen Z, Chen X, Herbert S (2010). Agronomic and physiological contributions to the yield improvement of soybean cultivars released from 1950 to 2006 in Northeast China. Field Crops Research 115:116-123.
Karmakar PG, Bhatnagar PS (1996). Genetic improvement of soybean varieties released in India from 1969 to 1993. Euphytica 90:95-103.
Kebere B, Ketema B, Prapa S (2006). Genetic gain in grain yield potential and associated agronomic traits in haricot bean (*Phaseolu vulgaris* L.). Kasetsart Journal (Natural Science) 40:835-847.
Ketema D (2007). Genetic variation for agronomic and root characters in Ethiopian lentil (*lens culinaris* Medikus) Landraces grown under moisture stress. M. Sc. Thesis. Haramaya University. Ethiopia.
Kusmenoglu I, Muehlbauer F (1998). Genetic variation for biomass and residue production in lentil (*Lens culinaris* Medik.). II. Factors determining seed and straw yield. Crop Science 38:911-915.
Majumder BM, Biswapati B, Prasanta K, Gangopadhyay A, Mani P, Kundu A, Mazumdar D (2008). Organic Amendments Influence Soil Organic Carbon Pools and Rice–Wheat Productivity. Soil Science Society of America Journal 72:775–785.
Melese D (2005). Morphological and random amplified polymorphic dna (rapd) marker variation analysis in some Ethiopian drought tolerant and susceptible chickpeA (*Cicer arientinum* L.) genotypes. An M.Sc. Thesis Submitted to the School of Graduate Studies, Addis Ababa University.
Mihret Y (2015). Genetic Gain in Lowland Sorghum [*Sorghum Bicolor* (L.) Moench] Varieties in Ethiopia. International Journal of Horticulture and Plant Breeding Sciences 2(1):1-13.
Pedersen JF, Kaeppler HF, Andrews DJ, Lee RD (1998). Sorghum. p. 344-354. In: Banga SS, SK Banga (eds.) Hybrid Cultivar Development. Narosa Publishing House, New Delhi, India.
SAS Institute (2004). SAS /STAT Guide for personal computers, version 9.0 editions. SAS Institute Inc. Cary, NC, USA.
Saleem MM, Tahir HN, Kabir R, Shahzad K (2002). Interrelationships and path analysis of yield attributes in chickpea (*Cicer arietinum* L.). International Journal of Agriculture and Biology 4(3):404-406.
Singh KB, Kumar J, Hawem MP, Btwf SS (1990). Diseases and pea resistance hrccding: Which way to go in thc ninctics? Pagcs 233-238 in Chickpea in the nineties: prnrccdings of the Second international Workshop on Chickpea Improvement, 4-8 Dec 1989, ICHJSAT Ccntcr, India (van Rheenen, H.A., and Saxcna, M.C:., eds.). Patanchcn~ 502 324, A.I'., India: Intcrnational Crops Rrscarch Institute for the Semi-Arid 'l'ropics.
Sinha SK, Aggrawal PK, Chaturvedi GS, Koundi KR, Khanna-Chopra R (1981). A comparison of physiological and yield characters in old and new wheat varieties. Journal of Agricultural Science (Camb.) 97:233-236.
Slafer GA (2003). Genetic basis of yield as viewed from a crop physiologist's perspective. Annals of Applied Biology 142:117-128.
Specht J, Williams J (1984). Contribution of genetic technology to soybean productivity - Retrospect and prospect. pp. 49-74. In: W.R. Fehr (ed.) Genetic Contributions to Yield Gains of Five Major Crop Plants. CSSA Special, Publication, 7. CSSA and ASA, Madison von Emil Strauss, Band 1, Bonn.
Tafese A (2011). Genetic gain in grain yield potential and associated traits of sesame (*Sesamum indicum* L.) in Ethiopia. M.Sc. Thesis Presented to the School of Graduate Studies of Haramaya University. Ethiopia. 87p.
Tamene T (2008). Genetic gain and morpho-agronomic basis of genetic improvement in grain yield potential achieved by faba bean breeding in Ethiopia. M.Sc. Thesis Presented to the School of Graduate Studies of Hawassa University, Hawassa, Ethiopia.
Temesgen A (2007). Genetic variability and association among seed yield and yield related traits in Kabuli chickpea (*Cicer arietinum* L.) genotypes. An M.Sc. Thesis Presented to the School of Graduate Studies of Haramaya University.
Tibebu B (2011). Genetic gain in grain yield and associated traits of Chickpea (*Cicer arietinum* L.) in Ethiopia. An M.Sc. Thesis Presented to the School of Graduate Studies of Haramaya University. Ethiopia. 121p.
Tigist D (2003). Genetic variability and associations of among yield and yield related characters in exotic lentil (*lens culinaris* medikus) lines. M.Sc. Thesis. Haramaya University. Ethiopia.
Wondimu F (2010). Assessment of genetic improvement in grain yield potential, malting quality and associated traits of barley (*Hordeum vulgare* L.) in Ethiopia. An M.Sc. Thesis Presented to the School of Graduate Studies of Haramaya University.
Yifru T, Hailu T (2005). Genetic improvement in grain yield potential and associated agronomic traits of teff (Eragrostistef). Euphytica 114:247-254.

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Diversity assessment of vanilla (*Vanilla* species) accessions in selected counties of Kenya using simple sequence repeats (SSRs) markers

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Diversity assessment of vanilla (*Vanilla* species) in Kenya is a key strategy for germplasm conservation and improvement. Production of vanilla crop in Kenya is limited due to inadequate knowledge on genetic diversity. This study was carried out to characterize 76 vanilla accessions from five counties of Kenya using 14 microsatellite DNA markers. POPGENE version 1.32 was used to compute variety factors. Amplicons ranged between 1 and 4. A total of 27 (96.43%) alleles were observed and their number ranged from 1.00 to 2.00 with a mean of 1.93. Effective allele values ranged from 1.00 to 1.99 with a mean of 1.63. Gene diversity ranged from 0 to 0.50 with a mean of 0.35, mean Shannon information index was 0.50 and Polymorphic information content values ranged from 0 to 0.38 with a mean of 0.35. Jaccard's similarity coefficient ranged from 0.08 to 1.00 with an average of 0.54. Unrooted phylogenetic tree was constructed in DARwin 6.0.8 using Unweighted Pair Group Method with Arithmetic Mean, clustering the samples into 3 main clusters (A 99.6%, B 98.96% and C 100%) and 6 sub-clusters (A1, A2, B1, B2, B3 and C1). Vanilla accessions grown in Kenya have a broad genetic background but low genetic diversity. Results inform the need to introduce other vanilla species as sources of genetic variation for breeding.

Key words: Genetic variation, DNA markers, breeding, Kenya.

INTRODUCTION

Vanilla (*Vanilla* species) belongs to the Orchidaceae family which comprises more than 25,000 species distributed in more than 800 genera (Govaerts et al., 2006). Vanilla is native to tropical forests of southeastern Mesoamerica (Hagsater et al., 2005). In the nineteenth century, vanilla species were introduced into other tropical countries like Asia and Africa from the original Mexican cultivated stock (Lubinsky and Risterucci, 2008). The crop was introduced in Kenya from neighbouring countries like Uganda. Vanilla is believed to have come from Madagascar and Reunion through Christian missionaries to Uganda (Mayawa, 2001). According to Bory et al. (2008), Madagascar and Reunion were the leading producers of vanilla

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since its introduction. In 2016, Madagascar, Reunion and Kenya produced 2,926, 2,304 and 15 tons year^{-1}, respectively (FAO*,* 2016).

Orchid species are grown for their flowers and vanilla is the only genus of the orchid family whose species produces a commercially important flavour (Stern and Judd, 1999). Stern and Judd (1999) studied the comparative vegetative anatomy and systematics of the species of vanilla and found that vegetative anatomical characters have some phylogenetic value. The basic chromosomal number of the vanilla genus is 16 (x=16), however, *Vanilla planifolia, Vanilla pompona* and *Vanilla tahitensis* are diploid with 2n=32 (Hurelpy, 1938; Purseglove et al., 1981).

Vanilla flavour is the second most expensive spice traded in the world market after saffron (Minoo et al., 2008). According to Oyugi (2018), vanilla crop has a huge monetary value. The crop can fetch a farmer as much as KES. 16,000 or more per vine if managed well (Oyugi, 2018). The high monetary value is on account of crop's many uses that include flavouring food, drinks, soaps, ointments, perfumes and cosmetics. A vine of vanilla can bear up to 80 beans. One vanilla bean measuring 10 to 12 cm long can fetch KES. 200 and a hectare can accommodate over 200 vines (Oyugi, 2018). Vanilla venture can act as a source of income to farmers and earn the country foreign exchange.

According to Shabbir (2018), vanilla crop is well grown in other countries such as Uganda, Comoros and Madagascar in both small and large scale. Most parts of Kenya have favourable climatic conditions for cultivating vanilla crop and there is both local and export markets ready upon its maturity. The major markets for vanilla beans include European countries and United States (Shabbir, 2018).

V. planifolia Andrews (syn. *V. fragrans*), *V. tahitensis* and *V. pompona* Scheide are commercially cultivated species for the production of natural vanilla flavour (Rao and Ravishankar, 2000). Natural vanilla flavour is made up of a large number of aromatic compounds with sweet fragrances (Sharp et al., 2012). Natural vanilla flavour is obtained as an extract from cured vanilla beans and is universally used as aromatic flavouring in food, beverages, pharmaceutical and cosmetic industries (Verpoorte and Korthou, 2007; Kaur and Chakraborty, 2013). Vanillin flavour is found to have antimutagenic, anticarcinogenic and antimicrobial properties. The antimutagenic property has ability to reduce chromosomal damage caused by X-ray and ultraviolet (UV) light (Keshava et al., 1998). Anticarcinogenic property have effects in a family of DNA-PK inhibitors (Duran and Karran, 2003) while antimicrobial property acts against the yeasts (Fitzgerald et al., 2003).

Limited knowledge on genetic diversity is the major bottleneck in wider breeding programmes, hybridization and dissemination of vanilla crop in Kenya because of unavailability of quality vanilla planting materials despite the fact that most parts of Kenya have favourable climatic conditions for cultivating vanilla crop (Shabbir, 2018). In most cases, when vanilla plant vines are transported from one agro-ecological zone to another they end up drying. A few vanilla vines may adapt to the new environment. According to Oyugi (2018), vanilla is one of the orphaned crops in Kenya and no information has been documented on available vanilla accessions found in Kenya. Studies of genetic variation in crop species is an important tool for germplasm conservation and crop improvement (Mason et al., 2015). It was necessary to assess the extent of diversity in vanilla accessions found in Kenya. The information will form a base for future vanilla crop improvement programmes. The objective of the present study was to determine the genetic diversity among vanilla accessions from selected counties of Kenya using simple sequence repeat markers.

MATERIALS AND METHODS

Experimental site

A total of 76 vanilla accessions were collected *in-situ* from five different agro-ecological zones in Kenya; Bungoma county (Latitude 0° 45' 16.799" N and Longitude 34° 30'09.270"E), Busia county (Latitude 0° 47' 11.113" N and Longitude 34° 24' 26.339"E), Kwale county (Latitude 4° 09' 58.223" S and Longitude 39° 34' 18.127"E), Kilifi county (Latitude 4°16' 53" S and Longitude 39° 44' 41.306"E) and Mombasa county (Latitude 4° 02' 12.7608"S and Longitude 39° 40'10.4556"E) (Figure 1). Genotyping was done in the Marker Assisted Breeding Laboratory at KALRO-FCRI, Njoro in Nakuru county.

Plant accessions collection

Purposive network sampling method was done *in-situ* due to the fewer number of vanilla farmers in Kenya. Global Positioning System (GPS) data was taken in each county of the study. Young vanilla leaves were sampled based on morphological distinction of the crops and stored in labelled falcon tubes containing silica gel (Xtrack) for DNA extraction. Plants with similar features growing in ecologically distinct county were assumed to be different; such plants were sampled and used in the analysis. All plant materials sampled were coded with slight modifications as previously described by Oyoo et al. (2015).

Coding modifications according to the description of Oyoo et al. (2015), involved reflection of the county and the number of accessions collected. Example; 'BGM 1, BGM 2, BGM 3…...BGM 8'.

Total nucleic acid extraction

Total DNA was extracted from dried young plant leaf samples using CTAB protocol according to Ibrahim (2011), with slight modifications. Modifications to Ibrahim (2011), involved introduction of a preliminary cleaning stage to remove phytates, increased centrifugation time for initial stages, this ensured that cell debris and the proteins were well decanted and hence minimized contamination. Final centrifugation time was reduced in final precipitation and washing stages to avoid pelleting of carryover impurities. Also, precipitation time was increased from

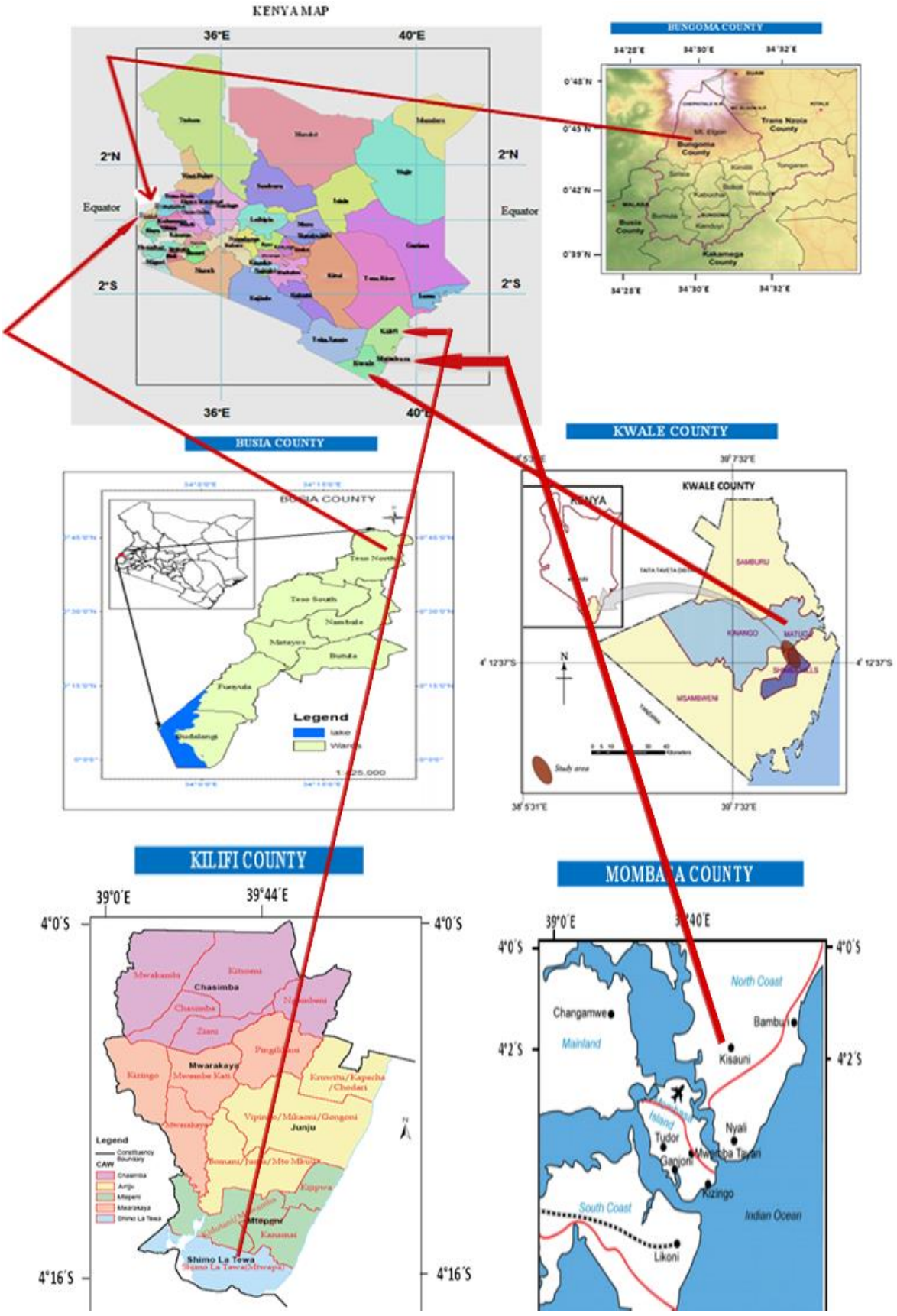

Figure 1. Map of the experimental site.

the reported 3 min to 18 h of Ibrahim (2011). This allowed ample time for DNA to precipitate and increased the concentration of DNA recovered.

DNA Quantification

The purity and concentration of DNA samples were determined using Nanodrop spectrophotometry and Agarose gel electrophoresis. DNA samples were run in a 1% agarose gel containing Ethidium bromide staining dye at voltage of 100 V and a current of 400 mA for 30 min and visualized on a UV Trans illuminator.

Selection of SSR markers and PCR genotyping

A total of 14 sets of SSR markers (Table 1) selected from earlier publications according to Bory et al. (2008), were amplified using PCR process. The SSR markers were selected based on coverage and distribution in all the linkage groups, high PIC values (>0.6) and maximum number of alleles detected. The primers for SSR were synthesized by Inqaba Biotech, South Africa.

Polymerase chain reaction (PCR) was done in a 96 universal gradient, 2720 thermal cycler (Applied Biosystems) in 20 µl final volume containing DNA template (20 ng) 2.0 µl, primer forward (10 µmol/L) 0.5 µl, primer reverse (10 µmol/L) 0.5 µl, 10x PCR buffer 2.0 µl, 10 mM dNTPs (2.5 mM each) 1.6 µl, $MgCl_2$ (2.5 mM) 1.2 µl, Taq DNA polymerase (HiMedia) (5 U/µl) 0.25 U (0.05 µl) and dd H_2O 12.15 µl. The amplification conditions for PCR profile were initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 30 s, specific annealing temperature for each SSR primer for 1 min, extension at 72°C for 2 min and final extension at 72°C for 10 min and infinite time at 4°C for storage. The PCR amplicons were mixed with 5 µl bromophenol blue DNA loading dye and run in a 2% agarose gel containing 3 µl ethidium bromide staining dye in a 1x Sodium Borate (SB) buffer at voltage of 80 V and a current of 400 mA for 1 h and visualized in a UV trans illuminator.

Scoring of the markers

Scoring of marker alleles of SSRs was done manually from the gel images. A simple numerical scoring method was used where 1 was used to represent presence of the expected band while 0 was used to represent absence of the band.

Phylogenetic analysis

The utility of markers was quantified in terms of number of amplicons per primer, percent polymorphism, polymorphic information content (PIC). [The PIC values of individual primers were calculated based on the formula PIC = 2 × F (1- F), where F is the frequency of the bands].

Genetic variation at each locus was characterized in terms of the number of alleles, observed heterozygosity (HO), expected heterozygosity (HE), Shannon's diversity index (I), gene flow (Nm), and gene differentiation coefficient (Gst) using the genetic analysis packages POPGENE Version 1.32 (Yeh, 2000). Gene diversity (GD) and polymorphic information content (PIC) were measured by calculating the shared allele frequencies (Weir, 1996) using PowerMarker 3.25 (Liu and Muse, 2005).

Nei's gene diversity (Nei, 1973) for the whole sample (Ht) was calculated to estimate the genetic diversity across the counties while genetic diversity within the populations (Hs) was used to determine diversity within counties. The proportion of genetic diversity among populations (Gst =1-(HS/Ht)) was also calculated for each primer combination and for all the primers. These estimates were partitioned into respective county population groups using the POPGENE software package Version 1.32.

Phylogenetic analysis was carried out to estimate evolutionary relationships among the vanilla accessions. All phylogenetic analyses were done in DARwin 6.0.8 using binary data from the gel image marker scores. Single data dissimilarity was calculated, and factorial coordinates calculated from the resulting dissimilarity data used to determine segregation of individual samples. Unrooted phylogenetic tree was constructed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) agglomerative hierarchical clustering method (Sokal and Michener, 1958) from distance matrices based on genetic distances.

RESULTS AND DISCUSSION

Quality and quantity of DNA

The quality and quantity of the isolated DNA was good for PCR. Sample DNA concentrations ranged from 62.5 to 3421.7 ng/µl while sample purity was between 1.71 and 2.09. All the primers produced expected product sizes. Some samples showed no bands (indicating absence of the target loci), one band (scored as homozygous at the amplified loci) while others are multiple bands (scored as heterozygous at the amplified loci). Representative gel images are as shown in Figure 2.

Polymorphism of the markers

Fourteen SSR primers used in the study resulted in amplified fragments which varied in size from 100 to 500 bp (Figure 2). The utility of markers was quantified in terms of observed number of alleles, effective alleles, gene diversity and Shannon's Information index (Kimura and Crow, 1964). Number of amplicons per primers ranged from 1 to 4 with only one marker giving one allele (mVplCIR031). A total of effective alleles were 22.76 and the number of alleles per primer ranged from 1.00 to 1.99 with a mean of 1.63. Observed number of alleles was 27 while number per primer ranged from 1 to 2 with a mean of 1.93. Based on the results, the effective number of alleles was lower than the observed number of alleles (Table 2) showing strong geographic differentiation in the population informing that the observed genetic diversity was mainly due to geography than the original evolution.

Gene diversity indices ranged from 0 to 0.50 while mean Shannon information index was 0.5 which suggested that the vanilla accessions studied had less genetic diversity. This finding shows that vanilla generally has a narrow genetic background as supported by the findings in India (Sreedhar et al., 2007), Mexico (Besse et al., 2004; Soto, 1996), and Reunion Island

Table 1. List of primer sequences of 14 microsatellite loci in the vanilla orchid (F- forward primer and R - reverse primer)

SSR locus	Primer sequences	Clone size (bp)	Annealing T (°C)
mVplCIR002	F-TGGATGTGCATTTGTG R-CGCATTCATTCACTTGT	222	53 - 60
mVplCIR003	F-TATAGATGCACACGAGC R- TCACATCCCTACATGC	349	53 - 60
mVplCIR005	F-TTTGCTTGAACGTATGTC R- GCAAACATAGAAATGCAC	259	53 - 60
mVplCIR010	F-GCACATAAATACCTTACACC R- GTTCACGTCAGTGTGCT	346	53 - 60
mVplCIR015	F-AGTGTCTTTGTGTGCCT R-TAGATAGTAAACCCATACTCAC	280	53 - 60
mVplCIR016	F- TATGTGTGAGAGGGTGC R- CAATTAGTCACATCCATAAAC	320	53 - 60
mVplCIR019	F- AAGTGCCCAATCTATC R-TGGATTCACCATGAC	222	53 - 60
mVplCIR022	F-CAAAACACAAGGAAATGC R-TGCAAGCCCACAAGT	197	53 - 60
mVplCIR025	F-GTGTAGCGGTTCATACAA R-CATTCATGGAAGTGGAG	231	53 - 60
mVplCIR026	F-GCACATACATGCTTATTG R-CATGTTCTTATTTGAGTGG	223	53 - 60
mVplCIR028	F-AACATGCACAAGAAAG R-TTTATGCACCTTGTTAG	190	53 - 60
mVplCIR031	F--ATTTCCTCCCTCACTGTA R-AATCTCAGGTGCTATTGG	346	53 - 60
mVplCIR047	F-CATGCTTACATCTTTGTGTT R-TAATGGACATGCACACTC	301	53 - 60
mVplCIR050	F-CTATGTGCGCTTTGG R-CACTCAAGAACATGCAAC	224	53 - 60

Bory *et al*. (2008).

(Cibrian, 1999) who worked in different regions with different technologies and found the results within the same range. Polymorphic information content of the markers ranged from 0 (mVplCIR031) to 0.375 (mVplCIR025). The primer with 0 PIC value was monomorphic showing that it lacked the ability to differentiate between the accessions while the primer with the highest PIC had the highest resolution. This is an indication that the markers used revealed little polymorphisms between the accessions.

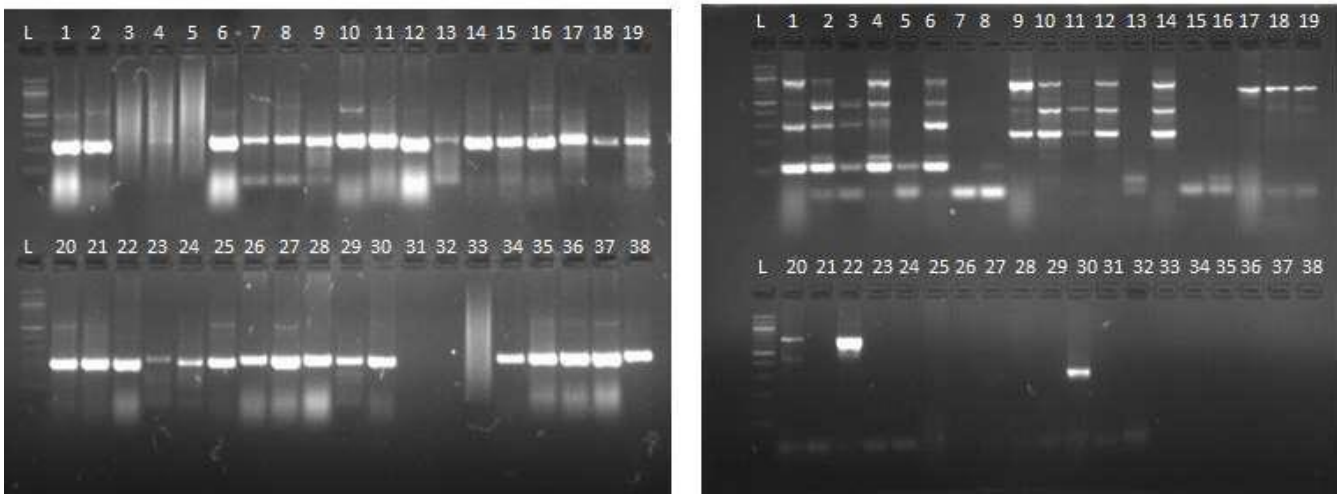

Figure 2. Representative gel images of L- Ladder (100bp), single and multiple bands of primers (*mVplCIR010*, *mVplCIR025* respective), (1 - 8 Bungoma), (9 - 25 Busia), (26 - 38 Mombasa) test samples.

Table 2. Summary statistics of genetic diversity indices of vanilla accessions studied in five counties in Kenya.

Locus	Sample size	na*	ne*	h*	I*	PIC	m
mVplCIR002	76	2.000	1.877	0.467	0.660	0.364	0.605
mVplCIR003	76	2.000	1.084	0.077	0.169	0.135	0.921
mVplCIR005	76	2.000	1.980	0.495	0.688	0.333	0.697
mVplCIR010	76	2.000	1.685	0.406	0.596	0.375	0.513
mVplCIR015	76	2.000	1.857	0.462	0.654	0.366	0.592
mVplCIR016	76	2.000	1.400	0.286	0.460	0.339	0.684
mVplCIR019	76	2.000	1.929	0.482	0.675	0.353	0.645
mVplCIR022	76	2.000	1.944	0.486	0.679	0.349	0.658
mVplCIR025	76	2.000	1.707	0.414	0.605	0.375	0.500
mVplCIR026	76	2.000	1.442	0.306	0.485	0.349	0.658
mVplCIR028	76	2.000	1.995	0.499	0.692	0.320	0.724
mVplCIR031	76	1.000	1.000	0.000	0.000	0.000	1.000
mVplCIR047	76	2.000	1.178	0.151	0.284	0.231	0.842
mVplCIR050	76	2.000	1.685	0.406	0.596	0.375	0.513
Mean	76	1.929	1.626	0.353	0.517	0.350	0.638

*na = Observed number of alleles, *ne = Effective number of alleles (Kimura and Crow, 1964), *h = gene diversity (Nei's, 1973), *I = Shannon's Information index (Lewontin, 1972), PIC = Polymorphic Information content, m = Major allele frequency.

A total of 27 (96.43%) alleles were identified and out of the 14 markers used in this study, 13 markers were polymorphic while one (mVplCIR031) monomorphic. This indicated that alleles with high frequencies contributed more to the effective number of alleles in this study. This frequency is higher than that reported by Gigant (2011), who observed 57.83% alleles using 19 similar primers in Comoros Island. This may be due to the large sampling area used in the present study and that perhaps the study used more effective sample collection strategies. Besse et al. (2004) identified 76.3% alleles using RAPD markers in Mexico. This shows that SSR markers have better resolution of distinguishing vanilla accessions than RAPDs. However, the polymorphic alleles identified in this study are significantly lower compared to the findings of Hu et al. (2019), who used SNP markers. SNP markers are generally more effective than SSR markers. mVplCIR031 was the most frequently observed allele 1.0 while mVplCIR025 was the least frequently observed marker because it had 0.5 chance of being observed as shown in Table 2. This shows that marker mVplCIR031 showed the least variation among the accessions studied while marker mVplCIR025 was the most variable.

Table 3. Nei's gene diversity indices for subdividing the populations.

Locus	Ht	Hs	Gst	Nm*
mVplCIR002	0.477	0.015	0.969	0.016
mVplCIR003	0.105	0.087	0.164	2.547
mVplCIR005	0.470	0.042	0.912	0.049
mVplCIR010	0.424	0.100	0.765	0.154
mVplCIR015	0.491	0.187	0.620	0.307
mVplCIR016	0.303	0.059	0.806	0.121
mVplCIR019	0.480	0.189	0.607	0.324
mVplCIR022	0.500	0.147	0.705	0.209
mVplCIR025	0.440	0.200	0.545	0.417
mVplCIR026	0.361	0.089	0.753	0.164
mVplCIR028	0.461	0.087	0.810	0.117
mVplCIR031	0.000	0.000	***	***
mVplCIR047	0.177	0.140	0.209	1.887
mVplCIR050	0.436	0.156	0.642	0.279
Mean	0.366	0.107	0.708	0.207
St. Dev	0.026	0.004	-	-

Ht=whole sample, Hs=within populations, Gst=degree of differentiation among populations, Nm* = estimate of gene flow from Gst.

Gene diversity

The average estimated haplotype diversity in the entire population (Ht= 0.37) was higher than the weighted average of estimated haplotype diversities in the subpopulations (Hs=0.11) and the differentiation among populations was high (Gst=0.71). The highest Ht was observed at locus mVplCIR022 (Ht=0.50) indicating that this was the most polymorphic and most informative locus within the population. The lowest haplotype diversity in the population was observed at locus mVplCIR031 (Ht=0.00) showing that this locus was monomorphic, and no diversity information could be deduced from it. All the other loci showed varied levels of genetic diversity ranging from 0.11 to 0.5 (Table 3).

There was low genetic diversity within subpopulations (Hs), and it ranged from 0.00 to 0.19. The highest diversity within subpopulations was at locus mVplCIR019 (Hs = 0.19) indicating that this locus had the highest resolution in segregating vanilla accessions from different counties and lowest at locus mVplCIR031 (Hs= 0.00) showing that this locus was not able to differentiate vanilla accessions based on the counties of origin (Table 3).

In the present study, the mean differentiation among populations was high (Gst =0.71). The highest Gst was recorded at locus mVplCIR002 (Gst = 0.97) while no Gst was calculated for locus mVplCIR031 because the primer was monomorphic and therefore had zero Hs and Ht values. Mean estimated gene flow was high for this study (Nm = 0.21). The highest Nm was recorded at locus mVplCIR003 (Nm = 2.56) while no gene flow was estimates at locus mVplCIR031 because of nil Gst value (Table 3).

In autogamous plant species, efficient gene dispersion is ensured by seeds while pollen is the source of gene diversity in allogamous species (Nybom, 2004). As such, populations of autogamous species are more strongly differentiated, but less variable than populations from allogamous species which are less differentiated but are more variable (Nybom, 2004). In our study, the calculated GST value in Kenyan *Vanilla* spp. (0.71) was higher than that reported by Gigant (2011) in Guadalupe for autogamous *Vanilla* spp. such as *Vanilla humblotii* (Gst = 0.520), *Vanilla barbellata* (Gst = 0.558) and *Vanilla claviculata* (GST = 0.623). The strong differentiation but less variability is characteristic of self-fertilized plants as is often in vanilla which exhibit genetic variability among populations rather than within, with high GST and low Hs (Nybom, 2004). Results presented herein, therefore confirmed autogamy as the major mating system in *Vanilla* spp. in Kenya. The high estimated gene flow (Nm = 0.21) observed in this study can therefore be explained to be as a result of inbreeding. This was demonstrated by Campagne et al. (2012) in *Vanilla mexicana* populations using reproductive biology experiments. This shows that vanilla accessions in Kenya have a small genetic diversity due to constant inbreeding and informs the need to introduce new cultivars to as sources of genetic variation for breeding and hybridization purposes.

Genetic of vanilla between distance counties

The highest genetic distance and lowest genetic

Table 4. Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

County	Bungoma	Busia	Mombasa	Kwale	Kilifi
Bungoma	***	0.937	0.501	0.464	0.775
Busia	0.065	***	0.510	0.576	0.712
Mombasa	0.691	0.673	***	0.751	0.673
Kwale	0.769	0.552	0.286	***	0.459
Kilifi	0.254	0.340	0.397	0.780	***

Table 5. Analysis of molecular variance (AMOVA) for the 76 vanilla accessions from 5 select counties of Kenya.

Source	Df	SS	MS	F	>F
Among groups	4	17.196	4.299	307.07	0.000
Within groups	71	0.962	0.014	-	-
Total	75	18.158	-	-	-

SS = sum of squares, MS = expected mean squares, F = F-statistics, >F = Significance level.

similarity were recorded between Kwale and Kilifi counties (0.78, 0.46) while the lowest genetic distance and highest genetic similarity were between Busia and Bungoma (0.07, 0.94). Vanilla accessions in Busia and Bungoma showed 94% identity showing that they are very close genetically and could have shared a common source since the counties border each other closely while accessions collected from Kilifi showed the least similarity to those from Kwale at 46% identity (Table 4).

Analysis of molecular variance (AMOVA) was used to partition among and within the genetic variation of the vanilla accessions. A total of 94.714% of the variation was observed among the accessions that were produced within the counties while the variation revealed within populations was 5.29% (Table 5) indicating that *Vanilla* spp. grown in Kenya has very little diversity and some variations observed may be due to microclimatic conditions.

Factorial analysis

Factor analysis was used to describe variability among the vanilla accessions and group them into clusters. The accessions were segregated into three distinct groups A, B and C according to their county of origin (Bungoma and Busia 'A', Kwale and Mombasa 'B' and Kilifi 'C'). Accessions from Busia and Bungoma counties were aggregated together (A) into indistinct group showing that vanilla species in these areas are closely genetically related. Cluster B was the most inclusive containing accessions from Mombasa and Kwale indicating that vanilla accessions in these counties have similar genetic makeups perhaps due to the same original sources. However, some accessions from Busia county were closely related to accessions from Mombasa and Kwale, moreover, they were found at the intersection of the two clusters A and B. This may be attributed to the fact that the accessions may have been transported by farmers or researchers to these counties. Accessions from Kilifi county segregated on their own showing that they were genetically distinct from other counties as shown in Figure 3.

Phylogenetic trees

The primers used clustered the samples into 3 main clusters (A, B and C) according to the county of origin and 6 sub-clusters (A1, A2, B1, B2, B3 and C1) as shown in Figure 4. Most accessions within a sub-cluster were probably duplicates, meaning they were genetically identical. The genetic closeness of vanilla accessions may result from asexual reproduction which is the most common mode of propagation and geographical locations closeness. This observation is supported by Divakaran (2006), who observed that clonal propagation of vanilla limits the genetic variability in the crop especially in countries where the crop was introduced. Low genetic diversity may also occur because of successful establishment of a few well adapted genotypes which are rapidly propagated despite multiple introductions (Li et al., 2006).

Cluster A comprised accessions from Bungoma (A1) and Busia (A2) counties. These were the accessions with the least diversity with average of 99.6% similarity index. Vanilla accessions from Mombasa, Kwale and a few from Busia (B1, B2 and B3) clustered together in cluster B and showed some level of diversity among the accessions ranging from 98.96 to 100% while vanilla accessions from Kilifi clustered on its own as C with 100% similarity index. The Jaccard's similarity

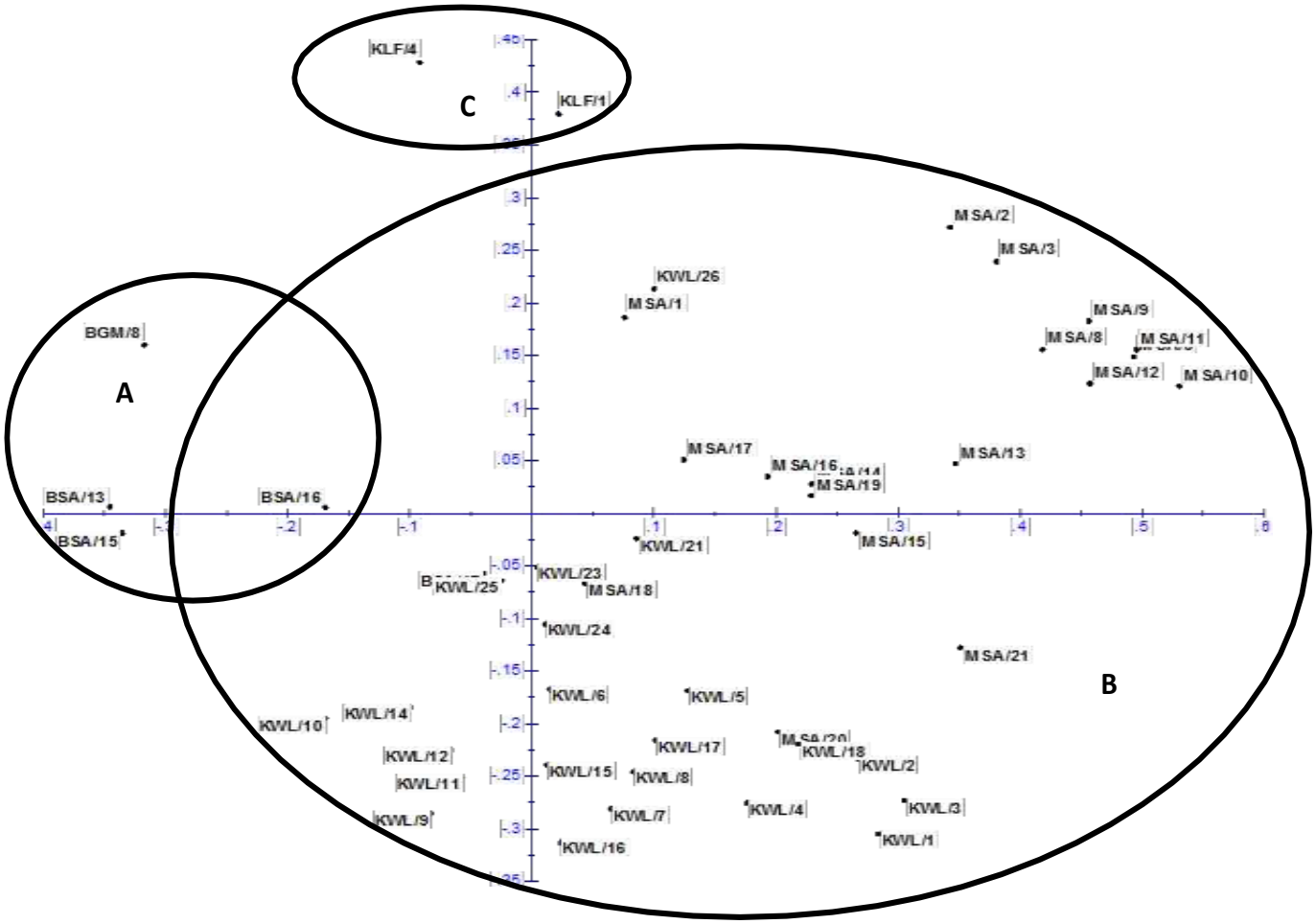

Figure 3. Factorial plot showing variability among the vanilla accessions, BGM-Bungoma, BSA-Busia, MSA-Mombasa, KWL-Kwale, KLF-Kilifi.

coefficient ranged from 0.083 to 1.00 with an average of 0.54 among all the 76 accessions used. The genetic relationship among the accessions was presented in the dendrogram which was constructed from the gel marker.

CONCLUSION AND RECOMMENDATION

Little information has been documented on vanilla crops in Kenya. This is the first study that used microsatellites to characterize vanilla species grown in Kenya. The study established that germplasm grown in the five counties of Kenya have low genetic diversity. The SSR analysis used in this study allowed delineation of vanilla accessions and provided a good detail of variations among the vanilla accessions indicating that SSR markers are suitable for characterizing germplasm and studying population genetics of vanilla species in Kenya.

Molecular characterization of vanilla species from select counties of Kenya showed that autogamy was the main system of reproduction. This is an important step for vanilla breeding programmes in Kenya. The study also lays the basis for use of more advanced molecular platforms such as genome wide sequencing in order to establish more diversity in cultivated vanilla accessions in Kenya.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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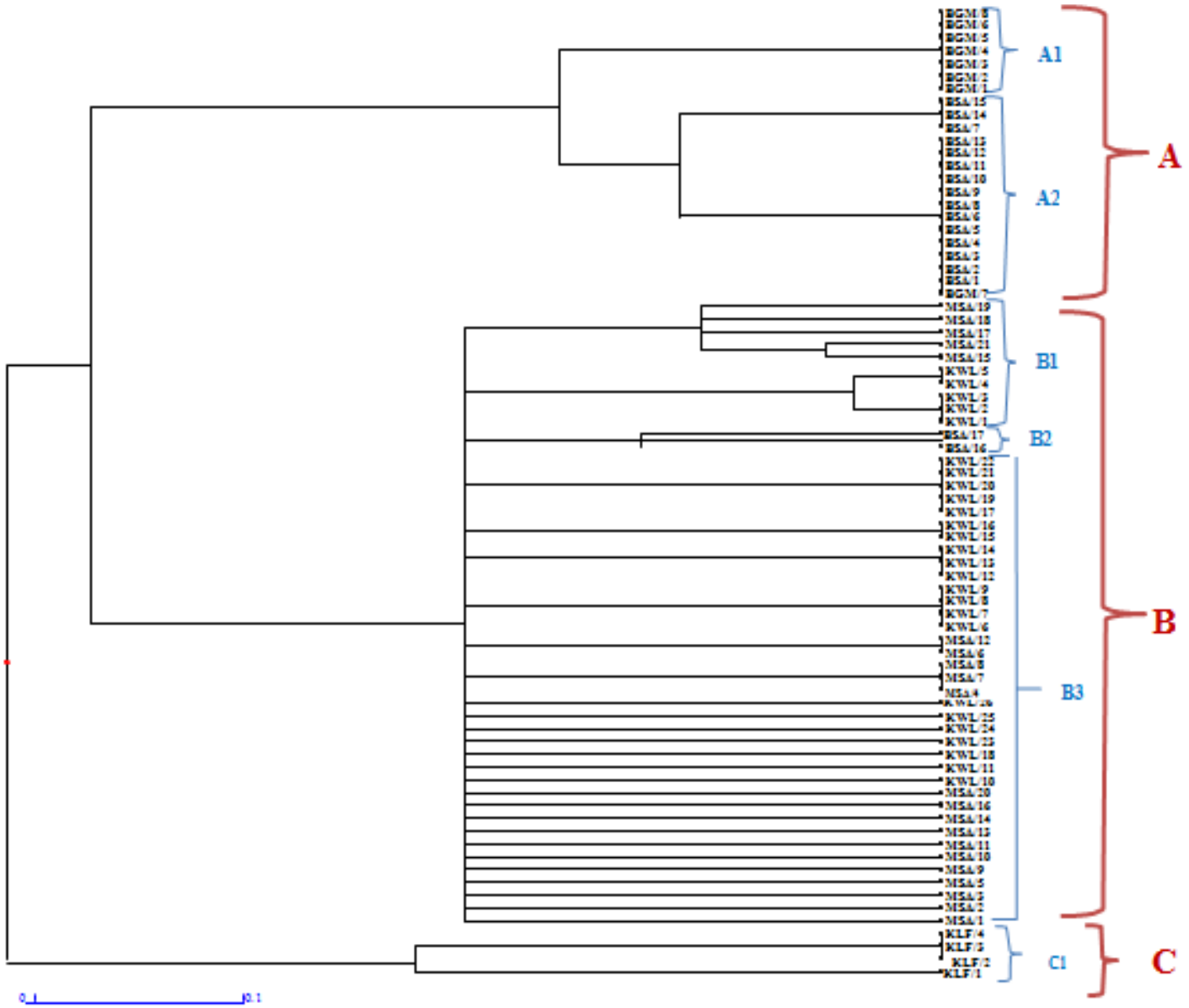

Figure 4. A dendrogram showing relationship among the vanilla accessions. BGM-Bungoma, BSA-Busia, MSA- Mombasa, KWL-Kwale, KLF-Kilifi

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REFERENCES

Besse P, DaSilva D, Bory S, Grisoni M, Lebellec F, Duval MF (2004). RAPD genetic diversity in cultivated vanilla: *Vanilla planifolia* and relationships with *Vanilla tahitensis* and *Vanilla pompona*. Plant Science 167(2):379-385.

Bory S, Lubinsky P, Risterucci AM (2008). Patterns of introduction and diversification of *Vanilla planifolia* (Orchidaceae) in Reunion Island (Indian Ocean). American Journal of Botany 95:805-815.

Campagne P, Smouse PE, Varouchas G, Silvain JF, Leru B (2012). Comparing the vanOosterhout and Chybicki Burczyk methods of estimating null allele frequencies for inbred populations. Molecular Ecology Resources 12:975-982

Cibrian JA (1999). Variation genetic of *Vanilla planifolia* in Mexico. Ph.D. Thesis, UNAM, Mexico City P 105.

Divakarana MNB (2006). Conservation of Vanilla species, *invitro*. Scientia Horticulturae pp. 175-180.

Duran S, Karran P (2003). Vanillins: A novel family of DNA-PK inhibitors. Nucleic Acids Research pp. 5501-5512.

Fitzgerald DJ, Stratford M, Narbad A (2003). Analysis of the inhibition of food spoilage yeasts by vanillin. International Journal of Food Microbiology 86(1-2):113-122.

Food and Agriculture Organization (FAO) (2016). Production data 2016. Retrieved on 19th January, 2019 from fao.org: http://www.fao.org.

Gigant AB (2011). Nineteen polymorphic microsatellite markers from

two African Vanilla species: across-species transferability and diversity in a wild population of *V. humblotii* from Mayotte. Conservation Genetics Resources pp. 121-125.
Govaerts R, CampacciM, Holland A, Baptista D (2006). World Checklist of Orchidaceae. Retrieved on 20th December, 2017 from http://www.kew.org/wcsp/ monocots/.
Hagsater E, Soto-Arenas MA, Salazar-Chavez GA, Jimenez-Machorro R, Lo´pez-Rosas MA, Dressler RL (2005). Las orqu ıdeas de Mexico. *Orqu Ideas Ygente*. Instituto Chinoin, Mexico pp. 38-71.
Hurel-Py G (1938). Etude des noyauxvégétatifs de *Vanilla planifolia*. Revue de Cytologieet de Cytophysiology Végétables 3:129-133.
Hu Y, Resende MFR, Bombarely A (2019). Genomics-based diversity analysisof Vanilla species using a *Vanilla planifolia* draft genome and Genotyping-By- Sequencing. Scientific Reports 9:3416.
Ibrahim R (2011). A modified CTAB protocol for DNA extraction from young flower petals of some medicinal plant species. Geneconserve 10(40):165-182.
Kaur B, Chakraborty D (2013). Biotechnological and molecular approaches for vanillin production: a review. Applied Biochemistry and Biotechnology 169:1353-1372.
Keshava C, Keshava N, Ong TM, Nath J (1998). Protective effect of vanillin on radiation-induced micronuclei and chromosomal aberrations in V79 cells. Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis 397(2):149-159.
Kimura M, Crow JF (1964). The number of alleles that can be maintained in a finite population. Genetics 49:725-738.
Liu K, Muse S (2005). PowerMarker: an integrated analysis environment for genetic marker analysis. Bioinformatics 21(9):2128-2129.
Li WG, Wang BR, Wang JB (2006). Lack of genetic variation of an invasive clonal plant Eichhornia crassipes in China as revealed by RAPD and ISSR markers. Aquatic Botany 84:176-180.
Lubinsky P, Risterucci AM (2008). Patterns of introduction and diversification of *Vanilla planifolia* (Orchidaceae) in Reunion Island (Indian Ocean). American Journal of Botany 95:805-815.
Mason AS, Zhang J, Tollenaere R, Teuber PV, Dalton-Morgan J, Hu L, Yan G, Edwards D, Redden R, Batley J (2015). High-throughput genotyping for species identification and diversity assessment in germplasm collections. Molecular Ecology Resources 15(5):1091-1101.
Mayawa (2001). Kilimo Bora cha Vanilla Mkoani Kagera. 4th Edition. pp. 233-240.
Minoo D, Jayakumar V, Veena S, Vimala J, Basha A, Saji KV, Peter KV (2008). Genetic variations and interrelationships in *Vanilla planifolia* and few related species as expressed by RAPD polymorphism. Genetic Resources and Crop Evolution 55(3):459-470.
Nei M (1973). Analysis of Gene Diversity in Subdivided Populations. PNAS pp. 3321-3323.
Nybom H (2004). Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Molecular Ecology 13:1143-1155.
Oyoo ME, Muhammed N, Githiri SM, Ojwang PO, Muniu FK, Masha E, Owuoche J (2015). *Insitu* morphological characterization of coconut in the coastal lowlands of Kenya. African Journal of Crop Science 9(2):65-74.
Oyugi Z (2018). Vanilla Farming Profit Booster. Retrieved on 14th August, 2018 from http:/www.farmbizafrica.com/profit booster/.
Purseglove JW, Brown CL, Robbins SRJ (1981). Spices. Longman, London pp. 644-735.
Rao RS, Ravishankar GA (2000). Vanilla flavour: production by conventional and biotechnological routes. Journal of the Science of Food and Agriculture 80(3):289-304.
Shabbir B (2018). World Vanilla Production. Retrieved on 14th August, 2018 from http:/www.farmbizafrica.com/profit booster/.
Sharp MD, Kocaoglu-Vurma NA, Langford V, Rodriguez-Saona LE, Harper JW (2012). Rapid discrimination and characterization of vanilla bean extracts by attenuated total reflection infrared spectroscopy and selected ion flow tube mass spectrometry. Food science 77:284-292.
Sokal R, Michener C (1958). A statistical method for evaluating systematic relationships. University of Kansas Science Bulletin 38:1409-1438.
Soto MA (1996). A monograph of Vanilla (Orchidaceae). PhD Thesis, UNAM University, Mexico City P 110.
Sreedhar RV, Venkatachalam L, Roohie K, Bhagyalakshmi N (2007). Molecular analyses of Vanilla planifolia cultivated in India using RAPD and ISSR markers. Orchid Science and Biotechnology 1(1):29-33.
Stern WL, Judd WS (1999). Comparative vegetative anatomy and systematic of Vanilla. Botanical Journal of the Linnean Society 131(4):353.
Verpoorte R, Korthou H (2007). Vanilla. In Berger R G, (Ed.) Flavours and Fragrances. Springer, Berlin pp. 203-217.
Yeh FC (2000). Population genetics. In: Young A, Boshier D, Boyle T (eds) Forest conservation genetics, principles and practice. CABI Publishing, Wallingford pp. 21-37.
Weir B (1996). Genetic Data Analysis II: Methods for Discrete Population Genetic Data. Sinauer Assoc., Inc.: Sunderland, MA, USA pp. 175-200.

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DNA isolation and optimization of PCR protocol for ISSR analysis of *Girardinia diversifolia*: A medicinal and economic plant species from Nepal Himalaya

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***Girardinia diversifolia* (Link) Friis is a perennial herb commonly known as Himalayan giant nettle which belongs to the family Urticaceae. The plant has cultural, medicinal and economic importance. The plant contains high concentration of polysaccharides, polyphenols and secondary metabolites which obstructs the process of isolation of the Deoxyribonucleic Acid (DNA) and inhibits downstream Polymerase Chain Reaction (PCR) amplifications. This study protocol developed a DNA extraction protocol from leaf-tissue based on the Cetyltrimethylammonium bromide, and optimized the PCR protocol for Inter Simple Sequence Repeat (ISSR) analysis. Genomic DNA extraction process was conducted using modified Doyle and Doyle method to obtain good quality DNA. The method yielded 445 ng/µL of DNA, where the purity ranged from 1.8-2.0 indicating minimum contamination of metabolites. The optimum condition for ISSR analysis was established using 4 mM $MgCl_2$, 0.6 mM dNTPs, 2.0 U *Taq* polymerase, 50 ng template DNA, and 0.7 µM primer. PCR program was optimized in the sequence of denaturation at 94°C for 3 min, subsequently followed by 45 cycles at 94°C for 30 s, annealing temperature at 45°C for 30 s, extension at 72°C for 2 min, and final extension at 72°C for 10 min. The modified technique was found to be ideal for isolation of genomic DNA and optimization of PCR process for ISSR analysis of *G. diversifolia*. The results of the research are beneficial for future molecular characterization and genetic diversity analysis of allied taxa.**

Key words: *Girardinia diversifolia*, Himalayan nettle, DNA isolation.

INTRODUCTION

*Girardinia div*ersifolia (Link) Friis is commonly known as Himalayan giant nettle and locally known as 'Allo' in Nepal. The plant belongs to family Urticaceae which contains approximately 54 genera and has more than 2000 species with high concentration of genera and species in tropical Asia (Wu et al., 2013). This plant is widely distributed in the subtropical and temperate Himalayas (Polunin and Stainton, 1984) and its habitat is found between the altitudes of 1,200 to 3,000 metres above sea level (Friis, 1981; Shrestha, 1997; Singh and

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Shrestha, 1988). Molecular genetic markers reflect the variation at the level of DNA (Anne, 2006). Inter Simple Sequence Repeat (ISSR)-PCR is a technique, which utilizes microsatellite sequence in polymerase chain reaction to generate multilocus markers (Reddy et al., 2002). This technique with great reproducibility has been widely used in classification and systematic comparison of species (Amom et al., 2018; Nilkanta et al., 2017), evolutionary relationship of species and identification of genetic varieties (Mohamad et al., 2017).

Himalayan giant nettle has great cultural, economic and medicinal significance among the indigenous people and local communities (IPLCs) of Nepal (Barakoti and Shrestha, 2008; Subedee et al., 2020). Fiber obtained from the stem of this plant is used to make clothes, fishing nets, bags, coats and many other textile products. The plant is used in traditional medicine for treating gastritis, joint pain, headache, and skin allergies (Barakoti and Shrestha, 2008; Subedee et al., 2020). Market demand of the products made from this plant is increasing year by year, and created a risk of over exploitation from its natural habitat. Generic phylogeny and character evaluation in Urticaceae including *G. diversifolia* from China has been studied based on analysis of nuclear ribosomal internal transcribed spacer (nrITS) and two plastid DNA regions *rbcL* exon and *trnL-F* spacer (Wu et al., 2013). However genetic diversity of *G. diversifolia* by using ISSR has not been conducted yet. Thus, there is a need to study molecular characteristics of this plant by using ISSR.

G. diversifolia contains many bioactive compounds such as β-sitosterol, 7- hydroxysitosterol and 3-hydroxystigmast-5-en-7-one (Njogu et al., 2011) trans syringin, linoleic and linolenic acid (Shrestha et al., 2020). Genetic analysis of plants provides broader knowledge on their diversity and basis to study important metabolites produced by them (Fernie and Klee, 2011). Evaluation of genetic diversity in plants requires high quality and quantity of DNA (Sá et al., 2011) for which *G. diversifolia* presented a great challenge. Secondary metabolites present in plants can interfere in genomic DNA extraction, purification and downstream applications (Friar, 2005). Urticaceae family members produce large amounts of exudate, which obstruct DNA extraction (Sarrazola and Alzate, 2019). Various methods of DNA extraction has been carried out on many species of Urticaceae family (Bharmauria et al., 2009; Wu et al., 2013) which has indicated that further modifications are essential to obtain good quality genomic DNA for PCR based analysis (Aboul-Maaty and Oraby, 2019). Despite high value, very few information is available on extraction of its genomic DNA. Isolation of DNA from leaf tissue was found to contain high degree of phytochemical while using standard protocol by Doyle and Doyle (1987). These phytochemicals inhibited PCR reaction. Hence, modification in the extraction protocol was a requisite to obtain good quality DNA. The objective of this study is to develop DNA extraction protocol and optimization of PCR protocol for ISSR-PCR analysis of *G. diversifolia*.

MATERIALS AND METHODS

Plant material

Seeds of *G. diversifolia* were collected from Naugad rural municipality of Darchula District, Far-western region of Nepal (29°47'34.9"N, 80°36'23.5"E). The seeds were thoroughly rinsed with distilled water, allowed to germinate on top of moist absorbent paper in plastic petri dishes using top-of-paper method (Rao et al., 2006) and transplanted to small pot after 12 days. The germinated plants were maintained at Truffle Research Centre, Coronation Garden of Tribhuvan University, Kirtipur, Kathmandu, Nepal (27°40'50"N, 85°17'26.5"E). Young fresh juvenile leaves of the plants were collected prior to extraction of DNA.

Reagents used in isolation of genomic DNA

Cetyltrimethylammonium bromide (CTAB) was modified which consists of extraction buffer 2% (w/v) CTAB (Sigma, Sintra, Portugal), 1 M Tris Hcl (pH 8.0), 0.5 M EDTA (pH 8.0), 5 M NaCl, with 5% PVP (w/v) Polyvinlypyrrolidone (Sigma, Sintra, Portugal), 5% β-mercaptoethanol, Ammonium acetate 7.5 M, (25:24:1) Phenol:Chloroform:Isoamyl alcohol (Sigma-aldrich). Ethanol 70% and 100% were used for isolation of DNA.

Isolation of genomic DNA

Freshly harvested young leaves (100 mg) were ground in liquid nitrogen into fine powder with the help of mortar and pestle. The study followed CTAB, DNA isolation protocol of Doyle and Doyle (1987), and modified the DNA extraction method. The modification of Doyle and Doyle protocol to extract genomic DNA was followed as described below.

Prior to DNA extraction, 5% β-mercaptoethanol and 5% PVP (Polyvinylpyrrolidone) was added in CTAB buffer. Freshly harvested young leaf samples (100 mg) were ground in liquid nitrogen in a pre-chilled mortar and pestle. CTAB buffer (500 µL) was added quickly and transferred into sterile 1.5 mL centrifuge tubes. The tubes were incubated at 55°C for 1 h with occasional shaking for every 10 min. Phenol:chloroform:isoamyl alcohol (25:24:1) was mixed well (500 µL) to form an emulsion by shaking tubes. Centrifugation was carried out at 14000 Revolution Per Minute (RPM). Phenol:chloroform:isoamyl alcohol step was repeated twice. The supernatant was carefully decanted and transferred to new tubes, pre-chilled 0.08 volumes of 7.5 M Ammonium acetate and 0.54 volumes of cold isopropanol was added and mixed well.

The samples were kept in -20°C for 1 h and centrifuged at 14000 RPM for 3 min. The pellet was washed twice with 70% and once in 100% ethanol. The supernatant was decanted and DNA pellet was air-dried at room temperature until the white pellet turned transparent. The DNA pellet was resuspended in 100 µL of TE buffer.

Quantification of extracted DNA and testing for purity

The yield of extracted DNA was measured in a nanospectrometer (Bio-spec nano) at 260 nm. The purity of DNA was measured by estimating the ratio of absorbance at 260 to 280 nm. The DNA purity was determined by running the sample in 0.8% agarose gel.

Table 1. Primers used in screening of ISSR-PCR of *G. diversifolia.*

S/N	Primers name	Primer sequence
1	UBC 812	5'-GAG AGA GAG AGA GAG AA-3'
2	UBC 834	5'-AGA GAG AGA GAG AGA GYT-3'
3	UBC 808	5'-AGA GAG AGA GAG AGA GC-3'
4	UBC 824	5'-TCT CTC TCT CTC TCT CG-3'
5	UBC 811	5'-GAG AGA GAG AGA GAG AC-3'
6	UBC 828	5'-TGT GTG TGT GTG TGT GA-3'
7	(ATC)6C	5'-ATC ATC ATC ATC ATC ATC C-3'
8	(ATC)6T	5' ATC ATC ATC ATC ATC ATC T-3'
9	UBC 826	5'- ACA CAC ACA CAC ACA CC-3'
10	UBC 817	5'-CAC ACA CAC ACA CAC AA-3'
11	UBC809	5'-AGA GAG AGA GAG AGA GG-3'
12	UBC 815	5'-CTC TCT CTC TCT CTC TG-3'
13	UBC 820	5'-GTG TGT GTG TGT GTG TT-3'
14	UBC 823	5'-TCT CTC TCT CTC TCT CC-3'
15	UBC827	5'-ACA CAC ACA CAC ACA CG-3'
16	(GA)9C	5'-GAG AGA GAG AGA GAG AGA-3'

The size of each fragments was estimated using 100 bp plus DNA ladder.

Optimization of Polymerase Chain Reaction (PCR)

The optimization of ISSR-PCR was carried out with the extracted genomic DNA from *G. diversifolia*. Five major factors; *Taq* DNA polymerase (Vivantis, Malaysia), dNTPs (Vivantis, Malaysia), primers (University of British Columbia, Canada), $MgCl_2$ (Vivantis, Malaysia), template DNA, and their concentrations were considered highly as shown in Table 4. The ISSR primer obtained from University of British Columbia (UBC) were used for optimization of PCR condition.

The reaction was carried out in the DNA thermocycler (Biorad T100). Total volume of 15 µL PCR reaction mixture was used which contained 1X buffer, (0.1-0.6) mM dNTPs, (1.5-4) mM $Mgcl_2$, (0.1-0.9) µM primer, (0.5-3.0) unit *Taq* polymerase and (25-100) ng of DNA template. The thermocycler was programmed and optimized by testing various conditions: 3 min at 93°C, followed by 45 cycles for 30 s at 93°C, 45 s at different annealing temperature (45-52)°C, extension at 72°C for 2 min, final extension at 72°C for 10 min and finally holding temperature at 4°C. After PCR reaction, electrophoresis of The PCR products were carried out in 1.8% agarose gel containing 10 mg/mL of ethidium bromide, 1X TAE buffer at 80 V for 1 h. DNA ladder of 100 base pair was used for determining the molecular weight. The DNA bands were observed under ultraviolet light using Gel documentation system.

RESULTS

Isolation and purity detection of DNA

The mean concentration and purity of DNA samples extracted from the leaves of *G. diversifolia* are presented in Table 2.

Yield of DNA from Doyle and Doyle method leaf tissue was found to have high degree of phytochemical and thus needed modification of the method. The modified method yielded 445 ng/µL of DNA, the purity ranged between 1.8 -2.0 indicating minimum contamination of metabolites.

High concentration of β-mercaptoethanol and PVP in extraction buffer played a major role in neutralization of polyphenols, tannins and oxidation of secondary metabolites (Porebski et al., 1997). The modified protocol showed high efficiency to extract good quality DNA by varying the concentration of NaCl, β-mercaptoethanol and PVP. The modified conditions are listed in Table 3.

Optimization of PCR-ISSR and screening primers

For optimization of ISSR-PCR, various concentrations were considered including template DNA, primers, *Taq* polymerase, dNTPs, $MgCl_2$, annealing temperature. The optimized conditions for ISSR-PCR protocol are given in Table 4.

The optimized condition for ISSR-PCR reaction for *G. diversifolia* was found to be 50 ng of genomic DNA, 4 mM Magnesium chloride, 0.6 mM dNTPs, 0.7 µM primer concentration, 2 U *Taq* polymerase in total 15 µL PCR reaction volume. The reproducible clear bands in agarose gel electrophoresis is shown in Figure 1.

DNA extracted from *G. diversifolia* using the optimized PCR parameters are shown in Table 4. Among 16 screened ISSR primers (Table 1), seven ISSR primers (UBC811, UBC812, UBC817, UBC824, UBC826, UBC827, UBC 834) showed the clear, reproducible bands and the experiment was repeated twice (Figure 2). Total volume of 15 µL PCR reaction mixture which contained 1× buffer, 0.6 mM dNTPs, 4 mM $Mgcl_2$, 0.7 µM primer, 2 unit *Taq* polymerase and 50 ng of DNA template were optimized

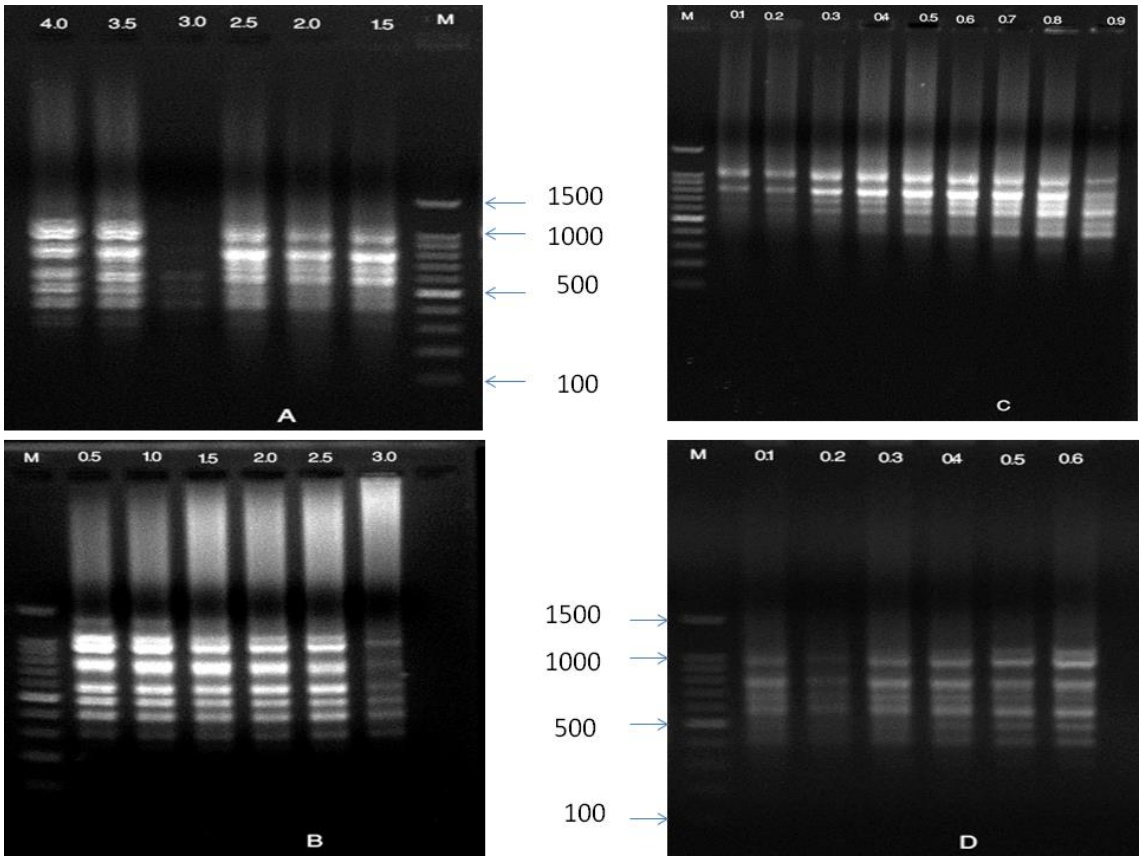

Figure 1. Optimization pattern of DNA sample of *Girardinia diversifolia* using the ISSR primer (UBC 817): **A**, Optimization of $MgCl_2$; **B**, Optimization of *Taq* polymerase; C, Optimization of primer; **D,** Optimization of dNTPs.

Table 2. Quantification and quality analysis of extracted DNA.

Protocol	Mean of A 260/280	Mean of A 260/230	Mean of concentration (ng/µL)	Color/Viscosity
Doyle and Doyle	2.208	1.10	331.39	Dark/viscous
Modified protocol	1.83	2.20	445	Clear/nonviscous

Table 3. Standardized condition of DNA extraction.

S/N	Parameters	Standardized condition	Inference
1	NaCl	5 M	Helped in removal of polysaccharides
2	PVP	5%	Absorbed polyphenols
3	β-mercaptoethanol	5%	Extracted clear DNA pellet
4	Ammonium acetate	7.5 M	Neutralized charges on the sugar phosphate backbone

(Figure 1 and Table 4). The thermocycler was optimized by testing conditions: 3 min at 93°C, followed by 45 cycles for 30 second at 93°C, 45 second at different annealing temperature 45°C, extension at 72°C for 2 min, final extension at 72°C for 10 min and finally holding temperature at 4°C. After PCR reaction, electrophoresis of the PCR products were carried out in 1.8% agarose gel containing 10 mg/mL of ethidium bromide, 1× TAE buffer at 80 V for 1 h. DNA ladder of 100 basepair was used for determining the molecular weight. The optimal

Table 4. PCR parameter tested and selected optimized condition.

S/N	PCR parameter	Tested range	Optimum condition
1	DNA template concentration (ng)	25,37.5, 50, 62.5, 75, 87.5, 100	50
2	Magnesium chloride (mM)	1.5, 2, 2.5, 3, 3.5, 4	4
3	Deoxynucleotide triphosphate(dNTPs) (mM)	0.1, 0.2, 0.3, 0.4, 0.5, 0.6	0.6
4	Primer concentration (µM)	0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9	0.7
5	Taq DNA polymerase (Units)	1, 1.5, 2, 2.5, 3	2
6	Annealing temperature (°C)	45-52	45

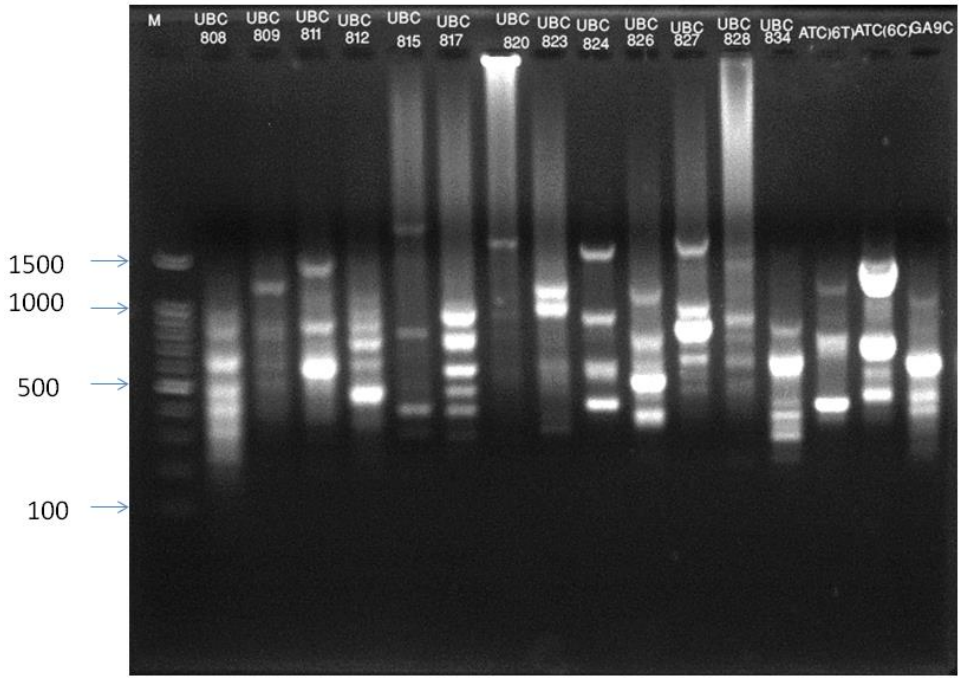

Figure 2. Amplification patterns of 16 ISSR primers for a specimen of *G. diversifolia*.

ISSR-PCR reaction conditions were used for the study of genetic diversity of *G. diversifolia* separately.

DISCUSSION

Genomic DNA isolation from *G. diversifolia* using Doyle and Doyle (1987) standard protocol was conducted. Many obstacles were encountered from the very first step of genomic DNA extraction, including cell lysis, DNA elution and subsequent PCR reactions. The biochemical composition in plant tissues of different species is expected to vary considerably and may not yield optimal DNA from one isolation protocol (Khanuja et al., 1999; Kumari et al., 2020). Due to the presence of PCR inhibitors, protocol of Doyle and Doyle (1987) did not show good quality DNA. It resulted in the absence of DNA band in electrophoresis technique. Thus, procedure described by Doyle (1987) was modified by altering the parameters: increased concentration of NaCl from 2 to 5M, increased concentration of β-mercaptoethanol, increased concentration PVP, addition of phenol to the deproteinization process.

The recommended values for the A260/A280 ratio ranged from 1.8-2.0 and absorption ratio at A260/230 is 2.0-2.22 for impurity free DNA (Arruda et al., 2017). Higher value of absorbance from 2.0 indicated the contamination of phenol in extracted DNA while lower value indicated the presence of proteins. The optimized protocol presented in the study showed a mean DNA concentration of 400 ng/µl extracted from the leaf of *G. diversifolia*. The method resulted in the mean value of 1.83 which confirmed the extraction of pure DNA at A260/A280 and A260/A230 ratios.

High concentration of NaCl (5 M) effectively removed polysaccharides during DNA extraction of *G. diversifolia*

(Table 3). Studies in other plants species like *Mangifera indica*, *Capsicum sp*. *Eclipta alba, Aegle marmelos*, *Grewia asiatica*, supported that this modification allowed an efficient elimination of polysaccharides (Devi et al., 2018; Kit and Chandran, 2010; Kumar et al., 2018; Mujeeb et al., 2017; Shukla et al., 2018). High concentration of NaCl helped in the elimination of polyphenols from the leaf of *G. asiatica* (Shukla et al., 2018).

Use of high concentration of PVP improved the quality of DNA by removing secondary metabolites during genomic DNA extraction process (John, 1992; Osena et al., 2017). The modified protocol used 5% PVP in 2% CTAB buffer which was effective in the elimination of polyphenols that resulted in the extraction of clear DNA pellets. Some studies also suggested the use of 5% PVP in CTAB buffer *Vigna* sp. (Choudhary et al., 2008) and *Mimosa tenuiflora* (Arruda et al., 2017) to obtain good quality DNA pellet.

β-mercaptoethanol enhances denaturation of protein (Tiwari et al., 2012). In the modified protocol, the concentration of β-mercaptoethanol was increased to 5%. The high concentration of β-mercaptoethanol is important for the reduction of polyphenols during extraction of genomic DNA of plants containing high content of secondary metabolites (Arruda et al., 2017). The modified protocol contains 5% β-mercaptoethanol instead of 2% as used by Doyle and Doyle (1987). The modified protocol helped to reduce brown coloured DNA pellet. Other study carried out in *Litchi chinensis* (Arruda et al., 2017; Puchooa, 2004) also reported that increase in the concentration of β-mercaptoethanol helped to extract clear DNA pellet. Polyphenols often damage extracted genomic DNA and make some enzymes inaccessible (Anerao et al., 2016). The use of phenol: choloroform: isoamyl alcohol effectively removed polyphenols and yielded pure genomic DNA.

For the identification of polymorphic characteristics and genetic diversity of plant species, ISSR acts as a powerful method since it depends on the quality and quantity of extracted DNA. Selection of primers is an important factor because the same primer may exhibit different amplification results in different species.

Conclusion

The Doyle and Doyle (1987) protocol was successfully modified to isolate genomic DNA by increasing the concentration of PVP, β-mercaptoethanol, NaCl and addition of Phenol:chloroform:isoamyl alcohol (25:24:1) in the extraction buffer. These changes made it possible to obtain high quality genomic DNA from *G. diversifolia*. The extracted DNA was used to optimize PCR based ISSR protocol which gave clear and amenable DNA bands. The obtained results confirm that the modified protocol is suitable with *G. diversifolia* and other plant species containing high concentration of secondary metabolites. The research is beneficial for future molecular characterization, genetic diversity analysis of allied taxa and genetic improvement works.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Aboul-Maaty NF, Oraby HAS (2019). Extraction of high-quality genomic DNA from different plant orders applying a modified CTAB-based method. Bulletin of the National Research Centre 43(1):25.

Amom T, Tikendra L, Rahaman, Nongdam P (2018). Evaluation of genetic relationship between 15 bamboo species of North-East India based on ISSR marker analysis. Molecular Biology Research Communications 7(1):7-15.

Anerao J, Jha V, Desai N (2016). Optimization of DNA Extraction Methods from *Garcinia* species for ISSR-PCR, RAPD-PCR and DNA Barcoding. Asian Journal of Biotechnology 9(1):35-42.

Anne C (2006). Choosing the right molecular genetic markers for studying biodiversity: From molecular evolution to practical aspects. Genetica 127:101-120.

Arruda SR, Pereira DG, Silva-Castro MM, Brito MG, Waldschmidt AM (2017). An optimized protocol for DNA extraction in plants with a high content of secondary metabolites, based on leaves of *Mimosa tenuiflora* (Willd.) Poir. (Leguminosae). Genetics and Molecular Research 16(3):1-9.

Barakoti TP, Shrestha KP (2008). Commercial utilization of Allo (*Girardinia diversifolia*) by the Rais of Sankhuwasabha for income generation. Banko Janakari 18(1):18-24.

Bharmauria V, Narang N, Verma V, Sharma S (2009). Genetic variation and polymorphism in the Himalayan nettle plant *Urtica dioica* based on RAPD marker. Journal of Medicinal Plants Research 3(3):166-170.

Choudhary K, Mathur N, Choudhary OP, Pillai U (2008). Protocol for Isolation of Genomic Dna from Dry and Fresh Leaves of *Vigna*

Species Suitable for Rapd and Restriction Digestion. Advances in Biological Research 2(5-6):83-89.
Devi AA, Brajendra N, Dinachandra M (2018). Genetic Diversity Analysis in Chilli (*Capsicum annuum* L.) Found in Manipur Using RAPD Markers. International Journal of Current Microbiology and Applied Sciences 7(10):257-262.
Doyle JJ, Doyle JL (1987). A Rapid DNA Isolation Procedure for Small Quantities of Fresh Leaf Tissue. Phytochemical Bulletin 19:11-15.
Fernie AR, Klee HJ (2011). The Use of Natural Genetic Diversity in the Understanding of Metabolic Organization and Regulation. Frontiers in Plant Science 2:59.
Friar EA (2005). Isolation of DNA from Plants with Large Amounts of Secondary Metabolites. In Methods in Enzymology 395:1-12.
Friis I (1981). A Synopsis of *Girardinia* (Urticaceae). Kew Bulletin 36(1):143-157.
John ME (1992). An efficient method for isolation of RNA and DNA from plants containing polyphenolics. Nucleic Acids Research 20(9):2381-2381.
Khanuja SP, Shasany AK, Darokar MP, Kumar S (1999). Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils. Plant Molecular biology Reporter 17(1):74.
Kit YS, Chandran S (2010). A simple, rapid and efficient method of isolating DNA from Chokanan mango (*Mangifera indica* L.). African Journal of Biotechnology 9(36):5805-5808.
Kumar A, Singh I, Badari NS (2018). Extraction of Genomic DNA from Dried mature leaves of *Eclipta alba* (L.) suitable for ISSR-PCR and other DNA fingerprinting and Barcoding techniques. International Journal of Molecular Biotechnology 4(2):23-30.
Kumari M, Jadhav AS, Chahande RV (2020). Isolation of genomic DNA from groundnut plant by modified, rapid and efficient protocol. Journal of Pharmacognosy and Phytochemistry 9(1):2268-2271.
Mohamad A, Alhasnawi AN, Kadhimi AA, Isahak A, Wan Yusoff WM, Che Radziah CMZ (2017). DNA Isolation and Optimization of ISSR-PCR Reaction System in *Oryza sativa* L. International Journal on Advanced Science, Engineering and Information Technology 7(6):2264.
Mujeeb F, Bajpai P, Pathak N, Verma SR (2017). Genetic Diversity Analysis of Medicinally Important Horticultural Crop *Aegle marmelos* by ISSR Markers. In L. Domingues (Ed.), PCR. Springer New York. pp. 195-211. Available at: https://link.springer.com/protocol/10.1007/978-1-4939-7060-5_14
Nilkanta H, Amom T, Tikendra L, Rahaman H, Nongdam P (2017). ISSR Marker Based Population Genetic Study of *Melocanna baccifera* (Roxb.) Kurz: A Commercially Important Bamboo of Manipur, North-East India. Scientifica 2017:1-9.
Njogu PM, Thoithi GN, Mwangi JW, Kamau FN, Kibwage IO, Kariuki ST, Mwalukumbi JM (2011). Phytochemical and Antimicrobial Investigation of *Girardinia diversifolia* (Link)Friis (Urticaceae). East and Central African Journal of Pharmaceutical Sciences 14(3):89-94.
Osena G, Nyaboga E, Amugune N (2017). Rapid and Efficient Isolation of High Quality DNA from Cassava (*Manihot esculenta* Crantz) Suitable for PCR Based Downstream Applications. Annual Research and Review in Biology 12:1-10.
Polunin O, Stainton A (1984). Flowers of the Himalaya. Oxford University Press.
Porebski S, Bailey LG, Baum BR (1997). Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Molecular Biology Reporter 15(1):8-15.
Puchooa D (2004). A simple, rapid and efficient method for the extraction of genomic DNA from lychee (*Litchi chinensis* Sonn.). African Journal of Biotechnology 3(4):253-255.
Reddy MP, Sarla N, Siddiq EA (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica 128(1):9-17.
Sá O, Pereira JA, Baptista P (2011). Optimization of DNA Extraction for RAPD and ISSR Analysis of *Arbutus unedo* L. Leaves. International Journal of Molecular Sciences 12:4156-4164.
Sarrazola JH, Alzate FA (2019). Obtaining DNA from Urticaceae: Overcoming the challenges associated with chemical compounds and herbarium specimens. International Journal of Molecular Biology 4(5):158-165.
Shrestha SS, Sut S, Ferrarese I, Barbon Di Marco S, Zengin G, De Franco M, Pant DR, Mahomoodally MF, Ferri N, Biancorosso N, Maggi F, Dall Acqua S, Rajbhandary S (2020). Himalayan Nettle *Girardinia diversifolia* as a Candidate Ingredient for Pharmaceutical and Nutraceutical Applications—Phytochemical Analysis and In Vitro Bioassays. Molecules 25:1563.
Shrestha R (1997). Cytological Studies in *Girardinia Diversifolia* (Link) Friis. Pakistan Journal of Botany 29(2):263-269.
Shukla R, Sharma DC, Pathak N, Bajpai P (2018). Genomic DNA Isolation from High Polyphenolic Content *Grewia asiatica* L. Leaf Without Using Liquid Nitrogen. Iranian Journal of Science and Technology, Transactions A: Science 42:347-351.
Singh SC, Shrestha R (1988). *Girardinia diversifolia* (Urticaceae), a non-conventional fiber resource in Nepal. Economic Botany 42:445-447.
Subedee BR, Chaudhary RP, Uprety Y, Dorji T (2020). Socio-ecological perspectives of Himalayan Giant Nettle (*Girardinia diversifolia* (Link) Friis) in Nepal. Journal of Natural Fibers 17(1):9-17.
Tiwari KL, Jadhav SK, Gupta S (2012). Modified CTAB Technique for Isolation of DNA from some Medicinal Plants. Research Journal of Medicinal Plant 6(1):65-73.
Wu ZY, Monro AK, Milne RI, Wang H, Yi TS, Liu J, Li DZ (2013). Molecular phylogeny of the nettle family (Urticaceae) inferred from multiple loci of three genomes and extensive generic sampling. Molecular Phylogenetics and Evolution 69(3):814-827.

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Investigation of lipid production and fatty acid composition in some native microalgae from Agadir region in Morocco

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Microalgae are a promising alternative source to produce biofuels and bio-products, as they consume carbon dioxide to grow, multiply quickly, and can be cultivated in domestic and industrial wastewater. The efficiency of microalgae-based biodiesel industry depends on the selection of appropriate strains with best lipid content yields and adequate fatty acids composition. The aim of this study was to select a potential microalga strain for lipid production by bioprospecting and screening lipid productivity and fatty acid compositions of some local microalgae isolated from different water bodies located in the region of Agadir, Morocco and cultivated in homemade photobioreactors. For this purpose, four freshwater microalgae *Parachlorella kessleri*, *Cyclotella* species, *Chlorella* species and *Closterium* species were isolated and cultivated. Lipid extraction was carried out using Soxhlet and fatty acids profiling was performed using Gas Chromatography. The highest lipid content was obtained with a diatom microalga *Cyclotella* spp. (33 wt%) after 15 days of residence time. The investigated strains produced high amount of oleic acid (41-54%) followed by linoleic (18-33%), palmitic (13-23%) and stearic acid (4-12%), respectively. Variations in lipid production and fatty acids composition as responses to changes in residence times form another important factor to feedstock selection for biofuel production.

Key words: Biofuel, biomass, culture time, fatty acid, freshwater microalgae, lipid.

INTRODUCTION

Biodiesel, as derived from vegetable oils or animal fats, is an alternative diesel fuel that is becoming accepted in a steadily growing number of countries around the world (Ganesan et al., 2020).

In fact, there is a huge interest in using microalgae to produce oil for biodiesel because of their potential for high productivity in shorter time and because they hold the potential to provide a scalable renewable feedstock, without harming the food supply (Chisti, 2007). However, the production costs are still not competitive compared

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to fossil fuels (Mizuno et al., 2013). Therefore, an increasing number of researches are in progress to enhance microalgae lipid production systems but there is barely any research dealing with the quality of this feedstock (Stansell et al., 2012). Since the late 1980s, biodiesel production has been increasing and standardization was a greater need than ever before. Two biodiesel standards that have served in the development of other standards around the world are the standards ASTM D6751 in the United States and EN 14214 in Europe. The development of those standards has accompanied the increasing interest in production and use of biodiesel (Knothe, 2009).

Some of the properties regulated by these two standards are difficult to be directly measured by most investigators due to the lack of necessary specialized equipment and, particularly in algal biodiesel pilot scale projects; the quantity of produced lipid is insufficient (Stansell et al., 2012). Therefore, many attempts have been undertaken to establish models that could predict some of the critical biodiesel properties from microalgae composition (Allen et al., 1999; Dunn, 2008; Imahara et al., 2006; Lapuerta et al., 2009; Lopes et al., 2008; Tong et al., 2010). These models provide preliminary assessments of the potential biomass of microalgae based on fatty acid (FA) composition (Stansell et al., 2012). These acids determine, in fact, the properties which influence the efficiency of the biodiesel feedstock such as cetane number, viscosity, cold flow, oxidation stability and lubricity (Knothe, 2009). In turn, the structural characteristics that influence the energetic properties of a FA are the length and the branching of the chain and the unsaturation degree (Knothe, 2008).

Most of reported studies support the concept that lipids rich in monounsaturated FAs (MUFAs) are suitable for biodiesel fuel, although the composition of the saturated FAs is also shown to be of great importance as well (Arguelles and Martinez-Goss, 2020). Therefore, the FAs composition should be modified to increase MUFA contents as well as enhancing lipid production.

Apparently there is a relationship between MUFA contents and algal class which potentially provides some basis for investigators to make initial selections of target classes of microalgae for bioprospecting (Stansell et al., 2012). Various studies on chemical and physical properties of biodiesel have suggested that biodiesel with a high level of MUFA may have excellent characteristics in regard to ignition quality, fuel stability, flow properties at low temperature, and iodine number (according to European biodiesel standard EN 14214) (Stansell et al., 2012; Ramos et al., 2009).

In Morocco, microalgae are less explored and further research is needed to assess the potential of these native microorganisms as a biofuel feedstock.

Recently, a study screened 57 marine microalgae isolated from the Moroccan coast to select favorable strains for biodiesel production (El Arroussi et al., 2017). The authors concluded that, based on biomass productivity, lipid contents and FAs composition, *Nannochloropsis* species, *Dunaliella tertiolecta, Isochrysis* species and *Tetraselmis* species are promising species for biodiesel application (El Arroussi et al., 2017).

In the context of evaluation and bioprospecting of microalgae from local habitat, this is the first study to screen some of the freshwater microalgae from Agadir region.

In this sense, the objective of the work was to evaluate the cultivation of four microalgae strains at pilot scale. The study was aimed to examine the suitability of our homemade 25 L rectangular glass photobioreactors (PBRs) for production of lipids from the selected microalgae. Three strains from 10 microalgae isolates were selected based on some criteria like morphological differences, robustness, growth, ease of culture and harvesting. Biomass, lipid productivity and FAs composition of three selected microalgae strains (*Cyclotella, Chlorella* and *Closterium* species) were compared by using different residence times inside the PBRs.

Parachlorella kessleri, is a microalga characterized by high biomass and lipid productivity (Mizuno et al., 2013; Takeshita et al., 2014). It has been investigated for its lipid productivity at laboratory scale (Mizuno et al., 2013; Takeshita et al., 2014; Fernandes et al., 2013) and at semi-industrial outdoor condition (Li et al., 2013), and is considered an ideal microalgae species for biofuel feedstock. For all these reasons, *P. kessleri* was selected as a control reference in this study to design our PBRs and to optimize the culture parameter including $NaHCO_3$ concentration and light intensity.

MATERIALS AND METHODS

Field collection

Water samples were collected about the same time from different water bodies that are located in the region of Agadir, Morocco. Sampling of large bodies of fresh water occurred at multiple sites along the waterfront. Collections were made for the top and bottom of the water at each location, with the goal of determining the dominant microalgae species in each area. All field samples were collected in 50 mL tubes and maintained at refrigerated condition while transferring to laboratory

Isolation and identification of microalgae

To isolate microalgae, serial dilutions of enriched flask were performed, and 50 µL samples from 1, 2, 4, and 6 dilution fractions were inoculated to a new 500 mL flask containing 250 mL of Bold Basal Medium (BBM) (Arif et al., 2020). A volume of 50 µL from different dilutions was transferred to wells of microtiter plates that had 200 µL BBM and were kept at 22°C under continuous illumination for 3 weeks of 850 Lux with a 16/8 h light/dark cycle using T8 led tube light (G13/9W 6000 K, Brilliant, Germany) (Cobos et al., 2017). The culture's purity was confirmed by repeated culturing and microscopic examinations.

In fact, the isolation of microalgae by serial dilution method does

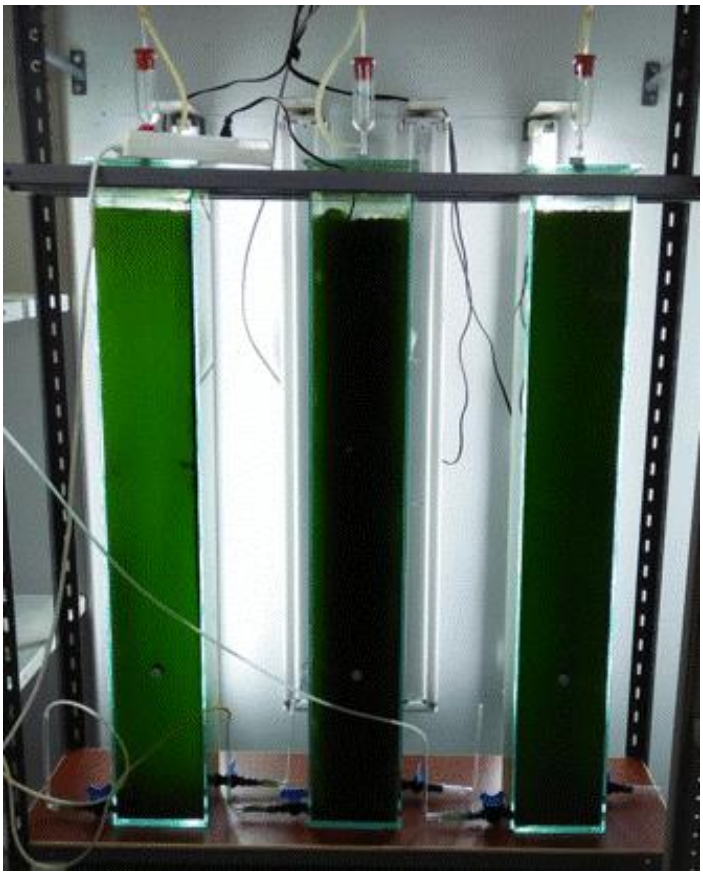

Figure 1. Homemade photobioreactors used in this study.

not necessarily ensure purity of cultures; smaller adherent heterologous microorganisms may not be detected by microscope examination.

Therefore, we have routinely tested our culture for purity by microscopic examination and if any contamination is detected, further dilutions were made. Culture purity assessments were based on conventional observations of cellular morphologies.

The morphological identification was done using botanical approaches (John et al., 2002). These approaches are based on classic morphological taxonomy, which includes the use of characteristics observable by light microscope such as shape, size, arrangement and pattern of cells.

The photobioreactor

Four glass vertical rectangular PBRs (110.0 cm in height, 15.0 cm in length, a closed system) with 24 L of working volume each (Musa et al., 2019), were developed and used for microalgae cultivation. The reactors contain three openings near the top, middle and bottom. The opening at the top was used as an exhaust for the disengaged bubbles, the bottom one was used to collect biomass, while de middle one was used for sampling. The wall thickness was 5 mm. For culture mixing, ambient air was introduced continuously with a constant air flow rate of 0.5 L/min through sterilized membrane into the bottom by a sparger fixed at the bottom of the PBR which converts the sparged air into tiny bubbles.

Fluorescent lamps were placed vertically and parallel to the front side of the PBRs with the same height as the PBR. They supply continuous illumination of 850 Lux with a 16/8 h light/dark cycle. The light intensity was measured at three fixed spots on the inside of the empty reactor using a light meter, and the average of three readings of light intensity was obtained.

Before all experiments, the PBRs were sterilized overnight with antiseptic detergent and rinsed many time with sterilized distilled water. Figure 1 shows the homemade PBRs used in this study.

Microalgae and cultivation conditions

P. kessleri, was obtained from GEPEA UMR CNRS 6144, Saint-Nazaire, France. *Cyclotella*, *Chlorella* and *Closterium* spp. were collected from different locations in the region of Agadir, Morocco. Growth media for *P. kessleri, Cyclotella* spp. and *Chlorella* spp. was based on BBM (Pruvost et al., 2009). For *Closterium* spp., culture medium was based on Bold 3N Medium (B3NM) and formulated based on UTEX recommendation (UTEX LB 2398).

These strains were easy to cultivate in PBRs without hindrances such as cell settlement, biofilm, or floc formation.

The culture medium BBM was prepared on distilled water and was composed of (per liter) 0.05 g $Na_2EDTA{\cdot}2H_2O$, 0.75 g $NaNO_3$, 0.225 g $MgSO_4{\cdot}7\ H_2O$, 0.025 g $CaCl_2{\cdot}2H_2O$, 0.014 g $FeSO_4{\cdot}7H_2O$, 0.123 g KH_2PO_4, 0.15 g K_2HPO_4, and 1.26 g $NaHCO_3$. Two micronutrients (1 ml each) were added to the culture medium.

The first micronutrient was composed of (per liter) 0.222 g $ZnSO_4{\cdot}7H_2O$, 0.044 g $Co(NO_3)2{\cdot}6H_2O$, 0.079 g $CuSO_4$, 2.86 g H_3BO_3 and 1.81 g $MnCl_2{\cdot}4H_2O$. The second micronutrient was composed of 0.219 g/L Na_2MoO_4.

Table 1. The effect of residence time in PBR on lipid and biomass production of *Parachlorella kessleri*.

Residence time (days)	Fresh biomass production (g/l/day)	Lipid content (%) of *Parachlorella kessleri*
5	2.15	13.14
15	0.98	18.20
30	1.10	5.00
45	1.14	1.75
60	0.38	3.80

The media was prepared by adding the components in order listed earlier. Each component was fully dissolved before adding the next one. The volume of 1 L of both micro-nutriments was prepared in advance and stored at 4°C.

Sodium bicarbonate was added to culture media after autoclaving the media through a 0.22 µm pore size filter.

The culture medium B3NM was prepared on distilled water and was composed of (per liter) 6 mL of P-IV metal solution, 0.75 g $NaNO_3$, 0.025 g $CaCl_2{\cdot}2H_2O$, 0.075 g $MgSO_4{\cdot}7H_2O$, 0.075 g K_2HPO_4, 0.175 g KH_2PO_4 and 40 ml of soil water. Vitamin B12 (0.135 mg/L) was added after autoclaving the culture medium.

P-IV metal solution was prepared on distilled water and was composed of (per liter) 0.75 g $Na_2EDTA{\cdot}2H_2O$, 0.097 g $FeCl_3{\cdot}6H_2O$, 0.041 g $MnCl_2{\cdot}4\ H_2O$, 0.005 g $ZnCl_2$, 0.002 g $CoCl_2{\cdot}6H_2O$ and 0.004 g $Na_2MoO_4{\cdot}2H_2O$.

A stock solution of P-IV metal was prepared and stored at 4°C prior to use. Soil water was composed of 1 tsp of Greenhouse soil, 1 mg of $CaCO_3$ and 200 mL of distilled water. It was prepared by combining and steaming the ingredients in a cover container for two consecutive days. The solution was cooled to room temperature and stored at 4°C.

For BBM and B3NM, pH was adjusted to 6.5 before being sterilized by autoclaving at 120°C for 20 min using a pH meter (BANTE instruments, China). All glassware used in the culture of microalgae was also autoclaved.

The volume of inoculum was chosen to give an initial density of 10^5 cells/ml. The room temperature of 22°C was regulated by air conditioner.

Samples were taken three times a week to analyze the change in pH, cell morphology and cell number using a counting chamber (hemocytometer).

Harvesting of microalgae

After the cultivation period, the supply of air was stopped; the microalgae were distributed into 5 L flocculation vessels and were allowed to settle in the dark overnight at room temperature. The sedimentation overnight was found to be the most appropriate to settle under the influence of gravity over 90% biomass from culture. The cell-free supernatants were gently discarded using a peristaltic pump. Thereafter the microalgae were harvested through centrifugation two times during 15 min at 5000 rpm (HERMLE LABORTECHNIK, Germany), lyophilized and stored at -20°C prior to lipid extraction.

Lipids extraction using Soxhlet

Approximately 5 g of lyophilized biomass were transferred to a cellulose cartridge and placed in the extraction chamber of a 250 mL Soxhlet extractor. The extractions were performed by a period of 5 h with an average cycle time of 15 min using 300 mL of Hexane. After extraction, the solvent was recovered by rotary-evaporation. The extracted lipid was then transferred to a bottle, and kept overnight under 40°C in an air-circulating oven to evaporate any residual amount of hexane. The next day, the sample was weighed to determine the extraction yield and stored at 4°C for chemical analysis.

The lipid content was calculated in relation to the dry weight of the lyophilized microalgae.

Determination of fatty acid composition

The FA composition of the total lipids was measured by Gas Chromatography (GC) analysis of FAME. Briefly, the lipids (0.3 g) were dissolved with 20 mL of iso-octane and gentle heating in a test tube. 1 mL of potassium hydroxide solution in methanol was then added and the tube was stirred thoroughly for 30 s. To neutralize potassium hydroxide, 5 g of sodium bisulfate monohydrate was added and the solution stirred. A volume of 1.5 µL of resulting FAME was then injected to GC (Agilent Technologies 6890, USA) equipped with a BPX70 capillary column (60 m × 0.32 mm × 0.25 µm). The temperature of the injector and FID detector was 220°C. GC oven temperature was set to 175°C. GC peaks were identified by FAME standard. The relative percentage of FAs was reported based on the peak area.

RESULTS

Effect of residence time on biomass and lipid production

In order to determine the best residence time of the culture inside the PBR for biomass and lipid production, microalga *P. kessleri* selected as a control strain and three native microalgae strains were isolated and cultivated in 25 L PBR under five different residence times.

As shown in Table 1, by increasing the residence time, fresh biomass productivity increases and reach a maximum of 51.16 g/l after 45 days of culture time, whereas 15 days of culture time in PBR was sufficient to have a maximum of 18.20% of lipid content.

Although the daily fresh biomass increases slightly when the residence times pass from 15 to 30 days then to 45 days, the total harvested fresh biomass increases significantly.

Furthermore, as shown in Table 1, an increase in residence time from 5 to 15 days causes a high lipid productivity during the exponential phase.

The reason for going up to 60 days of residence time is

Table 2. The effect of culture time in PBR on lipid production of various microalgae.

Residence time (days)	Lipid content (%) of *Parachlorella kessleri*	Lipid content (%) of *Cyclotella* spp.	Lipid content (%) of *Chlorella* spp.	Lipid content (%) of *Closterium* spp.
5	13.14	20.38	-	-
15	18.20	33.06	2.50	2.62
30	5.00	20.00	-	-

Table 3. Fatty acid composition of microalgae lipid of *Parachlorella kessleri* at different culture times in PBR.

Chain length: no of double bonds	Fatty acid	Lipid composition (w/total lipid)				
		5 days	15 days	30 days	45 days	60 days
C14:0	Myristic acid	0.00	0.12	0.12	0.13	0.12
C15:0	Penta-decyclic acid	0.00	0.05	0.05	0.05	0.05
C16:0	Palmitic acid	13.78	12.65	12.67	12.67	12.68
C16:1	Palmitoleic acid	0.00	0.18	0.20	0.20	0.19
C17:0	Margaric acid	0.00	0.07	0.08	0.08	0.07
C17:1	Heptadecenoic acid	0.00	0.03	0.03	0.03	0.03
C18:0	Stearic acid	3.50	5.36	5.38	5.39	5.40
C18:1	Oleic acid	70.15	47.88	47.97	47.94	47.96
C18:2	Linoleic acid	12.57	32.81	32.68	32.65	32.62
C18:3	Linolenic acid	0.00	0.09	0.11	0.11	0.11
C20:0	Arachidic acid	0.00	0.30	0.31	0.31	0.31
C20:1	Gadoleic acid	0.00	0.32	0.31	0.34	0.36
C22:0	Behenic acid	0.00	0.14	0.10	0.11	0.11

to study the effect of nutrient starvation on lipid accumulation by *P. kessleri*.

The results show that the productivity of *P. kessleri* is high and reach 2 g/l/day during the first 5 days of culture (Table 1). A slight increase of daily fresh biomass production was observed between 5 and 45 days of culture time. After 45 days of residence time, the productivity of *P. kessleri* decrease to 0.38 g/l/day.

Interestingly, 15 days is an optimal residence time of *P. kessleri* to accumulate lipids up to 18.20% (w/w) of the dry matter. When the residence time increases from 15 to 45 days, lipid production decreases to its minimum of 1.75% (w/w). At 60 days of residence time, lipid content increases to 3.80% which may be due to the decrease of biomass production rate.

Over all the investigated strains in this study, the biomass productivity in our homemade PBRs was around 1 g/l/day.

The lipid content of *Cyclotella* spp. was measured as well at different residence times. Three culture times were tested and the highest lipid content was 33.06% (w/w) after 15 days in PBR (Table 2). The optimal residence time that allows *P. kessleri* and *Cyclotella* spp. to produce the maximal of lipid productivity was 15 days. For *Chlorella* and *Closterium* spp., residence time was set to 15 days and the measured lipid productivity was comparable, 2.5 and 2.62% (w/w), respectively (Table 2).

Since the residence time of 15 days was optimal for having high lipid productivity for *P. kessleri* and *Cyclotella* spp., only this residence time was tested for *Chlorella* and *Closterium* spp.

Effect of residence time on fatty acids composition

The lipid profile using Gas Chromatography revealed that the FA compositions of the four strains are similar. Their profile shows a higher amount of oleic acid followed by linoleic, palmitic and stearic acids, respectively (Tables 3 to 5).

On the other side, residence time has an effect on FA proportions, mainly between 5 and 15 days. No changes of the FA compositions were observed over 15 days of residence time.

For *P. kessleri* (Table 3), when the residence time increases from 5 to 15 days, the FA proportions decrease from 70.15 to 47.88% and from 13.78 to 12.65% for oleic and palmitic acids, while they increase from 12.57 to 32.81% and from 3.50 to 5.36% for linoleic and stearic acids, respectively.

Table 4. Fatty acid composition of microalgae lipid of *Cyclotella* spp. at different culture times in PBR.

Chain length: no of double bonds	Fatty acid	Lipid composition (w/total lipid)		
		5 days	15 days	30 days
C14:0	Myristic acid	0.00	0.00	0.00
C15:0	Penta-decyclic acid	0.00	0.00	0.00
C16:0	Palmitic acid	18.15	15.37	21.03
C16:1	Palmitoleic acid	0.00	0.00	0.00
C17:0	Margaric acid	0.00	0.00	0.00
C17:1	Heptadecenoic acid	0.00	0.00	0.00
C18:0	Stearic acid	5.91	4.38	5.51
C18:1	Oleic acid	47.47	53.56	53.15
C18:2	Linoleic acid	28.46	26.69	20.31
C18:3	Linolenic acid	0.00	0.00	0.00
C20:0	Arachidic acid	0.00	0.00	0.00
C20:1	Gadoleic acid	0.00	0.00	0.00
C22:0	Behenic acid	0.00	0.00	0.00

Table 5. Fatty acid composition of microalgae lipid after 15 days of culture time in PBR for *Chlorella* and *Closterium* spp.

Chain length: no of double bonds	Fatty acid	Lipid composition (w/total lipid)	
		***Chlorella* spp.**	***Closterium* spp.**
C14:0	Myristic acid	0.00	0.47
C15:0	Penta-decyclic acid	0.00	0.67
C16:0	Palmitic acid	22.81	15.90
C16:1	Palmitoleic acid	0.00	1.62
C17:0	Margaric acid	0.00	0.48
C17:1	Heptadecenoic acid	0.00	1.13
C18:0	Stearic acid	12.39	5.29
C18:1	Oleic acid	46.80	40.81
C18:2	Linoleic acid	18.00	21.60
C18:3	Linolenic acid	0.00	9.18
C20:0	Arachidic acid	0.00	0.00
C20:1	Gadoleic acid	0.00	2.85
C22:0	Behenic acid	0.00	0.00

For *Cyclotella* spp. (Table 4), when the residence time increases from 5 to 15 days, the FA amounts decrease from 28.46 to 26.69%, from 18.15 to 15.37% and from 5.91 to 4.38% for linoleic, palmitic and stearic acids, respectively, for oleic acid the FA proportion increases from 47.47 to 53.56%.

For *Chlorella* spp. (Table 5), the highest fatty amount is oleic acid (C18:1) 46.80% followed with palmitic acid (C16:0) 22.81%, linoleic acid (C18:2) 18.00% and stearic acid (C18:0) 12.39%.

For *Closterium* spp. (Table 5), the highest fatty amount is oleic acid (C18:1) 40.81% followed with linoleic acid (C18:2) 21.60%, palmitic acid (C16:0) 15.90%, linoenic acid (C18:3) 9.18%, stearic acid (C18:0) 5.29% and gadoleic acid (C20:1) 2.85%.

DISCUSSION

Microalgae are among the most promising non-food-crop-based biomass for biodiesel application (Hannon et al., 2010). However, current technology does not economically support large-scale sustainable production (Musa et al., 2019). The biofuel production process includes the upstream and downstream processing stage, with several technological limitations (Musa et al., 2019).

In order for the microalgae biotechnology to be sustainable, realizable and economically affordable, it is necessary to develop efficient culturing systems for the production of higher amount of biomass (Khan et al., 2018). Among factors that are determinant in microalgae culture are nutrients ((N, P, K), temperature, pH, salinity, inorganic carbon, oxygen, light intensity, and CO_2 (Mata et al., 2010). Further factors could affect the success of cell growth such as stirring and mixing, the bioreactor geometry, culture time, and harvest repetition (Khan et al., 2018).

The lipid composition of most strains was published but mainly for cultures at the end of the stationary growth phase. It has not been studied with for different culture time.

This paper examines the effect of residence time of microalgae in PBR on lipid production and fatty acid composition, and to the best of our knowledge, this factor has never been reported on any study.

Our results show that the lipid contents and biomass productivity are inversely dependent, which is in concordance with reported studies and is associated by the amount of energy consumed as the result of lipid biosynthesis (Demirbas, 2009; Eloka-Eboka and Inambao, 2017).

The investigated microalgae, in this study, produce small amount of lipids during the first 5 days, because in the lag phase the microalgae, as reported by Jiang et al. (2012), are dividing actively, accumulating carbon in protein. In stationary phase (15 days), the reduction of available nutrients induces a gradual decrease of the cell division which causes an accumulation of cell products under lipid form.

Further, the microalgae lipids were extracted using hexane, however, using one solvent (polar or non-polar) is not potent enough to break the lipid bond with proteins, and the measured lipid content is underestimated. As reported by Halim et al. (2012), a mixture of polar solvent allows the formation of hydrogen bonds that enhance the lipid bond breaking with proteins.

Compared to our results, previous studies have reported relatively higher lipid content values using chloroform and methanol mixture as extraction solvents; 30% for *P. kessleri*, 30% for *Chlorella* spp., and 20% for *Closterium* spp. (Taleb et al., 2016; Karima et al. 2018). However, for *Cyclotella* spp., lower lipid content (25 %) has been reported using a similar mixture of extraction solvents (Graham et al., 2012)."

Of the organisms studied, lipid productivity varies greatly from one species to another and with culture time, and it seems that *Cyclotella* spp. strain featured, indeed, the highest lipid production among all the investigated microalgae.

Although the Soxhlet method using single solvent produces lower extraction yields than the ones obtained with a mixture of polar solvents (Li et al., 2014), it was used in this study to evaluate the efficiency of our homemade PBRs to produce biomass and to weigh out lipid content of some local microalgae strains.

It is obvious from our results that to achieve higher lipid productivity with an efficient photoconversion, the residence time of the culture inside the PBR should be optimum in a narrow range, otherwise the production decrease systematically.

Lipid compositions were also measured by changing the residence times. First of all, the dominant fatty acid in the investigated strains was oleic acid. The FA profile of *P. kessleri* was essentially identical after 15 days of residence time inside the PBRs. However, during growth phase (5 days), MUFA accounted for more than 70% of FAs, followed by SFA (17.28%) and PUFA (12.57%) (Table 3). It seems that there is a conversion from oleic acid to linoleic acid when residence time increases from 5 to 15 days.

In fact, oleic acid may play a role as a precursor for linoleic acid, hence growing phase convert the MUFA to a more highly unsaturated acid (Yuan and Bloch, 1961).

For *Cyclotella* spp. while the maximum MUFA was observed at a residence time of 15 days, the PUFA decreased with residence time (Table 4). However, SFA composition was not residence time dependent, it decreased from 24.06 to 19.75% and increased to 26.54% when residence time increased from 5 to 15 days and from 15 to 30 days.

Comparisons of fatty acid groups between *Chlorella* and *Closterium* spp. show that their MUFA compositions are essentially identical after 15 days of residence time (Table 5).

On the other hand, while *Chlorella* spp. produce high proportion of SFA (35.20%) than PUFA (18%), *Closterium* spp. contains low proportion of SFA (22.81%) than PUFA (30.78%).

Fatty acids composition of *P. kessleri* contains significantly higher proportions of C16:0 followed by C18:0 (Taleb et al., 2016), which is in contrast to our results where the higher FA was C18:1 followed by C18:2. Furthermore, the data in Table 6 show that in the investigated microalgae, the large amount of measured FA is generally the MUFA, which is in contrast to previous study that reported significantly higher PUFA content of the green algae (Stansell et al., 2012). In terms of MUFA content and based on Graham et al. (2012) work's, the investigated microalgae strain, mainly *Cyclotella* spp., shows a good balance between most crucial fuel properties such as cetane number and cold filter plugging point (Stansell et al., 2012).

The reason for the discrepancy between reported microalgae FA compositions and our findings is the extraction procedures (Certik et al., 1996). Indeed, the polarities of solvent as well as the extraction method have an effect on either the amount of double bonds or length of carboxyl chain.

Our results are in accordance with reported study based on the polarity of individual or mixtures solvents

Table 6. SFA, MUFA and PUFA composition of microalgae lipid after 15 days of culture time in PBR for *Parachlorella kessleri, Cyclotella, Chlorella* and *Closterium* spp.

Microalgae	SFA (%)	MUFA (%)	PUFA (%)
Parachlorella kessleri	18.69	48.41	32.90
Cyclotella spp.	19.75	53.56	26.69
Chlorella spp.	35.20	46.80	18.00
Closterium spp.	22.81	46.41	30.78

(Certik et al., 1996). Thus less polar solvents like hexane are appropriate for the extraction of fatty acids with longer carbon chains.

While the properties of biodiesel are determined by the composition of its FAs, the properties of single FA depend on its structure such as the chain length, the number of double bonds and branch chains (Cao et al., 2014). For example, the energetic value of biodiesel increases with FA chain length, but the fluidity decreases with chain length. The longer the chain, the greater the viscosity. Therefore, in order to get good quality biodiesel, an appropriate proportion must be between MUFA, SFA and PUFA on produced microalgae lipids.

According to reported works, the perfect biodiesel would be made mostly from MUFA and have fewer PUFA and SFA, respectively (Cao et al., 2014). From our results (Table 6), *Cyclotella* spp. represents, somehow a promising feedstock for biodiesel production.

Conclusion

This study with freshwater microalgae from Agadir region in Morocco revealed that *Cyclotella* spp. has potential for lipid-rich biomass production. The optimum residence time in PBR for this microalga to reach the maximum lipid productivity with higher MUFA was 15 days. The cultivation and harvesting of this strain should be regulated to facilitate its use for biofuel production. Parameters including light intensity, carbon dioxide, salinity stress, temperature and nutrient starvation or limitation should be optimized to enhance microalgae lipid production. Further investigations are necessary as well to enhance lipid production and the unsaturation of fatty acids using either nutritional or environmental stresses.

As outlined in previous studies (Li et al., 2014), the fatty acids profile and productivity are also highly dependent on cultivation conditions, biomass harvesting, cell lysis and strain selection in addition to solvent polarity and extraction methods.

The variation in lipid production and fatty acids profile as a result of changes in residence time represents an important factor to microalgae strain selection and should provide suggestions for further research.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Allen CAW, Watts KC, Ackman RG, Pegg MJ (1999). Predicting the viscosity of biodiesel fuels from their fatty acid ester composition. Fuel 78:1319-1326.

Arguelles ED, Martinez-Goss MR (2020). Lipid accumulation and profiling in microalgae *Chlorolobion sp*. (BIOTECH 4031) and Chlorella sp. (BIOTECH 4026) during nitrogen starvation for biodiesel production. Journal of Applied Phycology. Available at: https://link.springer.com/article/10.1007%2Fs10811-020-02126-z

Arif M, Wang L, Salama ES, Hussain MS, Li X, Jalalah M, Al-Assiri MS, Harraz FA, Ji MK, Liu P (2020). Microalgae Isolation for Nutrient Removal Assessment and Biodiesel Production. BioEnergy Research 2020:1-13.

Cao Y, Liu W, Xu X, Zhang H, Wang J and Xian M (2014). Production of free monounsaturated fatty acids by metabolically engineered *Escherichia coli*. Biotechnology for Biofuels 7:59-69.

Certik M, Andrasi P, Sajbidor J (1996). Effect of Extraction Methods on Lipid Yield and Fatty Acid Composition of Lipid Classes Containing y-Linolenic Acid Extracted from Fungi. Journal of the American Oil Chemists' Society 73:357-365.

Chisti Y (2007). Biodiesel from microalgae. Biotechnology Advances 25:294-306.

Cobos M, Paredes JD, Maddox JD, Vargas-Arana G, Flores L, Aguilar CP, Marapara JL, Castro JC (2017). Isolation and Characterization of Native Microalgae from the Peruvian Amazon with Potential for Biodiesel Production. Energies 10:224-239.

Demirbas A (2009). Progress and recent trends in biodiesel fuels. Energy Conversion and Management 50:14-34.

Dunn RO (2008). Crystallization behavior of fatty acid methyl esters. Journal of the American Oil Chemists' Society 85:961-972.

El Arroussi H, Benhima R, El Mernissi N, Bouhfid R, Tilsaghani C, Bennis I, Wahby I (2017). Screening of marine microalgae strains from Moroccan coasts for biodiesel production. Renewable Energy 113:1515-1522.

Eloka-Eboka AC, Inambao FL (2017). Effects of CO2 sequestration on lipid and biomass productivity in microalgal biomass production. Applied Energy 195:1100-1111.

Fernandes B, Teixeira J, Dragone G, Vicente AA, Kawano S, Bišová K, Přibyl P, Zachleder V, Vítová M (2013). Relationship between starch and lipid accumulation induced by nutrient depletion and replenishment in the microalga Parachlorella kessleri. Bioresource Technology 144:268-274.

Ganesan R, Manigandan S, Samuel MS, Shanmuganathan R, Brindhadevi K, Lan Chi NT, Anh Duc P, Pugazhendhi A (2020). A review on prospective production of biofuel from microalgae. Biotechnology Reports 27:e00509.

Graham JM, Graham LE, ZulkiXy SB, PXeger BF, Hoover SW, Yoshitani J (2012). Freshwater diatoms as a source of lipids for biofuels, Journal of Industrial Microbiology and Biotechnology 39:419-

428.
Halim R, Danquah MK, Webley PA (2012). Extraction from oil from microalgae for biodiesel production: A Review. Biotechnology Advances 30:709-732.
Hannon M, Gimpel J, Tran M, Rasala B, Mayfield S (2010). Biofuels from algae: challenges and potential. Biofuels 1:763-784.
Imahara H, Minami E, Saka S (2006). Thermodynamic study on cloud point of biodiesel with its fatty acid composition. Fuel 85:1666-1670.
Jiang Y, Yoshida T, Quigg A (2012). Photosynthetic performance, lipid production and biomass composition in response to nitrogen limitation in marine microalgae. Plant Physiology and Biochemistry 54:70-77.
John DM, Whitton BA, Alan J, Brook AJ (2002). The freshwater algal flora of the British isles. An identification guide to freshwater and terrestrial algae. Cambridge University Press.
Karima A, Silalahi MDS, Rinanti A (2018). Increasing content of lipid in tropical microalgae Chlorella sorokiniana and Closterium sp. with variation of nitrogen content and extraction temperature. MATEC Web of Conferences 197. Available at: https://www.matec-conferences.org/articles/matecconf/abs/2018/56/matecconf_aasec2018_13019/matecconf_aasec2018_13019.html
Khan MI, Shin JH, Kim JD (2018). The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. Microbial Cell Factories 17(1):36.
Knothe G (2008). "Designer" biodiesel: optimizing fatty ester composition to improve fuel properties. Energy Fuels 22:1358-1364.
Knothe G (2009). Improving biodiesel fuel properties by modifying fatty ester composition. Energy and Environnemental Science 2:759-766.
Lapuerta M, Rodrıguez-Fernandez J, Font de Mora E (2009). Correlation for the estimation of the cetane number of biodiesel fuels and implications on the iodine number. Energy Policy 37:4337-4344.
Li Y, Naghdi FG, Garg S, Adarme-Vega TC, Thurecht KJ, Abdul Ghafor W, Tannock S, Schenk PM (2014). A comparative study: the impact of different lipid extraction methods on current microalgal lipid research. Microbial Cell Factories 13:14.
Li X, Přibyl P, Bišová K, Kawano S, Cepák V, Zachleder V, Čížková M, Brányiková I, Vítová M (2013). The microalga Parachlorella kessleri - a novel highly efficient lipid producer. Biotechnology and Bioengineering 110:97-107.
Lopes JCA, Boros L, Krähenbühl MA, Meirelles AJA, Daridon JL, Pauly J, Marrucho IM, Coutinho JAP (2008). Prediction of cloud points of biodiesel. Energy Fuels 22:747-752.
Mata TM, Martins AA, Caetano NS (2010). Microalgae for biodiesel production and other applications: a review. Renewable and Sustainable Energy Reviews14:217-232.
Mizuno Y, Sato A, Watanabe K, Hirata A, Takeshita T, Ota S, Sato N, Zachleder V, Tsuzuki M, Kawano S (2013). Sequential accumulation of starch and lipid induced by sulfur deficiency in Chlorella and Parachlorella species. Bioresource Technology 129:150-155.
Musa M, Ayoko GA, Ward A, Rösch C, Brown RJ, Rainey TJ (2019). Factors Affecting Microalgae Production for Biofuels and the Potentials of Chemometric Methods in Assessing and Optimizing Productivity. Cells 8:851-874.
Pruvost J, Van Vooren G, Cogne G, Legrand J (2009). Investigation of biomass and lipids production with Neochloris oleoabundans in photobioreactor. Bioresource Technology 100:5988-5995.
Ramos MJ, Fernandez CM, Casas A, Rodriguez L, Perez A (2009). Influence of fatty acid composition of raw materials on biodiesel properties. Bioresource Technology 100(1):261-268.
Stansell GR, Gray VM, Sym SD (2012). Microalgal fatty acid composition: implications for biodiesel quality. Journal of Applied Phycology 24:791-801.
Takeshita T, Ota S, Yamazaki T, Hirata A, Zachleder V, Kawano S (2014). Starch and lipid accumulation in eight strains of six Chlorella species under comparatively high light intensity and aeration culture conditions. Bioresource Technology 158:127-34.
Taleb A, Kandilian R, Touchard R, Montalescot V, Rinaldi T, Taha S, Takache H, Marchal L, Legrand J, Pruvost J (2016). Screening of freshwater and seawater microalgae strains in fully controlled photobioreactors for biodiesel production. Bioresource Technology 218:480-490.
Tong D, Hu C, Jiang K, Li Y (2010). Cetane number prediction of biodiesel from the composition of the fatty acid methyl esters. Journal of the American Oil Chemists' Society 88:415-423.
Yuan C, Bloch K (1961). Conversion of Oleic Acid to Linoleic Acid, Journal of Biological Chemistry 236:1277-1279.

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Review

Symptoms, epidemiology and diagnosis: A mini-review on coronavirus

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There is worldwide concern about the rapid spread of the Covid19 (COVID-19) pandemic which now affects almost every country in the world. Generating accurate diagnosis of COVID-19 by testing hundreds of thousands of people per day, and the search for effective therapeutics and vaccines are currently the focus of intense research in large numbers of companies and academic institutions around the world. This review will describe the background, origin, epidemiology, symptoms of COVID-19 infection, and its methods of detection. Because of the rapidly changing news on the development of new therapeutic approaches (many of which have already been discarded), and the constantly breaking news - on a daily basis - about the development of vaccines (there are currently 22 separate vaccine programmes worldwide), we will leave the future therapeutic and vaccination options outside the scope of this review. We will address these topics later, once some clarity has prevailed surrounding the large number of putative therapeutic and vaccination options that are currently being explored and an evidence-based approach can be meaningfully applied to interpreting how patient management, and national epidemiological management, can benefit by these emerging breakthroughs.

Key words: Covid-19, corona, diagnosis, epidemiology, cytokine storm, CT scan, SARS.

INTRODUCTION

Coronaviruses are amongst a large group of viruses that may cause disease in animals and humans. There are four main sub-groups of coronaviruses, known as alpha, beta, gamma, and delta. Human coronaviruses (HCoVs) were first isolated in the 1960s from persons with upper respiratory tract infections (Tyrrell and Bynoe, 1965). HCoVs were detected in the alpha coronavirus (HCoV-229E and NL63) and the beta coronavirus (MERS-CoV,

SARS-CoV, HCoV-OC43 and HCoV-HKU1) (Tyrrell and Bynoe, 1965; Shuo et al., 2016). The common human coronaviruses are SARS-CoV, MERS-CoV, and SARS-CoV-2 (COVID-19). SARS-CoV caused severe acute respiratory syndrome (SARS) which emerged in November 2002 (Peiris et al., 2003) and disappeared by 2004 (https://www.who.int/csr/resources/publications). MERS-CoV transmitted from a camel reservoir which was identified in September 2012 and continues to cause sporadic and localized outbreaks (Stalin Raj et al., 2014; Assiri et al., 2013). COVID-19 emerged from China in December 2019 where the first cases of infection belong to people from fish market in the Chinese city of Wuhan (Rothan and Byrareddy, 2020; Zhu et al., 2020; Lu et al., 2020). It is thought that bats are the animal reservoirs for coronaviruses (Giri et al., 2020; Corman et al., 2018; Brook and Dobson, 2015). However, the reservoir animal that transmitted the COVID-19 virus to humans has not been determined so far (Ortiz-Prado et al., 2020).

On March 11, 2020, the World Health Organization (WHO) declared COVID-19 to be a pandemic disease. As of today, July 28, 2020, there are more than 16.7 million global confirmed cases with a death rate of approximately 4% (Li et al., 2020a). These numbers will undoubtedly increase greatly in the coming months, until intercepted by effective treatments, and mitigated through a successful vaccination strategy.

The diameter of coronavirus is approximately 125 nm and it has a relatively large ~31 kb positive-sense single stranded RNA genome. It is a spherical, enveloped, non-segmented and spiky virion which is an assembly of 3 proteins: Spike protein (S), Envelope protein (E) and Membrane protein (M). The name coronavirus has been given because of the crown-like spikes on its surface (protein S) (Li et al., 2020a; Lai and Cavanagh, 1997; Walla et al., 2020).

The Spike protein (S) is heavily glycosylated, which plays a role in enhancing neutralizing antibodies and mediating viral fusion with the host mucosa cell membrane via Hemagglutinin-esterase dimer protein (HE). Studies have shown that angiotensin-converting-enzyme2 (ACE2) is the receptor protein of S protein (Gheblawi et al., 2020). Envelope protein (E) works as a transport channel, whereas M protein is highly hydrophobic (Figure 1).

SYMPTOMS

The symptoms of COVID-19 infections can be classified into systematic disorders and respiratory disorders. Systematic disorders include: fever, dry cough, fatigue, sputum production, headache, hemoptysis, acute cardiac injury, hypoxemia, diarrhoea, dyspnea and lymphopenia. Respiratory disorders include rhinorrhoea, sneezing, sore throat, pneumonia, ground-glass opacities, RNAaemia and acute respiratory distress (https://www.ecdc.europa.eu/en/covid-19/latest/evidence/coronaviruses; Huang et al., 2020).

The incubation period before the symptoms appear was first reported as ~5.2 days (Li et al., 2020b). Others then showed the onset of the symptoms range from 6 to 41 days with a median of 14 days where the age and health background of the patient represent important factors that influence the symptom length and severity of this potentially fatal disease (Wang et al., 2020). This study demonstrated that people older than 70 years of age showed worse symptoms, including health complications of longer duration and greater severity than people younger than 70 years old (Wang et al., 2020). People with pre-existing medical problems such as high blood pressure, heart disease, cancer or diabetes are of high concern since up to 4% of people who have contracted the disease may succumb to it (Liang et al., 2020; Murthy et al., 2020). However, some people become infected yet remain asymptomatic. Most people (about 80%) spontaneously recover from the disease without any need for special treatment (Ortiz-Prado et al., 2020).

It is reported that the infection rate and severity of COVID-19 is relatively low in children compared to adults. The reason behind that could be due to immaturity of ACE2 protein in children. Furthermore, the innate immune system is less mature in children, and thus so is their adaptive immune response. Also, children are more susceptible to respiratory viral infections such as influenza, parainfluenza viruses, adenoviruses, respiratory syncytial viruses, and rhinoviruses. The production of antibodies created during these infections may cross-react with coronaviruses and thus could provide some acquired protection in this way (Li et al., 2020c).

Although the mechanism of action of COVID-19 is poorly understood, the similarity of the virus with the structure with SARS-CoV and MERS-CoV (Weiss and Navas-Martin, 2005; Ren et al., 2020) can offer clues as to the pathogenesis which may in turn help inform strategies surrounding the ongoing development of effective COVID-19 treatments and vaccination approaches (Lai and Cavanagh, 1997; Weiss and Navas-Martin, 2005).

EPIDEMIOLOGY

Coronaviruses are pathogens that target the human respiratory system. Previous outbreaks include both the

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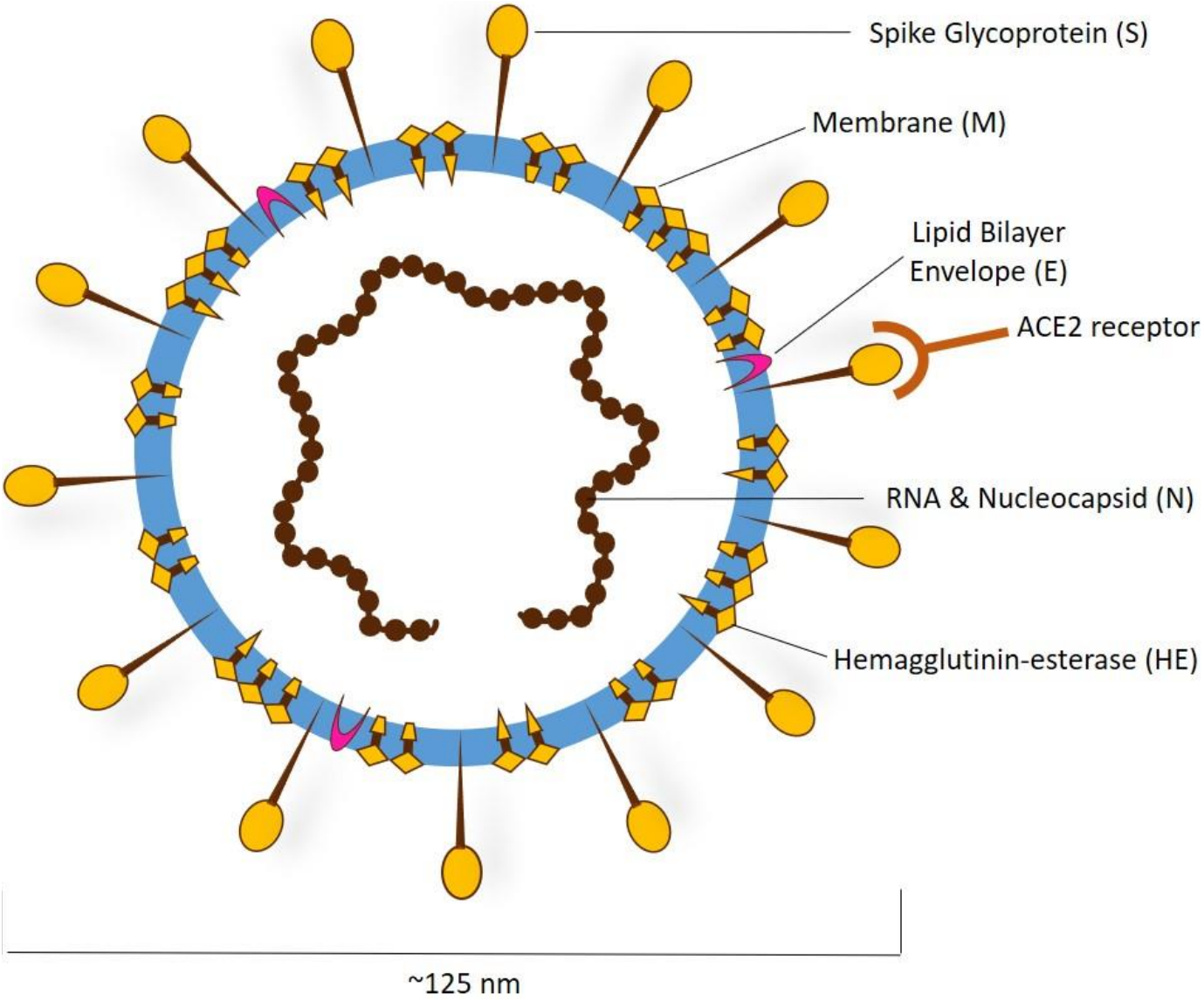

Figure 1. The general structure of coronavirus. The virion is comprised inner and outer layers. The inner layer contains nucleocapsid RNA and phosphorylated nucleocapsid and the outer layer contains phospholipid bilayers covered by the spike glycoprotein trimmer (S). The phospholipid bilayers consists of membrane (M) protein (a type III transmembrane glycoprotein) and the envelope protein (E).

severe acute respiratory syndrome (SARS)-CoV as well as the Middle East respiratory syndrome (MERS)-CoV, both of which were characterised as significant threats to public health. Throughout the second half of December 2019, a small number of patients were admitted to hospitals in China and initially diagnosed with pneumonia of an unknown cause. The infection of these first few patients was epidemiologically linked to a seafood and wet animal wholesale market located in Wuhan, Hubei province, China (Bogoch et. al., 2020; Lu et. al., 2020). Given the estimate of a reproduction number for the 2019 Novel (New) Coronavirus (COVID-19, designated as such by WHO on 11th February 2020) was significantly larger than 1 (estimates at that time were from 2.24 to 3.58), initial reports predicted a potential coronavirus outbreak (Zhao et.al, 2020; Lin et al., 2020).

The first five cases of COVID-19 were reported between 18 and 29th December 2019 (Du Toit, 2020). All five patients were hospitalised with acute respiratory distress syndrome with one patient dead (Ren et al., 2020). By 2nd January 2020, a total of 41 patients were confirmed to have been infected with COVID-19, and fewer than 50% of these patients had underlying diseases such as hypertension, cardiovascular disease and/or diabetes (Huang et al., 2020). These patients were presumed to have been exposed to the virus and infected in that hospital as a result of nosocomial infection, resulting in the conclusion that COVID-19 was

Table 1. Coronavirus population statistics as of **23.9**.2020.

Parameter	Cases of COVID-19	Deaths	Cured
Saudi Arabia	331359	4569	313789
Worldwide	32000000~	~980000	~22000000

Source: Saudi MOH website.

not spread by just one patient to many others. Instead, the increased number of cases was interpreted to have resulted when multiple patients became infected at numerous sites throughout the hospital. Additionally, the only people who were tested at that time were patients that were clinically sick, so therefore it is highly likely that many more people were actually infected. By 22nd January 2020, 571 cases of the 2019-new coronavirus (COVID-19) had been reported in 25 different provinces (districts and cities) in China (Lu, 2020). At this time, the China National Health Commission then reported the details of the 17 patients who had died from the virus. By 25th January 2020 almost 2000 confirmed COVID-19 cases were known to have occurred in mainland China, with 56 confirmed deaths (Wang et al., 2020). An additional report on 24th January 2020 estimated the cumulative incidence of COVID-19 infections in China to be 5502 patients (Nishiura et al., 2020).

As of 29th June 2020, over 16 million individuals worldwide have been confirmed to have been infected with COVID-19 with more than 600,000 of these infections resulting in death (Table 1). Laboratory-confirmed cases of COVID-19 have been reported in every continent other than Antarctica, and in over 180 countries (https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200308).

Over 270,000 COVID-19 cases have been confirmed in Saudi Arabia and this has resulted in more than 2700 deaths as of June 29th 2020.

In May 2020, parameter values to support public health preparedness and planning for the ongoing COVID-19 pandemic were released by the CDC and the Office of the Assistant Secretary for Preparedness and Response (ASPR). The 'best estimates' provided by these two organisations for the transmission of the virus, severity of disease as well as transmission by asymptomatic and presymptomatic individuals have been published (https://www.cdc.gov/coronavirus/2019-ncov/hcp/planning-scenarios.html). As mentioned previously, COVID-19 has a basic reproduction number (R0, otherwise known as R-naught) of 2.5 and of the people who become infected, 3.4% will become hospitalised and approximately 0.4% will die from the disease. Approximately 35% of patients infected with COVID-19 are asymptomatic; however, these patients are just as infectious as those who display symptoms. The average time to symptom onset from exposure to the virus is 6 days and there is a 40% chance of viral transmission occurring prior to the onset of any symptoms (https://www.cdc.gov/coronavirus/2019-ncov/hcp/planning-scenarios.html).

ROLE OF COMPUTED TOMOGRAPHY IN DIAGNOSIS

To date, the most sensitive tool used for confirming an infection with COVID-19 is the reverse transcriptase polymerase chain reaction (RT-PCR) test. Although computerised tomography (CT) has also been heavily used, its precise role for investigating patients with COVID-19 remained initially unclear. One group then reported they found 97% of 601 patients with COVID-19 were accurately diagnosed by CT (Ai et al., 2020). Another group reported that the diagnosis could be confirmed by CT in 50 of 51 patients (98%) compared to only 36 (71%) of these patients when using RT-PCR (Fang et al., 2020). With results of such striking significance, one may conclude chest CT should replace RTPCR in the routine testing for COVID-19, or at least to be the front line tool for screening, especially since CT is a much faster approach because results are immediately available once the scan is complete. However, while reviewing these two publications, Hope et al. (2019) criticized their entire research process and made the point that the consolidation and ground-glass opacity is not solely specific to COVID-19 pneumonia.

Hope et al. (2019) again addressed these methodological flaws in a further publication (Reptics et al., 2020) which repudiated the notion that CT was sufficiently sensitive and specific in diagnosing COVID-19 when used in the absence of an RT-PCR test, and added that doing this 'runs counter to current society guidelines'. They concluded that CT should just be 'reserved for evaluation of complications of COVID-19 pneumonia or for assessment if alternative diagnoses are suspected'.

When using CT, the images seen in cases of pneumonia that are caused by viruses from the same viral family appear essentially the same in those scans because of the similarities in the lung pathogenesis of these related pathogens. Another report showed that about 25% of the early COVID-19 cases detected negative in chest CT scans (Guan et al., 2020). Recently, the American College of Radiology (ACR) recommendations for the use of chest radiography and computed tomography (CT) for suspected COVID-19 infection (www.acr.org/Advocacy-and-Economics/ACR-Position-Statements/Recommendations-for-Chest-Radiography-and-CT-for-Suspected-COVID19-Infection. April 5 2020)

addressed the question of what that may be displaying on a chest CT scan is not specific to COVID-19, and can therefore also be related to other forms of infection. This helped define that a CT scan should not be considered for screening, nor used as front line diagnostic tool for COVID-19, but that chest CT may help in diagnosis of cases with advancing symptoms (World Health Organization, 2020).

CT is an imaging technique that involves a relatively high dose of radiation dose when compared with conventional x-ray techniques. In the early stages of a COVID-19 infection, isolation and supplementary medication are required and what is detected in a CT scan will not change the treatment yet would be exposing the patient to an unnecessary dose of radiation which itself is associated with tangible biological risks (Hall, 2002; Hong et al., 2019). A chest x-ray (CXR) on the other hand, involves exposure to far less radiation, and can be requested, instead of a CT, even on several occasions, to provide continuous information on the status of the lungs (Holshue et al., 2020). If, on occasions a CT scan becomes imperative, that is, if it outweighs patient benefits over patient risks, then low radiation output CT techniques should be performed rather than operating with high (or standard) dose levels (Kalra et al., 2004). By doing this, the radiation exposure concept of “As Low As Reasonable Achievable” (ALARA) can be maintained. Moreover, as COVID-19 is an airborne disease and highly contagious, ensuring a safe application of CT imaging when scanning COVID-19 patients is quite challenging, since any mistakes in following and ensuring good precautionary preventative regimes may result in transmission of the disease to staff and other patients.

ROLE OF ELISA IN DIAGNOSIS

The diagnostic tests used for the detection of COVID-19 infections have been an essential tool in tracking the spread of the disease during the current pandemic. The identification of the genetic sequence of COVID-19 allowed for diagnostic tests specific to SARS-CoV-2 to be developed rapidly (Wang et al., 2014).

Widely utilised to detect and quantify specific antibodies and antigens in samples, enzyme-linked immunosorbent assays (ELISA) share many qualities in common with the radioimmunoassay (RIA) from which they were derived (Gan and Patel, 2013). RIAs were first developed by Yalow and Berson to measure endogenous levels of insulin in plasma, leading to Yalow being awarded the 1977 Nobel Prize (Yalow and Berson, 1996). This assay was further developed resulting in the creation of a novel technique that could be used to detect the presence and quantify biological molecules in minute quantities of sample, which then lead to the analysis and detection of a wide range of other molecules such as proteins, hormones and peptides. Due to concerns about the safety of radioactivity these assays were adapted, leading to the replacement of radioisotopes with enzymes, and thus resulting in the creation of the ELISA (Gan and Patel, 2013). While the use of ELISA could assist in tracking antigen exposure, there are limitations with this approach, in particular the colour change mediated by the enzyme will react indefinitely and hence, if left for a sufficiently long time, the colorimetric change may falsely reflect the quantity of antibody present. Additional limitations include the requirement to generate a reciprocal antibody/antigen in order to detect the antibody/antigen of interest as well as non-specific binding of the antibody/antigen of interest to the ELISA plate thus leading to false-positive results (Gan and Patel, 2013).

Serological testing can also be used to assist in the investigation of an ongoing viral outbreak as well as in the retrospective assessment of the attack rate, or the extent of the outbreak. When validated serological tests are available these could be utilised using paired serum samples (in the acute and convalescent phase) to support diagnosis in the event that nucleic acid amplification tests (NAAT) assays return negative, yet a strong epidemiological link to COVID-19 infection remains (https://www.who.int/publications-detail/laboratory-testing-for-2019-novel-coronavirus-in-suspected-human-cases-2020).

ROLE OF PCR IN DIAGNOSIS

RT-PCR is a regularly used technique for the detection of the causative virus in respiratory secretions from patients suffering from acute respiratory infections. PCR is an enzyme-driven technique used to replicate DNA *in* vitro that can be utilised to produce a large enough quantity of DNA to detect and identify pathogens. As every pathogen has a unique DNA or RNA sequence, these molecules can be utilised as a molecular fingerprint to identify which pathogen is causing the disease in a patient. The clinical application of PCR was revolutionised by the invention of real-time quantitative PCR (qPCR) primarily due to the automation of analysis via removing the requirement for post reaction manipulation. Utilising probes with fluorescent reporter dyes for detection of the conserved genes ORF1ab and RT-PCR is the core technology used to detect the presence of SARS-CoV-2 in samples of the airways of patients. A positive result from one of these highly sensitive tests indicates the presence of viral RNA in the sample; however, a clinical correlation with a patient’s history is also required. It is important to bear in mind that NAAT tests such as RT-PCR, followed by nucleic acid sequencing when appropriate, are considered to be the gold standard for diagnosing COVID-19 as detailed by WHO.

There are many publications describing the process of

amplification of RNA known as reverse transcriptase polymerase chain reaction (RT-PCR) as the gold standard method for the detection of COVID-19 (Xiang et al., 2020). The immunological responses to COVID-19 have also been investigated by many researchers, and there are considerable variabilities in terms of sensitivity and specificity. Other laboratory parameters have been investigated in predicting cases with positive RT-PCR for COVID-19, a higher neutrophil (NEU) count, C-reactive protein (CRP), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and urea levels in serum have all been studied. In addition, patients with a positive RT-PCR have been reported to have a lower white blood cell (WBC) count, and serum albumin levels, compared to others. ALT, CRP, NEU, LDH, and urea showed very good accuracy in predicting cases with positive RT-PCR for COVID-19 as confirmed by the presence of RNA (Mardani et al., 2020).

Furthermore, typical pulmonary features of SARS pneumonia were not evident when studied in one report. Other tests of immunohistochemical staining showed an abnormal accumulation of CD4+ helper T lymphocytes and CD163+ M2 macrophages in lung tissue. Comparably, the case was investigated by SARS-CoV-2 infection RT-PCR and RNA *in situ* hybridization on surgically removed lung tissues (Zeng et al., 2020). The importance of RT-PCR is known for its rapidness, sensitivity and specificity for viral RNA detection as the case of COVID-19. The use of RT-PCR is also an essential tool for epidemiological studies, clinical management, and preventive medicine in general (Yip et al., 2020).

Essentially, to have an efficient RT-PCR method, a carefully selected target for amplification should be examined. In one study, four specific regions in the SARS-CoV-2 genome were identified. This allowed the design of sets of primers to be used for PCR (Yip et al., 2020).

Two other locations, orf1ab and S genes were also used to design primers for the detection assay (Yan et al., 2020). In addition, RdRp gene and E gene were utilized by two groups (Okamaoto et al., 2020; Son et al., 2020). Another group targeted the nucleocapsid (N), envelope (E), and open reading frame 1a or 1b genes to design primers for RT-PCR assay (Chan et al., 2020). The primer sets design should be performed by selecting the target for amplification and the region of the targeted sequence needs to be unique, specifically for COVID-19. This adequately allows RT-PCR assays in diagnostic virology to represent a highly sensitive approach (Yip et al., 2020).

Low copies of the virus may limit its detection; therefore, viral RNA is the best sensitive assay to use (Yip et al., 2020). Most viruses share a common region for the identity of the virus and some unique ones, and some very conserved ones for the strain of the particular virus itself. For this reason it is believed that COVID-19 has a conserved region (as discussed earlier) and studies have shown there is indeed a consistent, conserved region. Whilst the assay must deliver a satisfactory level of reproducibility, one crucial advantage of molecular testing is studying viral clearance assessed by RT-PCR when a drug is used for a cure (Singh et al., 2020). RT-PCR has also been used in the clustering of patients who were infected in a different geographical location to the Singapore study (Yong et al., 2020). A significant percentage (85%) of infected individuals, do not show any clinical symptoms which therefore makes RT-PCR the method of choice for the screening of asymptomatic carriers (Rivett et al., 2020).

Rivett et al. (2020) further studied the clustering of infected health care workers (HCWs) by performing viral genome sequencing for RT-PCR positive individuals who had been screened previously. A dominant lineage B1 was found in many of these HCWs (Yalow and Berson, 1996). The severity of acute disease was confirmed by RT-PCR in one study (Fang et al., 2020). Active disease can be monitored by RT-PCR where an investigator can follow the patients during the course of the viral infection, and some patients of course start to test negative as they recover from their illness (Han et al., 2020).

In the event of a new viral outbreak, a relatively quick establishment of RT-PCR testing for laboratory molecular diagnosis is a vital way to detect organisms of an important pathogenic and infectious nature. Practically, positive rates might be calculated in groups of patients for serological findings which may first be confirmed by RT-PCR (Jacobi et al., 2020). In reality, clinical suspicion of COVID-19 may be assessed by chest radiography (Schiaffino et al., 2020). The purpose here is to overcome the limitations of RT-PCR, especially when negative results have been obtained (Ma et al., 2020). In children with suspected COVID-19, negative results on RT-PCR were first noted in five cases but subsequent testing confirmed they had become positive for the virus.

Chest CT may improve the sensitivity for the diagnosis of COVID-19. However, exposure to radiation should be avoided as much as possible when there is an alternative diagnostic approach, especially for pregnant women and children. Nevertheless, portable chest radiography (CXR) has also been thought to offer a safe and efficient workflow, whilst counteracting possible false negative RT-PCR results. If there were any delay in the availability of RT-PCR to determine the diagnosis of COVID-19, then this would make CXR an attractive choice of approach. Disease severity may also be evaluated by CXR when there is no other way available to assess this (Hu and Wang, 2020).

Imaging has become an indispensable approach for the early detection of COVID-19, and for following the progress and outcome of the illness. Severity of the disease could be evaluated if a chest imaging modality is available (Kalafat et al., 2020). Some investigators

recommend the use of imaging modalities before employing RT-PCR since 60-93% of patients have positive chest computed tomographic findings that are consistent with COVID-19 (Di Micco et al., 2020).

In contrast, the utilization of urine specimens was not found to be informative for the RT-PCR detection of COVID-19. However, RT-PCR-negative patient at day 15 of infection was observed in patients who were positive after taking treatment regimen consisting of hydroxychloroquine (HCQ) and azithromycin (AZ). Therefore, we may conclude that it will probably become normal practice to utilize RT-PCR for the evaluation of the treatment of COVID-19. Further, Million et al. (2020) managed to perform a calculation of fatality rate by applying the RT-PCR assay within COVID-19 laboratory diagnosis.

CYTOKINE STORM

Cytokine storm is a term that indicates uncontrolled and generalized immune response (Ferrara et al., 1993). This term was initially used to describe the events that cause graft versus host reactions in transplantation. The state of cytokine storm is characterized by a powerful, exaggerated activation of the immune system. Cytokine storm is associated with a wide range of infectious and non-infectious conditions (Yuen and Wong, 2005).

One of the major devastating presentations of COVID 19 is acute respiratory distress syndrome (ARDS). This is associated with around 40% mortality and characterized mainly by the presence of bilateral lung infiltrations, and hypoxia. It may present with a wide range of symptoms: pneumonia, sepsis, pancreatitis, and thrombosis. The pathophysiology of this condition involves injury to the alveolocapillary membrane and this results in increased filtration of the lungs and exudation of protein rich pulmonary edema fluid into the airspaces, which eventually leads to respiratory failure (Bhatia et al., 2012).

Data from SARS and MERS-CoV has previously shown that these viruses cause an increased level of pro-inflammatory mediators (cytokines and chemokines) such as interferon γ, interleukin (IL) -1B, IL-6, IL-12, $CXCL_{10}$, and CCL_2. These mediators have been shown to be associated with extensive lung involvement and ARDS (Channappanavr and Perlman, 2017). Similarly, recent reports have shown increased levels of pro-inflammatory cytokines and chemokines in patients with COVID-19 (Huang et al., 2020). This indicates activation of TH1 cells. Of particular interest, is that in COVID-19 there are also increased levels of immunosuppressor mediators secreted by TH2 cells (IL-4, IL-10) (Zhang et al., 2020).

The clinical and laboratory findings in patients experiencing a cytokine storm, include cytopenias (thrombocytopenia and lymphopenia), coagulopathy (low platelet and fibrinogen levels, and elevated D-dimer levels), tissue damage/hepatitis (elevated LDH, aspartate aminotransferase, and alanine aminotransferase levels), and macrophage/hepatocyte activation (elevated ferritin levels). In addition, there may be fever, reduced (or absent) NK activity, elevated levels of CD25, sCD163 and the presence of hemophagocytosis (Crayne et al., 2019).

There are several proposed mechanisms for cytokine storm in patients with COVID-19 some of which are still ongoing. However, some theories for the predisposition to experience a cytokine storm in patients with COVID-19 including:

(1) Impaired viral clearance: Similar to what was thought in SARS and MERS-CoV that the virus exerts some strategies to resist the host defense mechanisms. These viruses are able to produce vesicles that have double membranes and that the virus can replicate inside these vesicles (Snijder et al., 2006). This eventually leads to an impaired immune response against the virus, which will cause additional accumulation of the virus and viral products. In this situation, the PCR test can be negative, yet the patient may experience a devastating effect from the viral inclusion bodies present inside the alveolar cells and macrophages (Xu et al., 2020).
(2) Low levels of type 1 interferon, which are important in viral clearance. It has been noted that patients with MERS-CoV show upregulation of pro-inflammatory cytokines and down-regulation of antiviral cytokines (Chan et al., 2015).
(3) Liu et al. (2019) suggested that antibodies against spike glycoprotein (anti-S-IgG) act as autoantibodies and hence promote a proinflammatory response in the lungs. This response is considered as pathological rather than protective and may act as a mediator of lung injury.

In general, viral escape mechanisms to avoid viral clearance, together with genetic, or acquired defects in the host defense may lead to further accumulation of the virus. This will eventually result in an impaired immune response, and an exaggerated immune activation, that will cause ARDS and multi-organ failure.

The disease course is variable, ranging from asymptomatic to severe life threatening/fatal disease. This is attributed to multiple factors including both genetic and host factors (Rouse and Sehrawat, 2010). This may explain why the mortality is high in some families/regions.

Several studies have shown the correlation between disease severity and inflammatory mediators. In one study, there was a positive correlation between IL-2R and IL-6 and the disease severity. These markers were higher in patients who are critically ill than in patients with a less severe disease course, or in those with no symptoms (Chen et al., 2020). One study reported that COVID-19 patients from ICU have increased serum level MCP-1, granulocyte colony stimulating factor (GCSF), and TNF-α (Huang et al., 2020). This indicates that a cytokine storm

is positively correlated with disease activity/severity in patients with COVID-19. Mortality studies, have reported that elderly patients with ARDS have pulmonary and interstitial tissue damage that was caused by nonspecific inflammatory mediators (Force, 2012).

Implications of understanding the cytokine storm in the specific context of COVID-19:

(1) Cytokines could be the target for treatment via the use of cytokine antagonists and immunomodulators.
(2) Cytokines could be used as markers of disease activity/severity.
(3) Disease monitoring and prediction of deterioration/ mortality.
(4) Individualized treatment should be considered because the presentation of the disease is variable among patients.

CONCLUSIONS

The inflammatory response starts with pathogen recognition and then recruitment of immune cells to the site of infection. Ideally this should ultimately lead to tissue repair and restoration of normal homeostasis. However, local excessive release of cytokines is the decisive factor that leads instead to clinical manifestations and pathological changes. In patients with severe COVID-19, the degree of cytokine increase is also closely related to increased mortality. Therefore, immunomodulation/suppression, and the use of cytokine antagonists, will enhance recovery, and improve survival, in COVID-19 patients. Since December 2019, COVID-19 diagnosis, therapy and research have become areas of great importance. Huge numbers of papers on these topics have already been published. However, further research is needed into the selection of optimal target cells, vectors, and therapeutic methods in order to successfully generate an effective, safe, non-toxic vaccine (or selection of vaccines).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Ai T, Yang Z, Hou H, Zhan C, Chen C, Lv W, Tao Q, Sun Z, Xia L (2020). Correlation of chest CT and RT-PCR testing in coronavirus disease 2019 (COVID-19) in China: a report of 1014 cases. Radiology, Published Online: Feb 26 2020.

American College of Radiology (ACR) (2020). ACR recommendations for the use of chest radiography and computed tomography (CT) for suspected COVID-19 infection. Accessed at www.acr.org/Advocacy-and-Economics/ACR-Position-Statements/Recommendations-for-Chest-Radiography-and-CT-for-Suspected-COVID19-Infection. April 5 2020.

Assiri A, Al-Tawfiq JA, Al-Rabeeah AA (2013). Epidemiological, demographic, and clinical characteristics of 47 cases of Middle East respiratory syndrome coronavirus disease from Saudi Arabia: a descriptive study. Lancet Infectious Diseases 13(9):752-761.

Bhatia M, Zemans RL, Jeyaseelan S (2012). Role of chemokines in the pathogenesis of acute lung injury. American Journal of Respiratory Cell and Molecular Biology 46(5):566-557.

Bogoch A, Watts A, Bachli T, Huber C, Kraemer MUG, Khan K (2020). Pneumonia of unknown etiology in Wuhan, China: potential for international spread via commercial air travel. Journal of Travel Medicine 27(2):taaa008.

Brook CE, Dobson AP (2015). Bats as 'special' reservoirs for emerging zoonotic pathogens. Trends in Microbiology 23(3):172-180.

CDC (2020). Coronavirus Disease 2019: Pandemic Planning Scenarios. CDC. Available @ https://www.cdc.gov/coronavirus/2019-ncov/hcp/planning-scenarios.html. May 20, 2020; Accessed: May 26, 2020.

Chan JF, Lau SK, To KK, Cheng VC, Woo PC, Yuen K-Y (2015). Middle East respiratory syndrome coronavirus: another zoonotic betacoronavirus causing SARS-like disease. Clinical Microbiology Reviews 28(2):465-522.

Chan WM, Ip JD, Chu AW, Yip CC, Lo LS, Chan K-H, Ng AC-K, Poon RW-S, To W-K, Tsang OT-Y, Leung W-S, Kwan MY-W, Chua GT, Chung TW-H, Hung IF-N, Kok K-H, Cheng VC-C, Chan JF-W, Yuen K-Y, To KK-W (2020). Identification of nsp1 gene as the target of SARS-CoV-2 real-time RT-PCR using nanopore whole-genome sequencing. Journal of Medical Virology 5:10.1002/jmv.26140.

Channappanavar R, Perlman S (2017). Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. Seminars in Immunopathology 39(5):529-539.

Chen L, Liu H-G, Liu W, Liu J, Liu K, Shang J, Deng Y, Wei S (2020). Analysis of clinical features of 29 patients with 2019 novel coronavirus pneumonia. Chinese Journal of Tuberculosis and Respiratory Diseases 43(0):E005.

Corman VM, Muth D, Niemeyer D, Drosten C (2018). Hosts and Sources of Endemic Human Coronaviruses. Advances in Virus Research 100:163-188.

Crayne CB, Albeituni S, Nichols KE, Cron RQ (2019). The immunology of macrophage activation syndrome. Frontiers in Immunology 10:119.

Di Micco P, Russo V, Carannante N, Imparato M, Rodolfi S, Cardillo G, Lodigiani C (2020). Clotting Factors in COVID-19: Epidemiological Association and Prognostic Values in Different Clinical Presentations in an Italian Cohort. Journal of Clinical Medicine 9(5):1371.

Du Toit A (2020). Outbreak of a novel coronavirus. Nature Reviews Microbiology 18(123):10.1038/s41579.

European Centre for Disease Prevention and Control (2020). Available @https://www.ecdc.europa.eu/en/covid-19/latest/evidence/coronaviruses Accessed: June 9, 2020.

Fang Y, Zhang H, Xie J (2020). Sensitivity of chest CT for COVID-19: comparison to RT-PCR. Radiology 2020: 200432 Published Online: Feb 19 2020, https://doi.org/10.114.

Ferrara JL, Abhyankar S, Gilliland DG (1993). Cytokine storm of graft-versus-host disease: a critical effector role for interleukin-1. Transplantation Proceedings 25(1 Pt 2):1216-1217.

Force T (2012). Acute respiratory distress syndrome: the Berlin definition. JAMA 307(23):2526-253.

Gan SD, Patel KR (2013). Enzyme immunoassay and enzyme-linked immunosorbent assay. Journal of Investigative Dermatology 133:e12.

Gheblawi M, Wang K, Viveiros A (2020). Angiotensin-Converting Enzyme 2: SARS-CoV-2 Receptor and Regulator of the Renin-Angiotensin System: Celebrating the 20th Anniversary of the Discovery of ACE2. Circulation Research 126(10):1456-1474.

Giri R, Bhardwaj T, Shegane M (2020). When Darkness Becomes a Ray of Light in the Dark Times: Understanding the COVID-19 via the Comparative Analysis of the Dark Proteomes of SARS-CoV-2, Human SARS and Bat SARS-Like Coronaviruses (BOOK). Cold

Spring harber Lab Publisher, doi: https://doi.org/10.1101/2020.03.13.990598. this version posted April 3, 2020.
Guan WJ, Ni ZY, Hu Y, Liang W-H, Ou C-Q, He J-X, Liu L, Shan H, Lei C-L, Hui DSC, Du B, Li L-J, Zeng G, Yuen K-Y, Chen R-C, Tang C-L, Wang T, Chen P-Y, Xiang J, Li S-Y, Wang J-L, Liang Z-J, Peng Y-X, Wei L, Liu Y, Hu Y-H, Peng P, Wang J-M, Liu J-Y, Chen Z, Li G, Zheng Z-J, Qiu S-Q, Luo J, Ye C-J, Zhu S-Y, Zhong N-S (2020). Clinical characteristics of 2019 novel coronavirus infection in China. medRxiv 2020. https://www.medrxiv.org/content/10.1101/2020.02.06.20020974v1. Published in New England Journal of Medicine. April 30 2020, 382:1708-1720.
Hall EJ (2002). Lessons we have learned from our children: cancer risks from diagnostic radiology. Pediatric Radiology 32:700-706.
Han H, Liu F, Lv Z, Wu K, Liu Y, Feng Y, Zhu C (2020). Positive rate of RT-PCR detection of SARS-CoV-2 infection in 4880 cases from one hospital in Wuhan, China, from Jan to Feb 2020. Clinica Chimica Acta 505:172-175.
Holshue ML, DeBolt C, Lindquist S (2020). Washington State 2019-nCoV Case Investigation Team. First Case of 2019 Novel Coronavirus in the United States. The New England Journal of Medicine 382:929-936
Hong JY, Han K, Jung JH (2019). Association of exposure to diagnostic low-dose ionizing radiation with risk of cancer among youths in South Korea. JAMA Network Open 2:e1910584.
Hope M, Raptis C, Henty T (2020). Chest Computed Tomography for Detection of Coronavirus Disease 2019 (COVID-19): Don't Rush the Science. Annals of Internal Medicine, April 8 2020: https://www.doi.org/10.7326/M20-1382.
Hu L, Wang C (2020). Radiological role in the detection, diagnosis and monitoringfor the coronavirus disease 2019 (COVID-19). European Review for Medical and Pharmacological Sciences 24(8):4523-4528.
Huang C, Wang Y, Li X (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet 395(10223):497-506.
Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao R, Jin Q, Wang J, Cao B (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet 395(10223):497-506.
Jacobi A, Chung M, Bernheim A, Eber C (2020). Portable chest X-ray in coronavirusdisease-19 (COVID-19): A pictorial review. Clinical Imaging 64:35-42.
Kalafat E, Yaprak E, Cinar G, Varli B, Ozisik S, Uzun C, Azap A, Koc A (2020). Lung ultrasound and computed tomographic findings in pregnant woman with COVID-19. Ultrasound in Obstetrics & Gynecology 55(6):835-837.
Kalra MK, Maher MM, Rizzo S (2004). Radiation exposure from chest CT: issues and strategies. Journal of Korean Medical Science 19:159-166.
Lai MM, Cavanagh D (1997). The molecular biology of coronaviruses. Advances in Virus Research 48:1-100.
Li G, Fan Y, Lai Y (2020a). Coronavirus infections and immune responses. Journal of Medical Virology 92(4):424-432.
Li Q, Guan X, Wu P (2020b). Early Transmission Dynamics in Wuhan, China, of Novel Coronavirus-Infected Pneumonia. The New England Journal of Medicine 382(13):1199-1207.
Li Y, Guo F, Cao Y (2020c). Insight into COVID-2019 for pediatricians. Pediatric Pulmonology 55(5):E1-E4.
Liang W, Guan W, Chen R, Wang W, Li J, Xu K, Li C, Ai Q, Lu W, Liang W, Li S, He J (2020). Cancer patients in SARS-CoV-2 infection: a nationwide analysis in China. Lancet Oncology 21(3):335-337.
Liu L, Wei Q, Lin Q, Fang J, Wang H, Kwok H, Tang H, Nishiura K, Peng J, Tan Z, Wu T, Cheung K-W, Chan K-H, Alvarez X, Qin C, Lackner A, Perlman S, Yuen K-Y, Chen Z (2019). Anti–spike IgG causes severe acute lung injury by skewing macrophage responses during acute SARS-CoV infection. JCI insight 4(4):e123158.
Lu H (2020). Drug treatment options for the 2019-new coronavirus (2019-nCoV). BioScience Trends 10.5582:bst.2020.
Lu H, Stratton CW, Tang Y-W (2020). Outbreak of pneumonia of unknown etiology in Wuhan, China: The mystery and the miracle. Journal of Medical Virology 92(4):401-402.
Ma H, Hu J, Tian J (2020). A single-center, retrospective study of COVID-19 features in children: a descriptive investigation. BMC Medicine 18(1):123.
Mardani R, Vasmehjani AA, Zali F, Gholami A, Nasab SDM, Kaghazian H, Kaviani M, Ahmadi N (2020). Laboratory Parameters in Detection of COVID-19Patients with Positive RT-PCR; a Diagnostic Accuracy Study. Archives of Academic Emergency Medicine 8(1):e43.
Million M, Lagier JC, Gautret P, Colson P, Fournier PE, Amrane S, Hocquart M, Mailhe M, Esteves-Vieira V, Doudier B, Aubry C, Correard F, Giraud-Gatineau A, Roussel Y, Berenger C, Cassir N, Seng P, Zandotti C, Dhiver C, Ravaux I, Tomei C, Eldin C, Tissot-Dupont H, Honoré S, Stein A, Jacquier A, Deharo JC, Chabrière E, Levasseur A, Fenollar F, Rolain JM, Obadia Y, Brouqui P, Drancourt M, La Scola B, Parola P, Raoult D (2020). Early treatment of COVID-19 patient's with hydroxychloroquine and azithromycin: A retrospective analysis of 1061 cases in Marseille, France. Travel Medicine and Infectious Disease 35:101738.
Murthy S, Gomersall CD, Fowler RA (2020). Care for Critically Ill Patients With COVID-19. JAMA 323(15):1499-1500.
Nishiura H, Jung SM, Linton NM, Kinoshita R, Yang Y, Hayashi K, Kobayashi T, Yuan B, Akhmetzhanov AR (2020). The extent of transmission of novel coronavirus in Wuhan, China, 2020. Journal of Clinical Medicine 9 p.
Okamaoto K, Shirato K, Nao N, Saito S, Kageyama T, Hasegawa H, Suzuki T, Matsuyama S, Takeda M (2020). An assessment of real-time RT-PCR kits for SARS-CoV-2detection. Japanese Journal of Infectious Diseases doi:10.7883/yoken.JJID.2020.108.
Ortiz-Prado E, Simbaña-Rivera K, Gómez-Barreno L (2020). Clinical, molecular, and epidemiological characterization of the SARS-CoV-2 virus and the Coronavirus Disease 2019 (COVID-19), a comprehensive literature review. Diagnostic Microbiology and Infectious Disease 98:1.
Peiris JSM, Lai ST, Poon LLM (2003). Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet 361(9366):1319-1325.
Reptics CA, Hammer MM, Short RG, Shah A, Bhalla S, Bierhals AJ, Filev PD, Hope MD, Jeudy J, Kligerman SJ, Henry TS (2020). Chest CT and COVID-19: a critical review of the literature to date. American Journal of Roentgenology, 2020. https://www.ajronline.org/doi/full/10.2214/AJR.20.23202.
Ren L-L, Wang Y-M, Wu Z-Q (2020). Identification of a novel coronavirus causing severe pneumonia in human: a descriptive study. Chinese Medical Journal 133(9):1015-1024.
Rivett L, Sridhar S, Sparkes D, Routledge M, Jones NK, Forrest S, Young J, Pereira-Dias J, Hamilton WL, Ferris M, Torok ME, Meredith L, CITIID-NIHR COVID-19 BioResource Collaboration, Gupta R, Lyons PA, Toshner M, Warne B, Bartholdson Scott J, Cormie C, Gill H, Kean I, Maes M, Reynolds N, Wantoch M, Caddy S, Caller L, Feltwell T, Hall G, Hosmillo M, Houldcroft C, Jahun A, Khokhar F, Yakovleva A, Butcher H, Caputo D, Clapham-Riley D, Dolling H, Furlong A, Graves B, Gresley EL, Kingston N, Papadia S, Stark H, Stirrups KE, Webster J, Calder J, Harris J, Hewitt S, Kennet J, Meadows A, Rastall R, Brien CO, Price J, Publico C, Rowlands J, Ruffolo V, Tordesillas H, Brookes K, Canna L, Cruz I, Dempsey K, Elmer A, Escoffery N, Jones H, Ribeiro C, Saunders C, Wright A, Nyagumbo R, Roberts A, Bucke A, Hargreaves S, Johnson D, Narcorda A, Read D, Sparke C, Warboys L, Lagadu K, Mactavous L, Gould T, Raine T, Mather C, Ramenatte N, Vallier AL, Kasanicki M, Eames PJ, McNicholas C, Thake L, Bartholomew N, Brown N, Parmar S, Zhang H, Bowring A, Martell G, Quinnell N, Wright J, Murphy H, Dunmore BJ, Legchenko E, Gräf S, Huang C, Hodgson J, Hunter K, Martin J, Mescia F, O'Donnell C, Pointon L, Shih J, Sutcliffe R, Tilly T, Tong Z, Treacy C, Wood J, Bergamaschi L, Betancourt A, Bowyer G, De Sa A, Epping M, Hinch A, Huhn O, Jarvis I, Lewis D, Marsden J, McCallum S, Nice F, Curran MD, Fuller S, Chaudhry A, Shaw A, Samworth RJ, Bradley JR, Dougan G, Smith KG, Lehner PJ, Matheson NJ, Wright G, Goodfellow IG, Baker S, Weekes MP (2020). Screening of healthcare workers for SARS-CoV-2 highlights the role of asymptomatic carriage in COVID-19 transmission. Elife. 2020 May 11:9.e58728.
Rothan HA, Byrareddy SN (2020). The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak. Journal of Autoimmunity

109:102433.
Rouse BT, Sehrawat S (2010). Immunity and immunopathology to viruses: what decides the outcome? Nature Reviews Immunology 10(7):514-526.
Schiaffino S, Tritella S, Cozzi A, Carriero S, Blandi L, Ferraris L, Sardanelli F (2020). SARS-CoV-2 Pandemic in Lombardy, Italy. Journal of Thoracic Imaging 35(4):W105-W106.
Shuo S, Gary W, Weifeng S, Jun L, Alexander CKL, Jiyong Z, Wenjun L, Yuhai B, George FG (2016). Epidemiology, Genetic Recombination, and Pathogenesis of Coronaviruses. Trends in Microbiology 24(6):490-502.
Singh AK, Singh A, Singh R, Misra A (2020). Hydroxychloroquine in patients withCOVID-19: A Systematic Review and meta-analysis. Diabetology & Metabolic Syndrome 14(4):589-596.
Snijder EJ, Van Der Meer Y, Zevenhoven-Dobbe J, Onderwater JJ, Meulen JVD, Koerten HK, Mommaas AM (2006). Ultrastructure and origin of membrane vesicles associated with the severe acute respiratory syndrome coronavirus replication complex. Journal of Virology 80(12):5927-5940.
Son HA, Hang DTT, Thuan ND, Quyen LTB, Thuong LTH, Nga VT, Quang LB, Hung TT, Son NT, Linh NT, Nam LV, Ba NV, Tien TV, Quyet D, Luong HV, Su HX (2020). Simple method for detection of a novel coronavirus (SARS-CoV-2) using one-stepRT-PCR followed by restriction fragment length polymorphism. Journal of Medical Virology 12:10.1002/jmv.26171.
Stalin Raj V, Osterhaus ADME, Fouchier RAM, Haagmans BL (2014). MERS: emergence of a novel human coronavirus. Current Opinion in Virology 5:58-62.
Tyrrell DA, Bynoe ML (1965).Cultivation of a novel type of common-cold virus in organ. British Medical Journal 1(5448):1467-1470.
Walla AC, Park Y-J, Tortorici AM (2020). Structure, Function, and Antigenicity of the SARS-CoV-2 Spike Glycoprotein. Cell 181(2):281-292.
Wang C, Yu H, Horby PW, Cao B, Wu P, Yang S, Gao H, Li H, Tsang TK, Liao Q, Gao Z, Ip DKM, Jia H, Jiang H, Liu B, Ni MY, Dai X, Liu F, Kinh NV, Liem NT, Hien TT, Li Y, Yang J, Wu JT, Zheng Y, Leung GM, Farrar JJ, Cowling BJ, Uyeki TM, Li L (2014). Comparison of patients hospitalized with influenza a subtypes H7N9, H5N1, and 2009 pandemic H1N1. Clinical Infectious Diseases 58(8):1095-1103.
Wang W, Tang J, Wei F (2020). Updated understanding of the outbreak of 2019 novel coronavirus (2019-nCoV) in Wuhan, China. Journal of Medical Virology 92(4):441-447.
Weiss SR, Navas-Martin S (2005). Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. Microbiology and Molecular Biology Reviews 69(4):635-664.
WHO - World Health Organization (2020b). Laboratory testing for 2019 novel coronavirus (2019-nCoV) in suspected human cases. Interim guidance, Available @ https://www.who.int/publications-detail/laboratory-testing-for-2019-novel-coronavirus-in-suspected-human-cases-20200117
World Health Organization (2020). "Coronavirus disease 2019 (COVID-19): situation report, 29". World Health Organization, February 19 2020. hdl:10665/331118 8 Rapid Expert Consultation Update on SARS-CoV-2 Surface Stability and Incubation for the COVID-19 Pandemic. The National Academies Press. 2020 March 27. doi:10.17226/25763. ISBN 978-0-309-67610-6. Retrieved May 18 2020.
World Health Organization (2020). Coronavirus disease 2019 (COVID-19) Situation Report – 48. World Health Organization. Available @ https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200308-Accessed: March 9, 2020.
Xiang F, Wang X, He X, Peng Z, Yang B, Zhang J, Zhou Q, Ye H, Ma Y, Li H, Wei X, Cai P, Ma W-L (2020). Antibody Detection and Dynamic Characteristics in Patients with COVID-19. Clinical Infectious Diseases doi:10.1093/cid/ciaa461.Epub ahead of print. PMID: 32306047; PMCID: PMC7188146.
Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, Liu S, Zhao P, Liu H, Zhu L, Tai Y, Bai C, Gao T, Song J, Xia P, Dong J, Zhao J, Wang F-S (2020). Pathological findings of COVID-19 associated with acute respiratory distress syndrome. Lancet Respiratory Medicine 8(4):420-422.
Yalow RS, Berson SA (1996). Immunoassay of endogenous plasma insulin in man. Obesity Research 4:583-600.
Yan C, Cui J, Huang L, Du B, Chen L, Xue G, Li S, Zhang W, Zhao L, Sun Y, Yao H, Li N, Zhao H, Feng Y, Liu S, Zhang Q, Liu D, Yuan J (2020). Rapid and visual detection of 2019 novel coronavirus (SARS-CoV-2) by a reverse transcription loop-mediated isothermal amplification assay. Clinical Microbiology and Infection 26(6):773-779.
Yip CC, Ho CC, Chan JF, To KK, Chan HS, Wong SC-Y, Leung K-H, Fung AY-F, Ng AC-K, Zou Z, Tam AR, Chung TW-H, Chan K-H, Hung IF-N, Cheng VC-C, Tsang OT-Y, Tsui SKW, Yuen K-Y (2020). Development of a Novel, Genome Subtraction-Derived, SARS-CoV-2-SpecificCOVID-19-nsp2 Real-Time RT-PCR Assay and Its Evaluation Using Clinical Specimens. International Journal of Molecular Sciences 21(7):2574.
Yong SEF, Anderson DE, Wei WE, Pang J, Chia WN, Tan CW, Teoh YL, Rajendram P, Toh MPHS, Poh C, Koh VTJ, Lum J, Suhaimi NM, Chia PY, Chen MI, Vasoo S, Ong B, Leo YS, Wang L, Lee VJM (2020). Connecting clusters of COVID-19: an epidemiological and serological investigation. Lancet Infectious Diseases 20(7):809-815.
Yuen KY, Wong SS (2005). Human infection by avian influenza A H5N1. Hong Kong Medical Journal 11(3):189-199.
Zeng Z, Xu L, Xie XY, Yan HL, Xie BJ, Xu W-Z, Liu X-A, Kang G-J, Jiang W-L, Yuan J-P (2020). Pulmonary Pathology of Early Phase COVID-19 Pneumonia in a Patient with a Benign Lung Lesion. Histopathology 6:10.1111/his.14138.
Zhang C, Wu Z, Li JW, Zhao H, Wang GQ (2020). The cytokine release syndrome (CRS) of severe COVID-19 and Interleukin-6 receptor (IL-6R) antagonist:tocilizumab may be the key to reduce the mortality. International Journal of Antimicrobial Agents Article 105954.
Zhao S, Lin Q, Ran J, Musa SS, Yang G, Wang W, Lou Y, Gao D, Yang L, He D, Wang MH (2020). Preliminary estimation of the basic reproduction number of novel coronavirus (2019-nCoV) in China, from 2019 to 2020: a data-driven analysis in the early phase of the outbreak. International Journal of Infectious Diseases (IJID) 92:214-217.
Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, Wang D, Xu W, Wu G, Gao GF, Phil D, Tan W, for the China Novel Coronavirus Investigating and Research Team (2020). A Novel Coronavirus from Patients with Pneumonia in China, 2019. The New England Journal of Medicine 382(8):727-733.

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Comparative study of maggots' production in brewery wet grains, dung and rumen content of cattle with or without addition of attractant

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The objective of this study was to compare maggots' production in different substrates following an experimental design of six treatments (S1, 1000 g of brewery wet grains - BWG; S2, 900 g of BWG and 100 g of blood; S3, 1000 g of dung; S4, 900 g of dung and 100 g of blood; S5, 1000 g of rumen content; and S6, 900 g of rumen content and 100 g of blood) repeated trice. 121 flies were collected and identified and 5 days after putting in place the experimental set up, larvae were collected. 6 families of flies, namely Calliphoridae (50.41%), Muscidae (18.18%), Sacrophagidae (12.40%), Strationomiydae (8.26%), Piophilidae (5.79%) and Syrphidae (4.96%) were identified. The highest fresh biomass (41.67±3.51 g) was obtained with S6 and S2 (21.33±15.63 g). The mean highest length (2.62±1.01 cm) was obtained with S3. The highest dry matter (DM) (23.89±2.90%) was obtained with S2 and the lowest DM with S6 (13.56±2.90%). The lowest values of crude proteins (CP) were obtained in S4 (32.82±10.05%) and S6 (33.82±0.18%). Highest fats contents were obtained in S2 (43.93±1.46%) and S4 (26.99±1.12%). In short, S2 can be used for maggots' production.

Key words: Identification, flies, production, larvae, insect breeding, bovine blood.

INTRODUCTION

In Africa, high costs of dietary protein sources constitute a real challenge on livestock development (Bouafou, 2000). This situation is largely due to the unavailability of inputs, competition between humans, animals and

industries over basic feed ingredients for livestock (Dronne, 2018). Gabon in particular is not on the sidelines of this observation and FAO (2016) pointed out that this country imports 96% of its needs in animal products and due to the increasing demand, the local livestock sector is facing many challenges to be addressed, among which the limited access to expensive animal feed. So, efforts have been made in order to valorize new and cheap locally and easily accessible protein sources for livestock feed, namely, flours from maggots, cockroaches, termites, grasshoppers, earth worms, etc., in order to boost the animal production sector (Hardouin et al., 2000). Among all these insects, maggots' production is easier and maggots' flour has an interesting nutritive value. Maggots are rich in reserve nutrients such as fats and proteins (47.50 and 52.23%) and potentially in essential amino acids (Bouafou et al., 2007). They can contribute in improving feeding in non-ruminants such as fishes, poultry and rodents (Mensah et al., 2002). Nutrient content in maggots depends on the type of substrate used (Sogbessan et al., 2006; Bouafou et al., 2008). All fast decaying organic matters can serve as substrates for fly laying and maggot development (Hardouin et al., 2000). Considering the high cost and the scarcity of protein sources commonly used in livestock feeding and in local markets, it seems that getting the right substrates of maggots' production can contribute in the reduction of animal production costs. The objective of this work was to determine best substrates for maggots' production.

MATERIALS AND METHODS

The substrates

The fresh cattle dung was collected at the stable of the National Higher Institute of Agronomy and Biotechnology (INSAB) of the University of Science and Techniques Masuku (USTM), at Franceville in the Haut-Ogooué province. This zone is situated at a latitude of 1°37'59'' south, a longitude of 13°35'00''and an altitude of 405 m above the sea level (FDNS, 2004).

The blood and the rumen content from cattle were collected fresh at a slaughter house of the town, and the BWG was also collected from a brewery industry of the same town. The blood is used as attractant meanwhile the BWG, the cattle dung and rumen content were used as basic substrates.

Experimental design

The completely randomized experimental design is made up of six treatments of 1000 g each, repeated 3 times in plastic basins of 86 cm of diameter and 14 cm of height. The substrates' composition according to the treatments is as follows: S_1, 1000 g of BWG; S_2, 900 g of BWG + 100 g of blood; S_3, 1000 g of cattle dung; S_4, 900 g of cattle dung + 100 g of blood; S_5, 1000 g of rumen content; S_6, 900 g du rumen content + 100 g of blood.

The breeding was conducted in a semi-hard building, isolated, ventilated, and sheltered from bad weather where flies could enter and leave freely. The entire design was placed in two boxes of 3.0 m length × 2.5 m width (7.5 m^2). The boxes were left open during 24 h for colonization (Bouafou et al., 2006). 24 h after putting in place the experimental set up, basins remained open to allow the flies to lay eggs on the substrates, then they were covered using lids (Figure 1). These lids had small aeration holes made with the help of a nail. The daily follow up of the experimentation was done trice (at 8 am, 12 am and 5 pm). The basins were open during the periods for a good aeration of the culture media.

Data collection

Identification of flies

For the identification of different colonizing flies in the substrates, 121 flies were collected with the help of a cage covered with a mosquito net treated with a RAMBO brand insecticide (Figure 2) mixed with Trafluthrin (0.25%) and Permethrin (0.25%) active ingredients. The cage contained plates in which were placed small quantities of substrates (cattle dung, rumen content, BWG) as well as the attractant (bovine blood).

Collection of maggots

Once the flies were trapped in the cage, they were killed by the insecticide, collected and conserved in alcohol at 70°C. Collected flies were identified by observation of morphological characters using an OPTIKA brand binocular loupe at X2 magnification. Harvesting of fly larvae was done 5 days after setting up the treatments. Two harvesting methods were used:

(1) The first method consisted of spreading the substrate on a harvesting device made up of a sieve and a receptacle, both exposed to sunlight. Maggots fleeing the sunlight fall to the bottom of the receptacle and are collected. This method is only for the substrate S_2 (BWG + blood). This substrate is very compact and makes manual sorting difficult.
(2) The second method is the manual sorting. It consists of soaking the substrates in hot water (60°C) in order to kill the maggots. Maggots' mobility makes the collection difficult. Once the substrates are soaked, parts of the killed larvae float in hot water and are then picked up with the help of a strainer, meanwhile the others (dead and alive) are trapped in the substrate. For the maggots' collection, 2 basins, one strainer, a plastic pot for each substrate and a filter were used. Trapped larvae were picked up by removing small quantities of substrates over time. After collection, all the maggots were transferred in 18 labelled pots according to different substrates. To kill the still alive larvae, hot water was introduced into the pots containing them.

Chemical analyses

For chemical analyses, dried maggots were ground manually with the help of a bowel and conserved in labelled plastic sachets

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Figure 1. Experimental design 24 h after setting up.

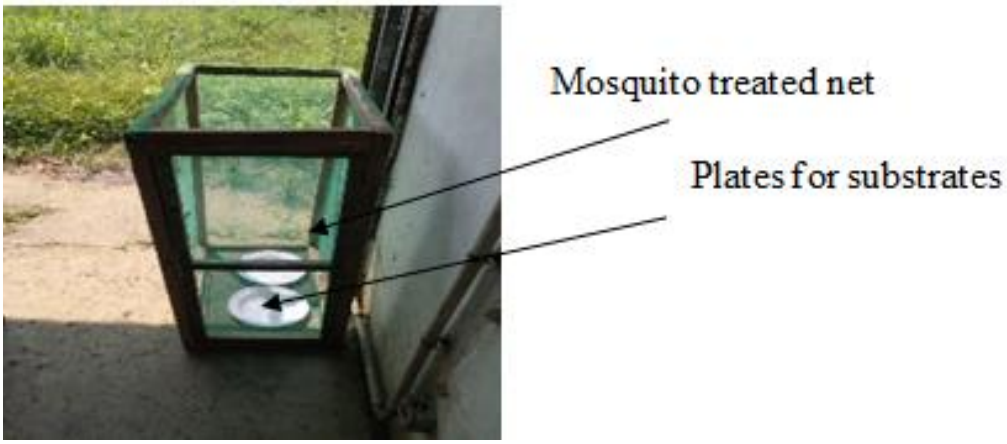

Figure 2. Fly trap.

(Figure 3). The analyses of flour samples were carried out in the laboratory to determine their chemical characteristics (water content (%); dry matter - DM (%), crude proteins - CP (%); fats content (%)) with two repetitions. The DM content was determined according to the method described by AOAC (1990). The water content was obtained via the determination of the humidity rate = [(fresh weight - dry weight) / fresh weight] × 100. The CP content was obtained using the Bradford method (1976) and the fats content using the extraction method with the help of the Soxhlet design.

Productivity of substrates: Fresh weight and dry weight

In order to determine the biomass of maggots produced, their fresh and dry weights were recorded according to substrates. After having removed water from pots, larvae are exposed on LOTUS® paper (Figure 4) during about 20 min before recording the fresh weight. This paper absorbs the water present on maggots' body. Weighing was done using a digital SARTORIUS® brand balance (capacity 500 g and precision 0.1 g).

After recording fresh weight, larvae were dried in an oven at 55°C during 24 h. After cooling, larvae were once weighed with the same balance.

Measurements in larvae

The length of a maggot was determined using 10 fresh maggots

Figure 3. Maggots' flour conditioned in labelled sachets.

Figure 4. Larvae exposed on LOTUS® paper.

randomly selected per substrate. The measurement is done using a ruler labelled in centimeters. A total of 60 maggots were measured for all the substrates.

Statistical analyses

Data on biomass, measurements and chemical composition of maggots were submitted to the analysis of variance at one factor (substrate) following the procedure of the general linear model (GLM proc.) with SPSS 20.0® software. In case of significant differences between the treatments, separation of means was done using the Waller Duncan test at 5% significant level (Steel and Torrie, 1980).

RESULTS

Results are related to the identification of colonizing flies

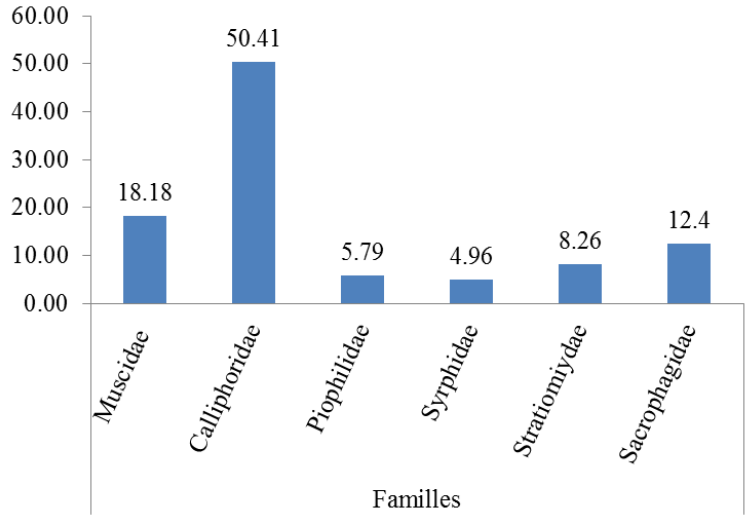

Figure 5. Proportions of identified flies according to families.

Table 1. Biomass and average measurements of maggots according to substrates.

Parameter		Substrates					
		S_1	S_2	S_3	S_4	S_5	S_6
Number		10	10	10	10	10	10
Biomass (g)	Fresh (g)	3.33± 2.52^{a}	21.33± 15.63^{b}	13.33± 3.21^{ab}	3.33± 1.53^{a}	11.67±6.11^{ab}	41.67± 3.51^{c}
	Dry (g)	0.58± 0.48^{a}	5.24± 4.81^{b}	1.91± 1.11^{ab}	0.65± 0.20^{a}	1.81± 0.48^{ab}	5.67± 0.98^{b}
Measurements (cm)		1.02± 0.14^{a}	0.90± 0.16^{a}	2.62± 1.01^{b}	1.52± 0.36^{a}	1.03± 0.20^{a}	1.28± 0.14^{a}

a, b, c : Means bearing the same letters on the same line are not significantly different (P>0.05). S_1: Simple brewery wet grains (BWG); S_2: BWG + blood; S_3: Simple cattle dung; S_4: Cattle dung + blood; S_5: Rumen content only; S_6: Rumen content + blood.

in substrates, biomass and mean measurements of maggots produced and their chemical composition.

Identification colonizing flies in substrates

The proportions of identified fly families are presented in Figure 5. This shows that the most represented family is Calliphoridae (50.41%) followed by Muscidae (18.18%). Less represented families are Piophilidae (5.79%) and Syrphidae (4.96%).

Biomass and average measurements of maggots produced

The biomass distribution in fresh and dry maggots as well as the respective mean length according to substrates is shown in Table 1. It reveals that the maggots from the substrate S_6 had the highest fresh matter content (41.67 ± 3.51 g) followed by maggots from the substrate S_2 (21.33 ± 15.63 g). There is no significant difference ($p>0.05$) for the values presented by the substrates S_1 and S_4 (3.33 ± 2.52^{a} versus 3.33 ± 1.53^{a}, $p>0.05$) and between the substrates S_3 and S_5 (13.33 ± 3.21^{ab} versus 11.67 ± 6.11^{ab} $p>0.05$). The same observation was made for the dried larvae whose weights are higher in the substrates S_2 and S_6 which are comparable, the couple of substrates S_3 and S_5, same for S_1 and S_4. So, except in S_3 (only cattle dung), the addition of blood caused an increase in fresh biomass from the substrates S_2 and S_6, respectively BWG + blood and the rumen content + blood. The growth in length of maggots has been more pronounced in the substrate S_3 compared to the maggots from the other substrates. The difference could be due to the quality of substrates: addition of blood could lead to the reduction in maggots' length against the substrate containing only dung.

Chemical composition of maggots' flour

The Table 2 presents the chemical composition of maggots' flour according to different substrates. Maggots

Table 2. Chemical composition of maggots' flour according to different substrates.

Parameter	Substrates					
	S_1	S_2	S_3	S_4	S_5	S_6
Water (%)	82.25±3.73^{ab}	76.11±6.66^{a}	86.38±4.44^{b}	79.30±4.50^{ab}	82.08±7.15^{ab}	86.44±12.77^{b}
DM (%)	17.76±2.90^{ab}	23.89±2.90^{b}	13.62±2.90^{a}	20.70±2.90^{ab}	17.92±2.90^{b}	13.56±2.90^{a}
CP (%)	53.81±0.96^{b}	55.09±2.25^{bc}	62.20±1.52^{c}	32.82±10.05^{a}	56.45±1.16^{bc}	33.82±0.18^{a}

a, b, c, d, e, f: Means bearing the same letters on the same line are not significantly different (P>0.05). S_1: BWG only; S_2: BWG + blood; S_3: Cattle dung only; S_4: Cattle dung + blood; S_5: Rumen content only; S_6: Rumen content + blood.

from the substrate S_6 had the highest water content (86.44 ± 12.77%) while the lowest water content (76.11 ± 6.66%) was observed in the substrate S_2. The addition of blood had no effect on the water content of maggots in all the substrates. The opposite trend is observed in terms of DM content, and inversely revealed that the substrate S_2 has the highest DM content (23.89 ± 2.90%), meanwhile the lowest DM content is from the substrate S_6 (13.56 ± 2.90%). These results showed that the addition of blood in the substrate made up of rumen content caused a decrease in DM content of maggots' flour. The CP content in substrates is ranked as follows: $S_5>S_2>S_1>S_6>S_4$. It can be concluded that the addition of blood in substrates (dung and rumen content) caused a decrease in CP content of maggots' flour. Concerning fats, the highest content was obtained with maggots' flour from the substrate S_2 (43.93 ± 1.46%) and the lowest content was obtained in the substrate S_6 (14.02 ± 0.51%). This means that in substrates made up of BWG and dung, the addition of blood led to an increase in lipid content unlike the level of substrate which basically constituted rumen content.

DISCUSSION

The most represented fly family was Calliphoridae with 50.41% of identified individuals. The same observation was made by Bouafou et al. (2006) during a study on fly inventory carried out on different by-products. The marked presence of this family could be justified by its large size, its social and cosmopolitan character. In fact, according to Byrd and Castner (2010), this family contains more than 1000 species and the members can be found worldwide; in addition, Calliphoridae like flying in groups and when a fly detects an excrement to lay on, it sends out pheromones to prevent the others (Claude and Daniel, 2013). Then, they arrive massively and firstly colonize the medium. Muscidae is the second most represented family (18.18%). The site chosen for this study and the great height of these species could justify that position. In fact, according to Byrd and Castner (2010), Muscidae are the flies belonging to a large synanthropic family, having a cosmopolitan distribution with more than 4000 species in the world. Both Piophilidae (5.79%) and Syrphidae (4.96%) are less represented. The height of Piophilidae as well as their uneven distribution in the world could justify this low rate. In fact, according to Byrd and Castner (2010), Piophilidae represents a small family with 69 species found in the world. As the family Syrphidae is concerned, the scarcity of flowers on the study site could justify their low abundance; in fact, the adults from this family live on flowers (Dussaix, 2009).

The addition of blood has caused an increase in fresh biomass from the substrates S_2 and S_6. These results are in the same line with those of Tendonkeng et al. (2017) who showed that the addition of 10% of fresh bovine blood in rumen content, in swine dung and in the mixture of both improves on the biomass of maggots produced. These authors obtained a fresh biomass of 66.10 g in the rumen content + blood versus 31.30 g in simple rumen content, 189.00 g in swine dung + blood versus 89.40 g in simple swine dung and 128.00 g in the mixture of both (rumen content + swine dung) + blood versus 58.50 g in the mixture of both (rumen content + swine dung) without blood. The fresh biomass obtained with the maggots from substrate S_6 (41.67 ± 3.51 g) is higher than that of Ndadi (2010), obtained with bovine rumen content (10.05 g) and of Keyi (2014) with simple fowl droppings; but close to that obtained by this last author (47.00 ± 5.4 g) with fowl droppings + blood. This difference could be explained by the type of substrates used (Ekoue and Hadzi, 2000; Bouafou, 2007). On the other hand, the substrate S_4 revealed less productive (3.33 ± 1.53 g); this low biomass obtained could be explained by the fact that, cattle dung being already attractive, addition of blood could have led to an increase in the number of flies on the substrate, hence, increasing the number of eggs laid and the number of maggots, hence limiting the nutrient content; so, many larvae die and the resistant ones do not develop well due to insufficient nutriments in the substrates. The addition of blood attracts not only flies but also improves on the nutritional value of the rumen content and the brewery wet grains, favoring the proper development of larvae.

The attractiveness of substrates and the nutrient availability could equally justify the results on measurements. It appears that the greatest length (2.62 ± 1.01 cm) was obtained with maggots from the substrate S_3.

The cattle dung being attractive, the quantity of eggs laid and the number of hatched eggs, proportional to the nutrient availability in the substrate could justify the good development of larvae. The mean lowest length (0.90 ± 0.16 cm) was recorded with the substrate S_2; this value is comparable to the norm indicated by Hardouin et al. (2000), Hardouin and Mahoux (2003), Bouafou et al. (2006), and Keyi (2014). According to these authors, larvae can measure averagely 1 cm (a length comprised between 0.4 and 1.5 cm according to their age in days). The greatest length (2.62 ± 1.01 cm) was recorded with the substrate S_3; this value is higher than that obtained by Tendonkeng et al. (2017) (1.22 cm); this difference could be explained by the good nutritional value in cattle dung and that consequently permits the increase in height of maggots over time (Ekoue and Hadzi, 2000; Bouafou et al., 2006).

The highest humidity rate (86.44 ± 12.77%) from maggots in the substrate S_6 was comparable ($p > 0.05$) to those from maggots in substrates S_5 (82.08 ± 7.15%), S_4 (79.30 ± 4.50%), S_3 (86.38 ± 4.44%) and S_1 (82.25 ± 3.73%) that were also comparable ($p > 0.05$) to the lowest rate obtained with maggots from the substrate S_2 (76.11 ± 6.66%). Generally, these values were lower than that recorded by Bouafou et al. (2007) (92.51%); this difference could be explained by the quantity of water present in these substrates. In another hand, this value of 86.44 ± 12.77%, obtained with maggots from S_6, was close to that obtained by Keyi (2014) who recorded a water content of 87.47 ± 4.49% with maggots from the substrate constituting swine dung + blood. The highest DM (23.89 ± 2.90%) was obtained with maggots' flour from the substrate S_2 and the lowest DM (13.56 ± 2.90%) with flour of maggots from the substrate S_6. It appears from all these values that the DM content is inversely proportional to the humidity rate.

The highest CP content (62.20 ± 1.52%) was obtained with maggots' flour from the substrate S_3; it was comparable ($p > 0.05$) to those in the maggots' flours from substrates S_2 (55.09 ± 2.25%) and S_5 (56.45 ± 1.16%); these values are superior to those found by Sogbessan et al. (2006), Bouafou et al. (2007) and Bouafou et al. (2008), respectively comprised between 47.50 and 54.00% and 52.23 and 50.17% but comparable to those recorded by Tendonkeng et al. (2017) and Keyi (2014), respectively 53.10 and 57.14%. Fats contents of maggots' flour were significantly different ($p < 0.05$) in all the substrates. The lowest value (14.02 ± 0.51%) was recorded with the maggots' flour from S_6. This value is inferior to those obtained by Sogbessan et al. (2006), Bouafou et al. (2007) and Bouafou et al. (2008), respectively 19.30, 24.43, and 35.41%. As far as CP and fats contents are concerned, the difference at the level of the results could be justified not only by the nutrient content in different substrates but also by the developmental stage of the larvae. In fact, Ekoue and Hadzi (2000) and Bouafou et al. (2007) outlined that the chemical composition of maggots' flour may depend on the developmental stage of the larvae. So, the most interesting stage is the maggot but the closest stage prior to its transformation into pupa so as to have all the nutritive elements (protides, lipids and minerals) (Hardouin et al., 2000).

Conclusion

At the end of this study, six families of flies namely, Calliphoridae, Muscidae, Sacrophagidae, Stratiomiydae, Piophillidae and Syrphidae were identified. The most represented family was Calliphoridae. The addition of blood has influenced the biomass, the average length and the chemical composition of maggots' flour.

Based on these results obtained, the substrate S_2 consist of brewery wet grains associated with blood which can be used to produce maggots for the non-ruminants' diets.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Association of Official Analytical Chemist (AOAC) (1990). Official method of analysis 15th edition. AOAC. Washington D. C. 10 p.

Bouafou KGM (2000). Putting in place Common External Tarification (C.E.T.) within U.E.M.O.A.: Influence on Ivorian aviculture. *Maîtrise* Dissertation 35 p.

Bouafou KGM, Kouame KG, Offoumou AM (2007). Nitrogen balance in growing rat fed dried maggots' flour. Tropicultura 25:70-74.

Bouafou KGM, Kouamé KG, Amoikon EK, Offoumou AM (2006). Potentials for maggots' production on by-products in Côte d'Ivoire. Tropicultura 24:157-161.

Bouafou KGM, Zannou-Tchoko V, Konan BA, Kouame KG (2008). A study of the nutritional value of dried maggots' flour on growing rats. Ivorian Review of Science and Technology 12:215-225.

Bradford M (1976). A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry (USA) 72:248-254.

Byrd JH, Castner JL (2010). Insects of forensic importance. Forensic entomology: The utility of arthropods in legal investigations pp. 43-79.

Claude W, Daniel C (2013). Treaty of forenstine entomology: Insects on the scene of crime. www.books.google.ga, consulted on 28/07/2018.

Dronne Y (2018). Raw agricultural materials for human and animal feeding: the world. INRA 31(3):165-180.

Dussaix C (2009). Syrphidae Diptera, ed. Diptera Insects *in* continental invertebrates in the la Loire – Greta country.

Ekoue SE, Hadzi YA (2000). Maggots' production as source of proteins for young poultry in Togo. Preliminary observations. Tropicultura 18:212-214.

Food and Agriculture Organization (FAO) (2016). Boost the livestock sector in Gabon by capacity reinforcement in cooperatives of breeders. www.fao.org, consulted on 12/09/2018.

FDNS (2004). Information for MSKU as of august 2004. https://www.fdsn.org/station_book/IU/MSKU/msku.html

Hardouin J, Mahoux G (2003). Livestock and utilization to the benefit of human and certain animals. Bureau for the Exchange and the Distribution of Information on Micro-Livestock (BEDIM). Gembloux 30:83-93.

Hardouin J, Dongmo T, Ekoue SK, Loa C, Malekani M, Malukisa M (2000). Technical guide of livestock n°7 on maggots. Bureau for the

Exchange and the Distribution of Information on Micro-Livestock (B.E.D.I.M.), ed. J. Hardouin BEDIM.
Keyi RP (2014). Comparative evaluation of production and nutritive value of maggots from fowl droppings and swine dung. Master of Science thesis. University of Dschang. 57p.
Mensah GA, Pomalegni SCB, Koudjou AL, Cakpovi JCG, Adjahoutonon KYKB, Agoundo A (2002). Maggots' flour from fly, a protein source well valorized in barbarie duck feeding. In: Report of the 1st UAC Colloquium of Science and Culture. Natural and Agronomic Sciences. Abomey-Calavi (Benin).
Ndadi NK (2010). Contribution to the study of adequate substrates for production of maggots as feed for poultry in Kinshasa, TFE in Zootechny, Faculty of Agronomic Sciences/Unikin 25p.
Sogbessan AO, Ajuonu N, Musa BO, Adewole AM (2006). Harvesting techniques and evaluation of maggot meal as animal dietary protein source for heteoclarias in outdoor concrete tanks. World Journal of Agriculture Science 4:394-402.
Steel JGD, Torrie JH (1980). Principles and procedures of statistics, 2nd. ed., McGraw-Hill, New York.
Tendonkeng F, Miégoué E, Lemoufouet J, Mouchili M, Matimuini NF, Mboko AV, Zogang BF, Mweugang NN, Zougou TG, Boukila B, Pamo TE (2017). Production and chemical composition of maggots according to the type of substrate. Livestock Research for Rural Development 29(67). Retrieved October 4, 2020, from http://www.lrrd.org/lrrd29/4/ften29067.html

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Quantification of indole-3-acetic acid from *Bambusa tulda* Roxb. seedlings using high performance liquid chromatography

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Indole-3-acetic acid (IAA) is an important plant growth regulator. As the very first endeavor, the study is aimed at extracting and quantifying IAA from seedlings of *Bambusa tulda* and evaluating its bioactivity. The extraction of IAA was performed in organic solvent followed by sample evaporation and TLC with a mobile phase composed of chloroform, methanol and formic acid (77:22:1 v/v) in isocratic mode. The extract obtained from preparatory TLC was subjected to HPLC with acetic acid and methanol (75:25 v/v) as the mobile phase in isocratic mode at a flow rate of 0.8 ml/min and operation pressure of 54 MPa at 30°C, and detection was monitored at 280 nm. Bioassay of the extracted IAA was carried out in *Abelmoschus esculentus* seedlings. The similar Rf value (0.412) of the extract during TLC analysis and similar peak of HPLC chromatogram with retention time 28.71 min to that of standard IAA indicated the presence of IAA in *B. tulda* seedling extract. The extracted IAA was quantified to be 10.28 µg/ml. In bioassay experiment, the extracted IAA significantly enhanced root length, root fresh weight, root dry weight, shoot length, shoot fresh weight, shoot dry weight and significant increase of total chlorophyll and protein in *A. esculentus* leaves. Therefore, *B. tulda* seedlings could be a potential source of IAA, and it can be utilized for production of bio-fertilizer at a commercial scale.

Key words: *Abelmoschus esculentus,* Bamboo, High performance liquid chromatography, indole-3 acetic acid.

INTRODUCTION

The demand for agricultural yield has increased tremendously with an increase in the world's population and thereby leading to a large scale production of chemical fertilizers. Since the use of chemical fertilizers and pesticides in the agricultural fields has caused degradation of soil quality and fertility, thus the expansion of agricultural land with fertile soil is nearly impossible. Hence researchers and scientists have shifted their attention for a safer and productive means of agricultural practices (Gouda et al., 2018). Auxin is a critical phytohormone for plant growth and orchestrates many developmental processes in plants. Complex and redundant regulation of IAA abundance, transport, and response allow an intricate system of auxin utilization that

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achieves a variety of purpose in plant growth and development (Woodward and Bartel, 2005). It is a low molecular weight compound highly involved in the control of plant growth and development (Porfírio et al., 2016). Although there are several naturally occurring compounds comprising auxin activity, IAA is the most abundant auxin (Simon and Petrasek, 2011), only a minor part of which resides in plant in free form, the majority being conjugated to amino acids or sugars (Ludwig et al., 2009).

IAA is considered to be the main biologically active plant hormone of the auxin class and is a product of L-tryptophan (L-Trp) metabolism. It stimulates cell elongation by modifying certain conditions, like increase in cell osmotic contents, increase in water permeability into the cell, decrease in wall pressure, increase in cell wall synthesis, and inducing specific RNA and protein biosynthesis. It promotes antioxidant activity, inhibits or delays abscission of leaves, induces flowering and fruiting (Zhao, 2010). It is a mobile signaling molecule that can be transported among cells to form auxin gradients and auxin maxima that are essential for plant development (Petrasek and Friml, 2009). The widespread use of auxin in plant propagation protocols and physiological studies (Strader et al., 2010) has led to many efforts towards the development of analytical methods for the quantification of the very low auxins levels in plants. Simultaneous quantitative profiling of IAA provides a basis for defining additive, synergistic or antagonistic hormone activities and identifying hormone networks regulating plant functions. For quantitative measurement of endogenous plant hormones in crude plant extracts, HPLC–MS/MS provides high sensitivity, specificity, accuracy and reproducibility (Gomez et al., 2002).

Being a regulating agent in numerous plant growth phenomena, in an attempt to assess its role in plant growth and development, its quantification is crucial a step. The ability to rapidly quantify IAA in small amounts of tissue has great value in the study of auxin biology. As an essential signaling molecule, quantitative information about IAA levels has been a valuable aspect. Guney et al. (2016a) assessed the effect of hormonal applications on seed germination and seedling morphological traits in *Lilium artvinense*. The application of IAA and other phytohormons was recorded to be influential in germination percentage of *Lilium martagon* seeds (Guney et al., 2016b). Similarly, auxin has been found to enhance rooting effect in *Ficus benjamina* L. (Topacoglu et al., 2016) and *Schefflera arboricola* (Sevik et al., 2015). Therefore, IAA has been quantified from tobacco flowers (Liu et al., 2002), aerial parts of *Arabidopsis thaliana* L. (ecotype Columbia) plants, leaves of *Triticum aestivum* L., cv. Jara and *Nicotiana tabacum* L., cv. Bright Yellow 2*, N. tabacum* L., cv. Wisconsin 38 (Dobrev et al., 2005), *Acer mono*, *A. negundo* and *Zea mays* (Zhang et al., 2008) and *A. thaliana* (Barkawi et al., 2010), and leaves and crown of *T. aestivum* cultivars, winter cv. Samanta and spring cv. Sandra (Kosova et al., 2012). There is scanty of research on quantification of IAA from bamboo seedlings. In this research, the IAA from seedlings of *B. tulda* was quantified through HPLC method and its bioassay in *Abelmoschus esculentus* was performed with the hypothesis that bamboo seedlings could be potent source of IAA.

MATERIALS AND METHODS

Sample preparation

Samples were prepared according to Barkawi et al. (2010), Pan et al. (2010), Liu et al. (2012) and Porfírio et al. (2016) with slight modifications. Seeds of *B. tulda* were obtained from the Department of Forest Resources, Janakpur, Nepal. The seeds were surface sterilized in 70% ethanol with 0.1% Tween 20 (polyoxyethylene sorbitan monolaurate) working in a laminar flow hood (Barkawi et al., 2010). The samples were vortexed intermittently for 5 min, and the solution was removed using a sterile Pasteur pipet, taking care to remove as much liquid as possible and seeds were dried overnight in laminar flow hood. About 25 to 35 germinating seeds were placed in sterile filter paper in petri-plates. Experiments were repeated five times and replicated three times making a total of 15 petri plates and they were randomly kept in growth chamber at 28±2°C adjusted to 16 h light and eight hours dark period for 18 days (Figure 1). Autoclaved distilled water was applied to the experiment in the morning (11:00 am-12:00 noon) to avoid water stress. After 18 days, the seedlings were harvested using sterilized forceps; blotted on a laboratory tissue paper and the separated upper portion of the seedlings were stored at -80°C in liquid nitrogen until the time of further analysis. 250 mg stored sample was ground in liquid nitrogen, then it was mixed with methanol (HPLC grade) (4 ml per gram of fresh weight) followed by centrifugation at 4°C for 15 min at 10,000 rpm. The pallet was sonicated for five minutes and re-extracted with methanol. The pH of the solution was acidified to pH 2.5 with 1 M HCL and extracted twice with methanol. The upper organic phase was transferred to a beaker, filtered by syringe filter ($SFPS_{25}X$, 0.45 µm × 25 mm) and evaporated in rotatory evaporator at 35°C. The extract was dissolved in 100 ml methanol and stored at -20°C. The reference (control) solution was obtained by dissolving 5 mg standard IAA (HiMedia) in 5 ml of methanol.

Detection of IAA by thin layer chromatography (TLC)

A thin mark was made 1 cm above the bottom of the TLC plate ($60GF_{254,}$ 20 × 20 cm, Merck) to apply the sample and control (standard IAA) spots (Abubakar et al., 2016). Methanol fraction of 25 µl of test sample and 15 µl of control solution were spotted equidistantly (1 cm) on the TLC plate using capillary tubes and developed in the mixture of chloroform, ethyl acetate and formic acid (77:22:1 v/v) as mobile phase. The mobile phase was poured into the TLC chamber to a leveled few centimeters above the chamber bottom. The solvent was allowed to saturate the container. The plate was then immersed in such way that the sample spots were well above the level of mobile phase, but not immersed in the solvent for development. After migration of the principal components, detection was performed in UV light (254nm) by spraying with the mixture of 2 ml of 0.5 M $FeCl_3$ in 98 ml of 35% $HClO_4$ (Rahman et al., 2010), and the spots with retardation factor (R_f) value identical to standard IAA (HiMedia) was calculated.

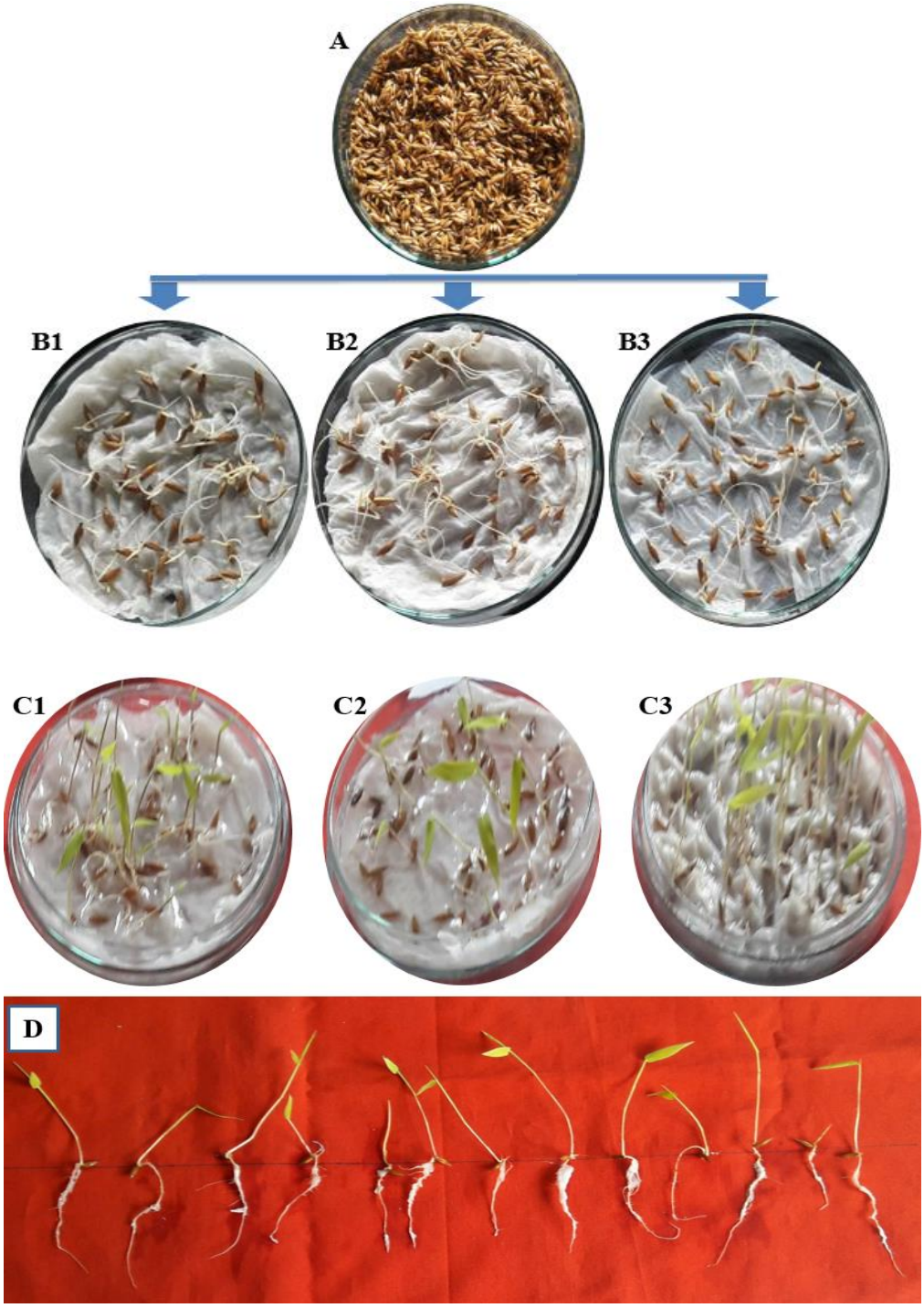

Figure 1. Preparation of seedlings sample; A - Sterilized *B. tulda* seeds; B1, B2 and B3 - Sprouting seedlings, C1, C2, C3 and D - 18 days old *B. tulda* seedlings.

Similarly, preparative TLC was carried out to obtain the purity of sample for HPLC. The analysis was performed in triplicates.

Quantification of IAA by HPLC

HPLC was performed to quantify the IAA in reverse-phase (RP) HPLC using an RP-C18 column (5 µm, 250 × 4 mm) with UV absorbance at 280 nm. 15 µl extract of bamboo seedlings and standard IAA was injected on RP-C18 column (Shimadzu Lab solutions). The mobile phase was composed of acetic acid: Methanol (75:25 v/v) at pH 3.8 of HPLC grade at isocratic phase. The flow rate was 0.8 ml/min and the operation pressure of 54 MPa at 30ºC. Quantity of standard IAA and test sample was individually

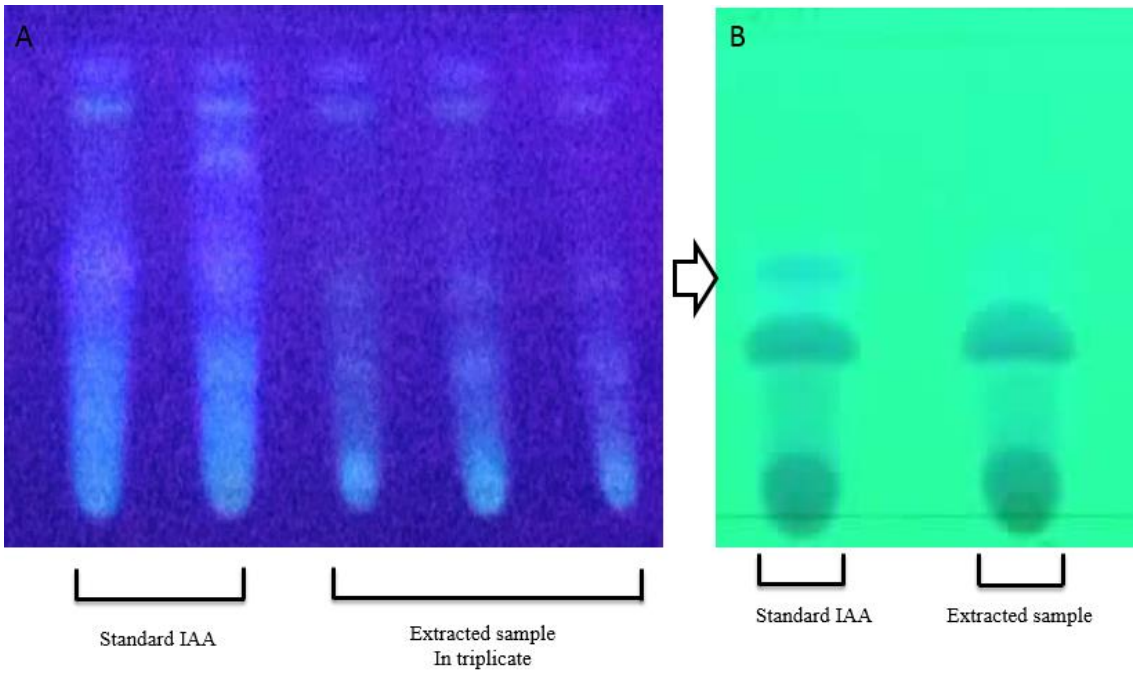

Figure 2. Thin layer chromatography (TLC). (A) Standard IAA and extracted sample in triplicate, and (B) Standard IAA and extracted sample after purification using preparative TLC.

adapted in order to estimate the quantity of IAA in bamboo seedlings. Standard calibration curves were obtained by plotting the peak areas of standard concentration of IAA (0.975-500 µg/ml) using a serial dilution and equation was generated to quantify IAA from bamboo seedlings. Experiments were repeated three times with three replication.

In-vitro bioactivity of extracted IAA on ladies' finger (*Abelmoschus esculentus*) seeds

Bioactivity of extracted IAA from bamboo seedlings was demonstrated by inoculating seeds of *A. esculentus* in in-vitro under auxenic condition. *A. esculentus* was selected for this experiment because it is one of the most preferred vegetables in context of Nepal. *A. esculentus* seeds were collected from local agricultural cooperative, Kathmandu, Nepal. The seeds were then surface sterilized with 1% sodium hypochlorite for one minute and washed three times with autoclaved distilled water. Then the seeds were immersed in 1 mg/ml stock solution of extracted IAA, standard IAA (HiMedia) as positive control and distilled water as negative control. Seeds were dried overnight in laminar hood and 10 germinating seeds were kept in sterilized filter paper in petri plates. Each treatment was repeated three times with three replicates thus making a total of 27 petri plates which were placed in growth chamber at 30±2°C for 14 days. Autoclaved distilled water was applied to the experiment in the morning (10:00 am -11:00 am) in the interval of 48 h. After 14 days, the growth parameters like root length, shoot length, root fresh weight, root dry weight, shoot fresh weight, shoot dry weight, and total chlorophyll and protein content of *A. esculentus* seedlings were recorded for all the treatments. Total chlorophyll and protein content of the seedlings under in-vitro assay were estimated by reading optical density at 645, 652 and 663 nm on spectrophotometer (Shimadzu UV-1800). The amount of total chlorophyll pigments was determined by the equation of Arnon (1949) and protein by Folin reaction (Lowry et al., 1951).

Statistical analysis

Analysis of variance (ANOVA) was performed to compare growth characters of *A. esculentus* using IBM Statistical Package for the Social Science (SPSS). Mean values of growth characters of *A. esculentus* were analysed by Tukey's Honestly significant difference (HSD) at the significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Qualitative analysis of IAA by TLC

IAA was extracted from bamboo seedlings using methanol solvent. TLC results showed that this method may be used efficiently to detect target compounds. The methanol extract of bamboo seedlings affirmed the presence of IAA with Rf value similar to the standard IAA (0.412) indicating that IAA was present in *B. tulda* seedling extract (Figure 2A and B). IAA has been implicated in almost every aspect of plant growth and development from embryogenesis to senescence (Perry et al., 2005).

Plant growth regulators are difficult to analyze because they occur in very low amounts in plant extracts which are rich in interfering substances, especially secondary metabolites (Dobrev et al., 2005). Biochemical experiments regarding quantification of IAA as we have performed from bamboo seedlings are the very first attempt in context of Nepal. Similar research in relation to isolation and quantification of plant growth regulators like IAA in bamboo seedlings as well as in other plants were unavailable to compare our research in context of Nepal.

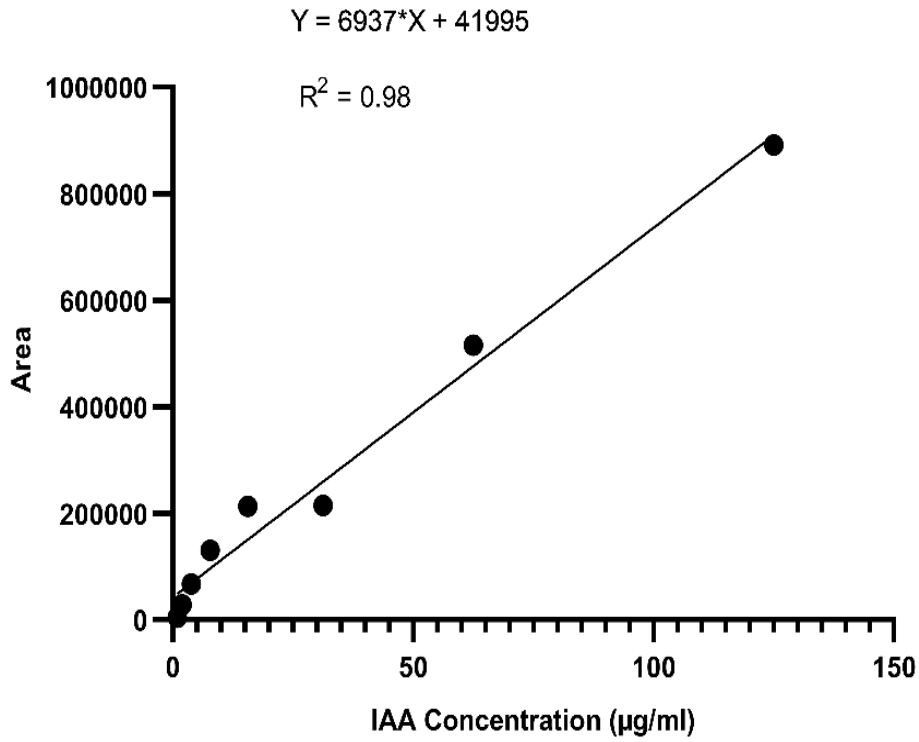

Figure 3. Regression curve of standard IAA.

Sporadic efforts performed to analyze IAA in other plants most commonly in abroad have been gathered to discuss with our results.

We used TLC followed by HPLC for quantification of our target bioactive compounds (IAA) from *B. tulda* seedlings. Although a wide variety of methods are available for auxin isolation and analysis, chromatographic methods are still the predominant and established technique within the laboratory (Porfírio et al., 2016). The IAA on a reversed phase HPLC column was quantified under isocratic program. Extracted IAA from *B. tulda* seedlings showed similar peak to that of standard IAA (HiMedia) in HPLC analysis. The retention time of extracted IAA was found to be 28.71 min (Figure 4A and B). There was a linear relationship between the concentration of standard IAA and area of HPLC peak and an equation Y= 6937x + 41995 (R^2= 0.98) (Figure 3) was obtained. Amount of IAA production by *B. tulda* seedlings was quantified to be 10.28 µg/ml. Sharma et al. (2014) mentioned that the goal of HPLC is to promote the measurable IAA and reduce the amount of unexpected substances in sample. Junior et al. (2011) mentioned that HPLC detected IAA levels which are undetectable in colorimetric analysis. The amount of IAA extracted from *B. tulda* seedlings by HPLC was 10.28±0.1µg/gm in this experiment. Kim et al. (2006) reported the 20.4±6.1 and 16.2±11 nmol/gm from *Zea mays* between 1 and 2 cm of primary root tip. Similarly, Nakurte et al. (2012) reported 14.03±1.84 ng/gm IAA from shoot extract of *Hordeum vulgare* while Hussain and Hasnain (2011) reported 293.33 ng/gm IAA from seedling extract of *Triticum aestivum* var. Uqab 2000 during ultra-performance liquid chromatography coupled to a tandem mass spectrometer through electrospray interface. Higher level of extracted IAA in this experiment may be attributed to rapid growth nature of bamboo during in-vitro growth condition. However, the growth conditions of *Hordeum vulgare* (Nakurte et al., 2012) at 22°C for 14 days and that of *Triticum aestivum* var. Uqab 2000 (Hussain and Hasnain, 2011) incubated under autoclaved calcinated sand for 14 days were quite different than was set in this experiment. The amount of IAA may vary depending on the types of plants and growth condition in in-vitro experiments.

In-vitro bioactivity of extracted IAA on ladies' finger (*Abelmoschus esculentus*) seeds

Analysis of growth characters such as shoot length (6.37±0.37), shoot fresh weight (25.93±0.78), shoot dry weight (8.78±0.18), root length (3.4789±0.08), root fresh weight (9.90±0.05) and root dry weight (2.79±0.11) of *A. esculentus* seedlings inoculated with extracted IAA were found significant as compared to negative controls ($P < 0.05$) (Table 1). However, these growth parameters of *A. esculentus* treated with standard IAA (HiMedia) were not significantly different ($P < 0.05$) to the seedlings

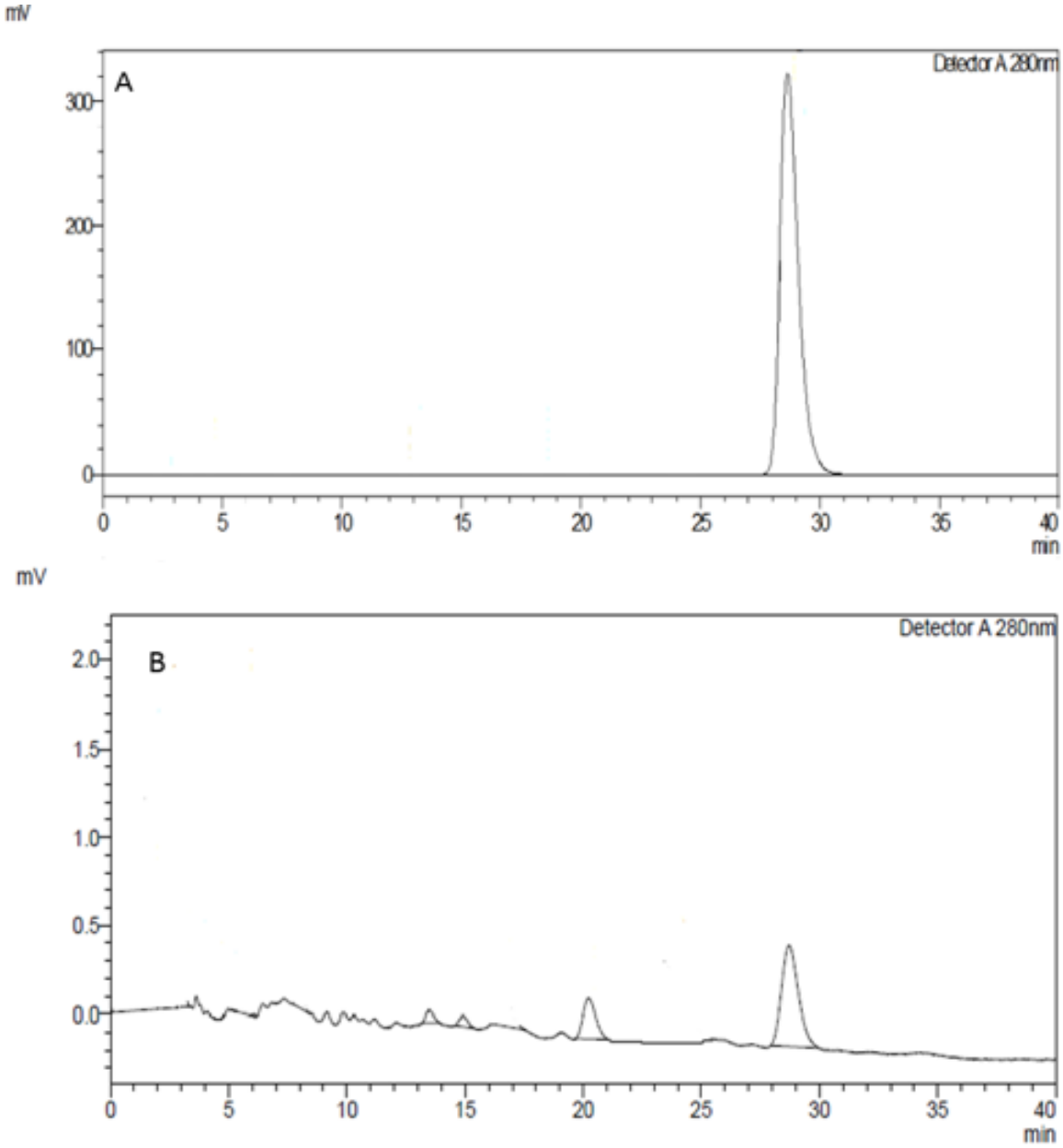

Figure 4. HPLC Chromatogram; (A) Standard IAA, and (B) TLC purified sample.

inoculated with extracted IAA. Similarly, extracted IAA also contributed increased biosynthesis of protein content (0.78±0.1 mg /gm) and total chlorophyll (0.88±0.02) in *A. esculentus* leaves. K.C. et al. (2020) reported marked influence of IAA on production of total chlorophyll in *B. tulda* seedlings. The results revealed an increase of growth performance of *A. esculentus* seedlings inoculated with extracted and standard IAA than the negative control. Enhanced chlorophyll content may be an indication of interaction that stimulates the chlorophyll related enzymes for increased biosynthesis of chlorophyll (Kang et al., 2014). The explicit role of IAA has been witnessed in almost every aspect of plant growth and development from embryogenesis to senescence (Perry et al., 2005).

Conclusion

The study quantified and highlighted the importance of IAA for growth and development of plants. Bioassay of extracted IAA from *B. tulda* seelings in *A. esculentus* were found to be effective in growth of shoot length, shoot biomass, root length, root biomass, and

Table 1. Bioactivity of extracted IAA on growth performance of *A. esculentus* seedlings in in-vitro experiment.

Treatments	RL (cm)	RFW (mg)	RDW (mg)	SL (cm)	SFW (mg)	SDW (mg)	Protein (mg/gm)	Total chlorophyll (µg/mg)
Extracted IAA	3.47±0.08^{a}	9.90±0.05^{c}	2.79±0.11^{e}	6.37±0.37^{g}	25.9±0.78^{m}	8.78±0.18^{p}	0.78±0.10^{f}	0.88±0.02^{k}
Standard IAA	3.85±0.30^{a}	11.27±0.52^{c}	3.22±0.22^{e}	6.77±0.09^{g}	29.76±0.18^{m}	9.78±0.19^{p}	0.96±0.02^{t}	1.25±0.009^{j}
Water	3.04±0.10^{b}	2.63±0.38^{d}	1.23±0.20^{f}	3.62±0.13^{h}	18.08±0.93^{n}	6.76±0.09^{s}	0.52±0.01^{v}	0.82±0.04^{k}

Levels not connected by same letter in the same column are significantly different at P < 0.05 according to Tukey's HSD test at the significance level of P <0.05. Experiment was repeated three times with three replicates for each treatment (RL- Root length, RFW- Root fresh weight, RDW – Root dry weight, SL – Shoot length, SFW – Shoot fresh weight, SDW – Shoot dry weight).

biosynthesis of total chlorophyll and protein in leaves. This IAA deserves the potential of being used as exogenous source of phytohormone although it requires further purification. An assessment of growth response of this extracted IAA in agronomically important crops under greenhouse and field conditions are warranted. Bamboo seedlings are an important but less explored source of plant growth regulators. Therefore, similar types of follow up research in seeds of other bamboo species as well as juvenile culms are essential.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interests.

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REFERENCES

Abubakar N, Shehu K, Yahaya MM, Tafinta IY, Imonikhe MA (2016). Phytochemical Screening and Thin Layer Chromatographic Studies of *Guiera senegalensis* GF Gmel (Egyptian mimosa). Annals of Biological Sciences 4(1):26-30.

Arnon DI (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in beta vulgaris. Plant Physiology 24(1):1-15.

Barkawi LS, Tam YY, Tillman JA, Normanly J, Cohen JD (2010). A high-throughput method for the quantitative analysis of auxins. Nature Protocols 5(10):1609-1618.

Dobrev PI, Havlicek L, Vagner M, Malbeck J, Kaminek M (2005). Purification and determination of plant hormones auxin and abscisic acid using solid phase extraction and two-dimensional high performance liquid chromatography. Journal of Chromatography A 1075(1-2):159-166.

Gomez-Cadenas A, Pozo OJ, Garcia-Augustin P, Sancho JV (2002). Direct analysis of abscisic acid in crude plant extracts by liquid chromatography-electrospray/tandem mass spectrometry. Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques 13(4):228-234.

Gouda S, Kerry RG, Das G, Paramithiotis S, Shin HS, Patra JK (2018). Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture. Microbiological Research 206:131-140.

Guney K, Cetin M, Sevik H, Guney KB (2016a). Effects of some hormone applications on germination and morphological characters of endangered plant species *Lilium artvinense* L. Seeds, New Challenges in Seed Biology-Basic and Translational Research Driving Seed Technology, Dr. Susana Araújo. *InTech 4*:97-112.

Guney K, Cetin M, Sevik H, Guney KB (2016b). Influence of germination percentage and morphological properties of some hormones practice on *Lilium martagon* L. seeds. Oxidation communications 39(1):466-474.

Hussain A, Hasnain S (2011). Interactions of bacterial cytokinins and IAA in the rhizosphere may alter phytostimulatory efficiency of rhizobacteria. World Journal of Microbiology and Biotechnology 27:2645-2654.

Junior RFG, Pedrinho EAN, Castellane TCL, de Macedo Lemos EG (2011). Auxin-producing bacteria isolated from the roots of Cattleya walkeriana, an endangered Brazilian orchid, and their role in acclimatization. Revista Brasileira de Ciencia do Solo 35(3):729-737.

K.C. BM, Gauchan DP, Khanal SN, Chimouriya S, Lamichhane J (2020). Extraction of indole-3-acetic acid from plant growth promoting rhizobacteria of bamboo rhizosphere and its effect on biosynthesis of chlorophyll in bamboo seedlings (Online published). Indian Journal of Agricultural Research Article Id: A-5578.

Kang SM, Radhakrishnan R, You YH, Joo GJ, Lee IJ, Lee KE, Kim JH (2014). Phosphate solubilizing *Bacillus megaterium* mj1212 regulates endogenous plant carbohydrates and amino acids contents to promote mustard plant growth. Indian Journal of Microbiology 54:427-433.

Kim YJ, Oh YJ, Park WJ (2006). HPLC-based quantification of indole-3-acetic acid in the primary root tip of maize. Journal of Nanobiotechnology 3(1):40-45.

Kosova K, Prasil IT, Vítamvas P, Dobrev P, Motyka V, Flokova K, Novak O, Tureckova V, Rolcik J, Pesek B, Travnickova A (2012). Complex phytohormone responses during the cold acclimation of two wheat cultivars differing in cold tolerance, winter Samanta and spring Sandra. Journal of plant physiology 169(6):567-576.

Liu BF, Zhong XH, Lu YT (2002). Analysis of plant hormones in tobacco flowers by micellar electrokinetic capillary chromatography coupled with on-line large volume sample stacking. Journal of Chromatography A 945(1-2):257-265.

Liu X, Hegeman AD, Gardner G, Cohen JD (2012). Protocol: high-throughput and quantitative assays of auxin and auxin precursors from minute tissue samples. Plant Methods 8(1):31.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951).

Protein measurement with the Folin phenol reagent. Journal of biological chemistry 193:265-275.
Ludwig-Muller J, Decker EL, Reski R (2009). Dead end for auxin conjugates in *Physcomitrella*? Plant Signaling and Behavior 4(2):116-118.
Nakurte I, Keisa A, Rostoks N (2012). Development and validation of a reversed-phase liquid chromatography method for the simultaneous determination of indole-3-acetic acid, indole-3-pyruvic acid, and abscisic acid in barley (*Hordeum vulgare* L.). Journal of analytical methods in chemistry 2012:1-6.
Pan X, Welti R, Wang X (2010). Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography-mass spectrometry. Nature protocols 5(6):986-992.
Perry J, Dai X, Zhao Y (2005). A mutation in the anticodon of a single tRNAala is sufficient to confer auxin resistance in Arabidopsis. Plant physiology 139(3):1284-1290.
Petrasek J, Friml J (2009). Auxin transport routes in plant development. Development 136(16):2675-2688.
Porfírio S, da Silva MDG, Peixe A, Cabrita MJ, Azadi P (2016). Current analytical methods for plant auxin quantification-A review. Analytica Chimica Acta 902:8-21.
Rahman A, Sitepu IR, Tang SY, Hashidoko Y (2010). Salkowski's reagent test as a primary screening index for functionalities of rhizobacteria isolated from wild dipterocarp saplings growing naturally on medium-strongly acidic tropical peat soil. Bioscience, Biotechnology and Biochemistry 74:2202-2208.
Sevik H, Guney K, Topaçoglu O, Unal C (2015). The influences of rooting media and hormone applications on rooting percentage and some root characters in *Schefflera arboricola*. International Journal of Pharmaceutical Science Invention 4(2):25-29.
Sharma S, Verma PP, Kaur M (2014). Isolation, Purification and Estimation of IAA from Pseudomonas sp. using High-performance liquid Chromatography. Journal of Pure and Applied Microbiology 8:3203-3208.
Simon S, Petrasek J (2011). Why plants need more than one type of auxin. Plant Science 180(3):454-460.
Strader LC, Culler AH, Cohen JD, Bartel B (2010). Conversion of endogenous indole-3-butyric acid to indole-3-acetic acid drives cell expansion in *Arabidopsis* seedlings. Plant Physiology 153(4):1577-1586.
Topacoglu O, Sevik H, Guney K, Unal C, Akkuzu E, Sivacioglu A (2016). Effect of rooting hormones on the rooting capability of *Ficus benjamina* L. cuttings. Sumarski list 140(1-2):39-44.
Woodward AW, Bartel B (2005). Auxin: regulation, action, and interaction. Annals of Botany 95(5):707-735.
Zhang FJ, Jin YJ, Xu XY, Lu RC, Chen HJ (2008). Study on the extraction, purification and quantification of jasmonic acid, abscisic acid and indole-3-acetic acid in plants. Phytochemical Analysis 19(6):560-567.
Zhao Y (2010). Auxin biosynthesis and its role in plant development. Annual Review of Plant Biology 61:49-64.

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Improvement of the key flavour compounds of fermented garlic by fermentation process optimization

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Flavour compounds in fermented garlic were identified by physicochemical analysis and gas chromatography (GC) after pre-treatment. Reducing sugars, acids, polyphenols and 5-hydroxymethyl furfural (5-HMF) were detected in fermented garlic, and 5-HMF was identified to be the key aroma compounds. To further improve the flavour quality of fermented garlic, the influence of pre-treatment method, fermentation temperature and fermentation humidity on the 5-HMF formation was studied, and then single factor tests and response surface methods were employed to optimize the fermentation process. The results showed that the best flavour was obtained when the pre-treatment was done by low temperature freezing, the fermentation temperature was 61.8°C and the humidity was 40.2%. Under the optimum condition, the content of 5-HMF reached 3.6 mg/g with a high quality score of 19.

Key words: Flavour enhancement, 5-hydroxymethyl furfural (5-HMF), response surface methodology (RSM).

INTRODUCTION

As a common condiment in dietary cooking, garlic is not only rich in amino acids, vitamins and various trace elements, but also contains 35 kinds of sulphur-containing organic matter (Kimura et al., 2017; USDA, 2018). However, the unique spicy and pungent odour of fresh garlic seriously restricts the development of the garlic industry in China. To improve the taste as well as the nutritional value of the garlic, the fermented garlic gained much attention in the recent years.

Fermented garlic is also called black garlic, or fermented black garlic. Compared with fresh garlic, fermented garlic has stronger antioxidant capacity, which attributes to its richness in protein, sugars, vitamins and various minerals (Kim et al., 2013). In the fermented garlic producing process, a large number of proteins are converted into the essential amino acids, which is easier for absorption of the human body, and has a good promoting effect on enhancing human immunity, alleviating fatigue, and maintaining physical health (Kim et al., 2016; Qiu et al., 2020). Moreover, as an ideal health food, fermented garlic has a sweet35-36 and sour taste, no spicy taste, and can be eaten directly (Wang et al., 2014).

Fermented garlic, as a deeply processed product of garlic, has great economic value due to its unique health-care effect. In recent years, much work had been done to

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investigate the quality changes of functional ingredients and flavour components. However, the quality factors of fermented garlic have not been determined, and there is no standardized production method for fermented garlic. As a result, the production quality of fermented garlic is uneven. It greatly hindered the development of fermented garlic industry.

In this paper, the content of reducing sugar, acid, polyphenols, 5-HMF and water in different batches of finished fermented garlic was tested to analyse the formation rule of fermented garlic quality, further to identify the key aroma substances of black garlic. The single factor experiment and response surface method were used to optimize the garlic fermentation process. Thus, the flavour of black garlic can be improved, the good quality of black garlic can be guaranteed. This is beneficial to improving the commercial nature of black garlic and provide theoretical basis for the industrial production of black garlic.

MATERIALS AND METHODS

Experimental materials

The fresh garlics from the white variety were purchased in the market, which is from Wuhu, Shandong province. The cleaned garlics with skin were placed in the freezer for pre-treatment. And the pre-treatment methods included low temperature freezing, normal temperature preservation, high temperature boiling. Then the garlics were placed in the fermentation cabinet with high temperature and constant humidity for 15 days. The temperature range was controlled from 50 to 90°C, and the humidity range was controlled from 30 to 70%. Next, the garlics were placed in the fermentation cabinet and dried at 55°C by hot air for 3 days. Finally, the finished products were packed (Liang et al., 2015). After fermentation, different batches of black garlic were selected and classified into excellent, good, qualified and unqualified according to the taste comfort (Zhang et al., 2015; Jiang, 2016). The content distribution of reducing sugar, total acid, total phenol, 5-HMF and water in each grade of black garlic was determined respectively to study the influence on taste comfort.

Determination of reducing sugar content

For solution A, Copper sulfate (15 g) and methylene blue (0.05 g) were diluted with distilled water and then transferred to a 1000 mL volumetric flask.

For solution B, potassium sodium tartrate (50 g), sodium hydroxide solution (75 g) and potassium ferrocyanide (4 g) were diluted with distilled water and then transferred to a 1000 mL volumetric flask.

Determination of reducing sugar content was carried out following the Fehling's solution method in GB/T 5009.7-2016. The garlic sample (5 g) was chopped, ground and diluted with distilled water, and then transferred to a 100 mL volumetric flask for filtration. 5 ml of A, 5 ml of B, and 10 ml of distilled water were added to the conical flask. After the solution was heated to boiling, one group was dropped the sample solution until the blue just fades away as the end of the pre-titration. The other group was dropped the sample solution 1 ml less than the pre-titration, and the titration was continued at the boiling point until the blue just fades away. The volume consumed of the sample solution was recorded.

Determination of total acid content

The total acid content was determined according to acid-base titration in GB/T 12456-2008. The garlic sample (5 g) was chopped, ground and diluted with distilled water, and then transferred to a 100 mL volumetric flask for filtration. 5 ml filtrate was placed in a 250 ml trigonometric bottle, then 50 ml distilled water was poured into it, and 3 drops of phenolphthalein indicator solution was added. Titration was conducted with 0.1 mol/L sodium hydroxide solution until the solution appeared red, and the volume number of sodium hydroxide standard solution consumed was recorded.

Determination of total phenol content

The heated garlic sample (5 g) was chopped, ground and transferred to a 100 ml volumetric flask with distilled water. Filtrates (0.5 mL), 0.5 mL of Folin Ciocalteu reagent, 1.5 mL of sodium carbonate solution (100 g/L 1, w/v) and 7.5 mL of distilled water were mixed in a 10 mL volumetric flask. The mixture was incubated at 75°C for 10 min, and then placed for 2 to 3 h. The absorbance of the solution was measured at 760 nm.

Determination of 5-HMF content

The spectrophotometry was used to determine the content of 5-HMF (Li et al., 2015). The sample extract (5 mL) was transferred to two tubes containing 5 ml of 4-methylaniline solution. One milliliter of barbituric acid was added to the first tube, while 1 ml of diluted water was added to the second tube as a blank control. Absorbance at 550 nm was measured immediately. The 5-HMF content was calculated as per gram dry weight of the black garlic sample.

Determination of water content

Black garlic samples of a certain weight were dried in a drying oven at 105°C to a constant weight, and the weight difference before and after drying was determined, namely the water content.

Determination of volatile flavour substances

To identify the volatile flavour substances in the finished fermented garlic, an Agilent GC6890 plus system (Agilent Technologies, USA) was used. The specific operations were as follows:

1. Sample treatment: A 5 g sample of black garlic was weighed and ground thoroughly. Then it was put in a 100 mL volumetric flask for filtration. 30 mL of filtrate was drained, mixed with 30 mL of distilled water. And then the solution was extracted 45 mL for later use. Extraction was performed with 20 mL, 25 mL and 30 mL dichloromethane for 40 min, and the remaining raffinate was combined in each extraction. Then a rotary evaporation was used to evaporate and concentrate to 1 mL for standby.
2. Chromatographic column: Capillary column of db-wax (60 m × 0.35 mm × 0.25 μm). Column temperature: the initial temperature was 40°C for 2 min, followed by elevation by 5°C/min to a temperature of 60°C. Then the temperature increased to 100°C at a rate of 10°C/min, and rose to 240°C at a rate of 18°C /min for 6 min. The carrier gas was nitrogen, the split injection ratio was 1: 40, the injection volume was 1 μL, the temperature of the injection port was 220°C, and the temperature of the detector was 240°C.

Table 1. Factors and levels in Box-Behnken central composite design.

Level	A pre-treatment method	B fermentation temperature (°C)	C fermentation humidity (%)
-1	low temperature freezing	60	40
0	normal temperature preservation	70	50
1	high temperature boiling	80	60

Improvement of the key favour compounds by RSM

The influence of pre-treatment method (low temperature freezing, normal temperature preservation, high temperature boiling), fermentation temperature (50, 60, 70, 80 and 90°C), and fermentation humidity (30, 40, 50, 60 and 70%) on the 5-HMF formation was studied by single factor tests. On this basis, the Box-Behnken central combination design method was used to design a response surface experiment with 3 factors and 3 levels to optimize the fermentation process (Yang et al., 2020). The factors and levels were listed in Table 1.

Statistical analysis

The Box-Behnken central combination design method was performed, and the multiple fitting was conducted by using Design Expert 8.0 to establish a parametric regression model and analyse the fermentation process of fermented garlic.

RESULTS AND DISCUSSION

Determination of flavour component and the establishment of quality evaluation system

The fermentation of fresh garlic to fermented garlic is mainly the results of enzymatic Browning and non-enzymatic Browning, among which the most important one is non-enzymatic Browning, namely Maillard reaction. In the reaction process, reducing sugars in fresh garlic interact with amino acids to produce carbonyl ammonia reaction, which produces melanoidins, darkens the garlic cloves of fermented garlic, and produces aroma (Nakagawa et al., 2020). In addition, sugars produce a certain amount of organic acids under the action of certain enzymes and microorganisms such as lactic acid bacteria in the fermented process. These metabolites produced in the process of biological transformation from the unique food sensory characteristics of fermented garlic products. The purpose of scientific production can be achieved through the data analysis of these metabolites and the monitoring of the production process. Through the accumulation of experience in production practice, it was determined that reducing sugars, acids, polyphenols, 5-HMF and water were the key quality components of fermented garlic. The source of reducing sugars in fermented garlic is the hydrolysis of starch in fresh garlic. As the substrate of Maillard reaction, reducing sugars directly affect the process of Maillard reaction in fermented garlic and determine the accumulation of melanoidins. At the same time, the high content of reducing sugar can mask the bitter, astringent and sour taste of fermented garlic, increase the sweet taste of the product, and improve the flavour of the product from many aspects. Acid substances are the main cause of the sour and sweet taste of fermented garlic and are generated by carbohydrate metabolism. However, if the total acid content is too high, the sour taste is too heavy and difficult to swallow. If the total acid content is too low, it cannot give fermented garlic sweet and sour flavour. Phenolic substances are an important component of fermented garlic. When fermented garlic is heated and fermented at high temperature, polyphenolic substances decompose into many small substances and release a large amount of phenolic hydroxyl, which indirectly increases the total phenol content, thus giving fermented garlic strong antioxidant ability. The content of phenolic substances directly affects the function and function of fermented garlic (Kim et al., 2012). 5-HMF is a heat-induced degradation substance, mainly derived from Maillard reaction and thermal decomposition of sugars. 5-HMF has antioxidant properties and beneficial effects, but excessive 5-HMF has some toxic effects, such as carcinogenesis, mutagenesis etc. So it must be controlled within a certain range. Studies have shown that the variation of 5-HMF content can be used as an important indicator to predict the blackening rate of garlic samples. And in the food industry, 5-HMF is also used as a standard indicator for determining food quality in the food industry (Zhang et al., 2015; Lee et al., 2020). In addition, 5-HMF has the function of flavour enhancement and colour blending. With the continuous reaction of 5-HMF, a lot of brown substances and aromatic substances will be produced, giving black garlic unique flavour (Zhang et al., 2012). And according to the research report, the furfural content in black garlic is the most significant (Molina-Calle et al., 2017). Therefore, it was considered as the key aroma component that can reflect the quality of black garlic. Water affects the taste and quality of black garlic. At the same time, as an important participant in biological transformation, it affects the production process. Through the data analysis of the content distribution of characteristic indexes in different grades of fermented garlic, it could be found that when the content of reducing sugar was higher than 40%, fermented garlic showed bright black brown with obvious clear sweet taste. When the reducing sugar content was between 34 and 40%, the skin of fermented garlic had a slight yellow colour and

tastes sweet. When the content of reducing sugar was between 28 and 34%, the skin of fermented garlic was dark, brown and generally sweet, with slight astringency and bitterness. When the content of reducing sugar was lower than 28%, fermented garlic surface was mostly uneven yellowish brown, with poor sweetness and obvious bitterness. Therefore, in the fermentation process of fermented garlic, the content of reducing sugar should be increased as much as possible, which will give fermented garlic excellent taste and improve the quality of fermented garlic.

Acid substances are an important cause of fermented garlic's sweet and sour taste. After data analysis of its content distribution, it was found that when the total acid content was between 3.2 and 3.8%, fermented garlic was suitable for sweet and sour taste and has no peculiar smell. When the total acid content was between 2.5 and 3.2%, the sour taste of fermented garlic was not obvious and the taste was sweet. When the total acid content was between 3.8 and 4.4%, fermented garlic had obvious sour taste and sour taste. When the total acid content was lower than 3.2%, fermented garlic had no obvious sour taste and smells of garlic, while when it was higher than 4.4%, fermented garlic had too much sour taste and is difficult to eat.

Polyphenols impart antioxidant ability to fermented garlic and affect its function. Through the data analysis of the content distribution of total phenols in fermented garlic, it was found that when the content of total phenols was higher than 10 mg/g, fermented garlic had a good taste and a bright dark brown colour. When the total phenols content was between 8 and 10 mg/g, fermented garlic tasted good and had a slight yellow skin. When the total phenols content was between 6 and 8 mg/g, fermented garlic was brown with a slightly bitter taste. When the content of total phenol was lower than 6 mg/g, the colour of fermented garlic was uneven and the bitterness was obvious.

As a precursor of melanin-like substance, 5-HMF can reflect the Maillard reaction process. At the same time, as the key aroma component of fermented garlic, 5-HMF can give black garlic unique aroma, but too much 5-HMF will give fermented garlic a certain bitter taste and has some toxic effects, such as carcinogenesis, mutagenesis etc. Through the data analysis of the content distribution of 5-HMF in fermented garlic, it was found that when the content of 5-HMF was between 8 and 12 mg/g, the taste of fermented garlic was sweet and sour with obvious aroma and no bitterness. When the content of 5-HMF was between 12 and 15 mg/g, fermented garlic was suitable in taste with no bitterness, but the aroma was not obvious. When the content of 5-HMF ranged from 15 to 18 mg/g, fermented garlic had a sweet and sour taste and a bitter taste. When the content of 5-HMF was higher than 18 mg/g, fermented garlic had a sour and sweet taste but a severe bitter taste. While when the content is lower than 3 mg/g, the Maillard reaction is not sufficient and there is no sweet and sour taste.

Water is the key factor in the processing of black garlic. If the content is too high, it will be soft, rotten and sticky. If the content is too low, it will be dry and hard. Through the data analysis of the water content distribution of black garlic, it was found that when the water content was between 35 and 45%, the texture of black garlic was soft and non-sticky. When the water content is between 45 and 55%, the texture of black garlic is soft and slightly sticky. When the water content is between 25 and 35%, black garlic is hard in texture. When the moisture content is higher than 55%, the texture is soft and rotten, and the teeth are very sticky. When the water content is lower than 25%, the black garlic is very dry and hard.

According to the above analysis, reducing sugars, total acid, total phenols, 5-HMF and water content could be divided into four scoring criteria (full score was 20 points). The specific results were shown in Table 2, which will provide theoretical reference for quality control and process optimization in future fermented garlic production.

After heat treatment in the fermentation process, the cell structure of garlic is destroyed, resulting in the release of alliinase. It leads to the degradation of sulphur compounds such as allicin, and the generation of thiophene, furan, furfural and other melanin-like substances, making fermented garlic has a unique flavour. The specific volatile flavour components in fermented garlic are shown in Figure 1, 15 volatile flavour compounds in black garlic were detected by GC , including acetone, 5 - methyl thiophene - 2 carboxylic methyl ester, 2,5-diethylthiophene, 4-methyl-4-hydroxy-pentanone, 5-methylfurfural, nonanal, furfural, ethyl acetate, 3,5,5-trimethylcycloethylene-2-enone, 4-benzoyl-2-hydropyran-3-one, 6-methyl-2-mercaptopyrimidine-1-oxide, 2-hydroxy-2 (p-methoxyphenyl) - 3-hydroxycarbonyl-3-methyl-butane, 2-acetylfuran, 3-methyl-2-carboxyamidothiophene, 1,3-dimethyl-1,3-dihydro-imidazol-2-thione.

It was found that diallyl disulphide and diallyl trisulphide were the main factors causing the pungent and spicy odour of garlic. They both had a pungent odour, in addition, diallyl trisulphide also had burning smell and sulphur smell. However, thioether compounds could not be detected in fermented garlic. This was because the unstable C-S bond of allyl-thioethers broke during the hot processing, resulting in propenyl radicals and alkyl-sulphide groups. Some of free radicals were converted into aromatic thiacycloalkanes during the heating process, while others reacted with low molecular compounds to form dipropenyl sulphide (Molina-Calle et al., 2017). The decrease of diallyl disulphide content significantly reduced the pungent odour and greatly improved the flavour of mature fermented garlic. In addition, pyrazines and alkyl-sulphides that were not present in fresh garlic have been found in fermented garlic. As a kind of aromatic substances, pyrazine is

Table 2. Quality index scoring standard.

Index score	Reducing sugar (%)	Total acid (%)	Total phenol (mg/g)	5-HMF (mg/g)	Water (%)
4	>40	3.2-3.8	>10	3-8	35-45
3	34-40	2.5-3.2	8-10	8-13	45-55
2	28-34	3.8-4.6	6-8	13-18	25-35
1	<28	<2.5,>4.6	<6	<3,>18	<25,>55

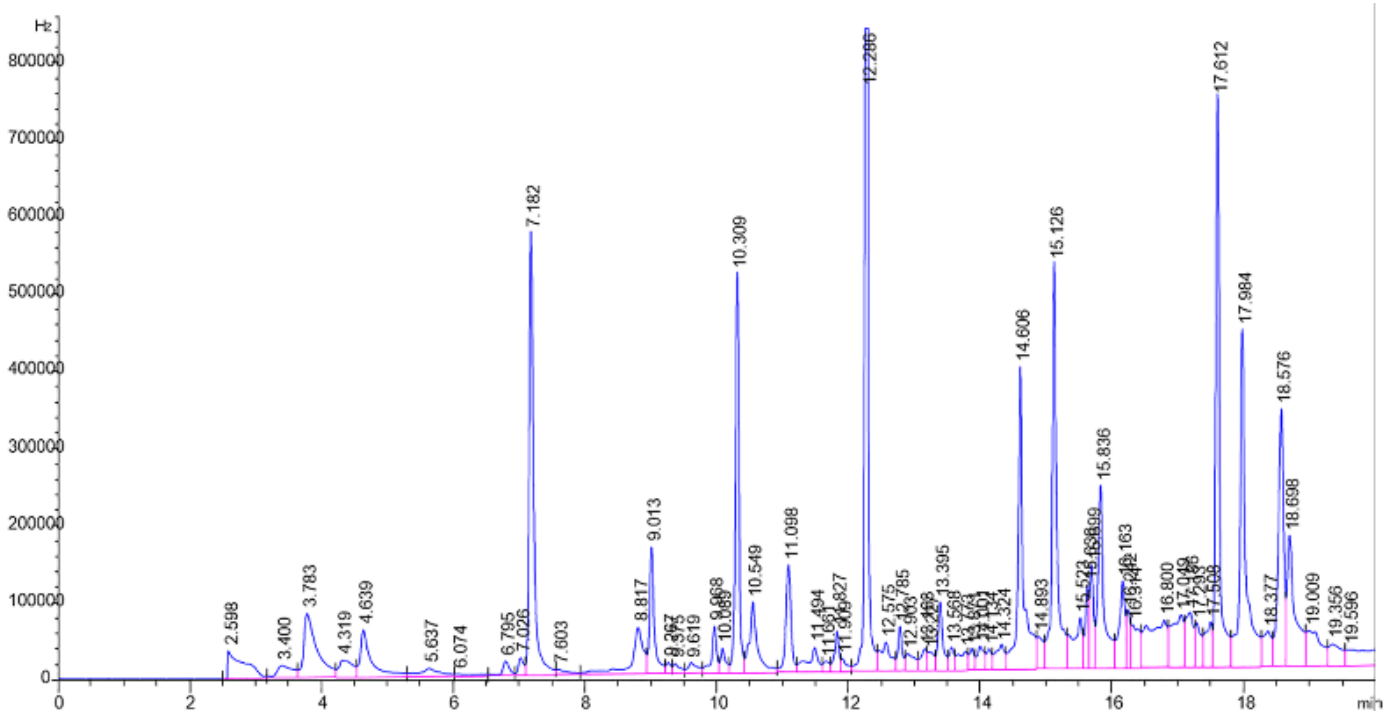

Figure 1. Volatile flavouring substance in black garlic.

generated by the Maillard reaction of non-sulphur amino acids and fructose during the fermentation of garlic, which had a roasted aroma, and had an important contribution to the aroma performance of fermented garlic (Li et al., 2005).

Effect of pre-treatment methods on the 5-HMF formation

As shown in Figure 2, in order to improve the flavour of fermented garlic and explore the influence of pre-treatment method on the 5-HMF formation, it was found that the final 5-HMF content in fermented garlic was significantly different under three different pre-treatment methods. By comparison, it could be found that the content of 5-HMF pre-treated by low-temperature freezing and high temperature boiling was lower than that stored at normal temperature, and the effect of low-temperature freezing was the most obvious, with a reduction of 5.4 mg/g. In the early stage of fermentation, the formation of 5-HMF was relatively slow in the high temperature boiling and low-temperature freezing treatment, which delayed the formation time and speed of 5-HMF. In addition, the content of 5-HMF could be reduced in the later stage of low-temperature freezing, which could reduce the bitterness brought by too much 5-HMF and improve the flavour of fermented garlic.

Effect of fermentation temperature on the 5-HMF formation

Fermentation temperature was also an important factor affecting the flavour formation of fermented garlic. As shown in Figure 3, after exploring the influence of different fermentation temperatures on the generation of 5-HMF, it could be found that when the fermentation

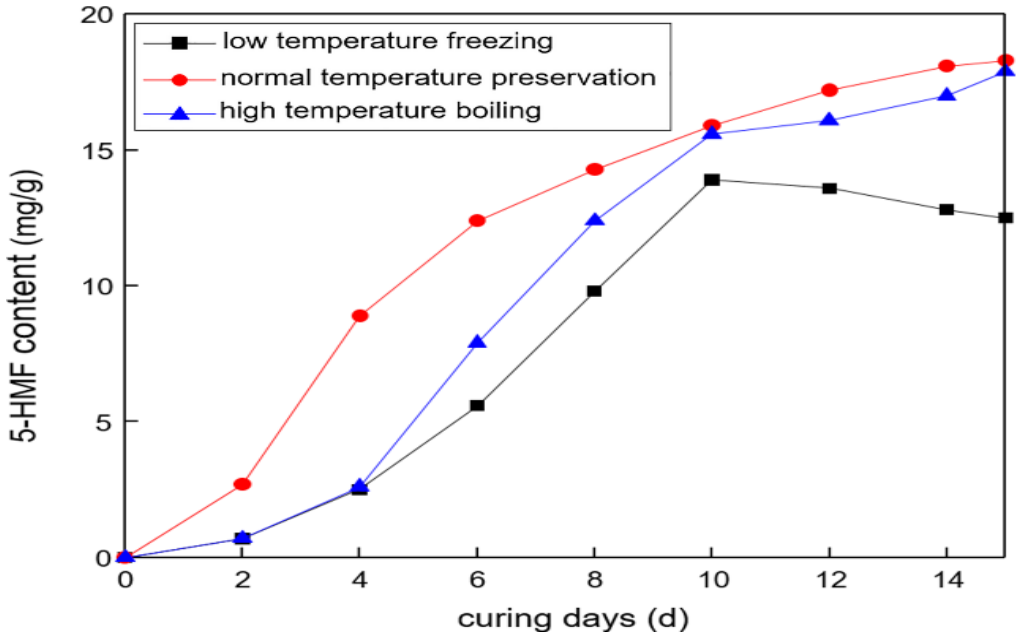

Figure 2. The influence of pre-treatment method on the 5-HMF formation.

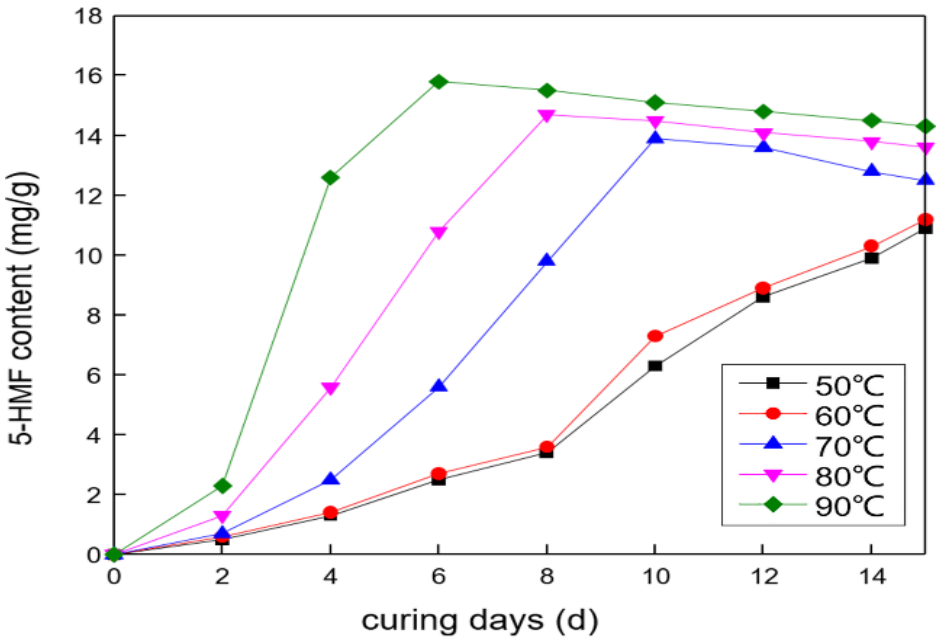

Figure 3. The influence of fermentation temperature on the 5-HMF formation.

temperature was 50 and 60°C, 5-HMF was always on the rise, and the final content was 10.5 and 10.9 mg/g, respectively. When the fermentation temperature was higher than 60°C, the content of 5-HMF increased first and then decreased. And with the increase of temperature, the less time it took to reach the maximum value, the higher the maximum value was. The higher temperature in the early stage of fermentation, the more sufficient the Maillard reaction and the more beneficial to the accumulation of 5-HMF. The lower temperature in the later period, the faster the consumption of 5-HMF, the more conducive to the preservation of the flavour of fermented garlic. Therefore, the temperature range needed to be controlled reasonably, 60, 70 and 80°C

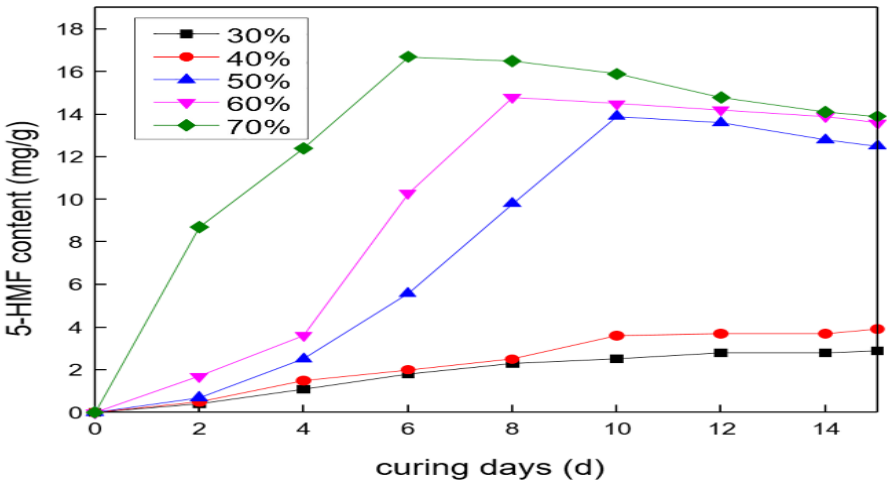

Figure 4. The influence of fermentation humidity on the 5-HMF formation.

were selected as the ranges of response surface.

Effect of fermentation humidity on the 5-HMF formation

In addition to the two factors mentioned previously, fermentation humidity was also the key condition to enhance the flavour of black garlic. By exploring the effect of fermentation humidity on the 5-HMF formation, it was found that when the fermentation humidity was too low, the content of 5-HMF in fermented garlic is always on the rise, and the increase was not obvious. When the fermentation humidity was too high, the content of 5-HMF increased first and then decreased. With the increase of humidity, the time to reach the maximum value was less and the maximum value was higher. If the fermentation humidity was too low, the texture of fermented garlic was relatively dry and hard, and the tissue cells were in a compact state, so it was not easy to carry out Maillard reaction. Therefore, the production of 5-HMF in the early stage of fermentation was relatively small, and the amount of 5-HMF used to synthesize melanin after fermentation was also relatively small, so the content of 5-HMF showed a small increase. High fermentation humidity and soft texture of fermented garlic were conducive to the release of reducing sugar and amino acids for Maillard reaction, and the content of 5-HMF in the early fermentation period was greatly increased. Due to the improvement of the reaction environment, the amount of 5-HMF used in the late fermentation to produce melanin-like compounds was also relatively large, so the content of 5-HMF decreased slightly. As can be seen from Figure 4, high humidity fermentation in the early stage of fermentation could promote the generation of 5-HMF, providing a good preparation for the accumulation of melanoidins. Low humidity fermentation in late fermentation could inhibit the formation of 5-HMF, reduce the content of 5-HMF, reduce the bitter taste of black garlic, and contribute to the formation of good flavour of fermented garlic. Therefore, it was necessary to control the humidity range reasonably, 40, 50 and 60% were selected as the value range of the response surface.

Optimization of the fermentation process for the 5-HMF formation using RSM

Response surface analysis was used to optimize the fermentation process of black garlic, and the Design Expert 8.0 software was used to design a 3-factor 3-level experiment. The experiment scheme and the response results are shown in Table 3.

The Design Expert 8.0 software was used to perform multivariate fitting on the experimental data in Table 3, and regression analysis was carried out on its response value. On the basis of the regression analysis, the parametric regression model was established. The regression equation of the model for the 5-HMF formation was as follows:

$$Y = 18.40 - 1.00A + 1.71B + 4.49C - 0.01AB - 0.18AC - 0.050BC - 4.09A^2 - 0.11B^2 - 3.84C^2$$

The variance analysis of the regression model of fermentation parameters of fermented garlic are shown in Table 4. In order to verify the reliability of the parameter regression models, the variance analysis was carried out for the regression equation. The results are shown in Table 4: the model was highly significant ($P < 0.01$), and the look of fit value was insignificant ($P > 0.05$), indicating that the regression model had good fitting effect and the experiment scheme was operable. R^2=0.9919 indicated that the model was highly reliable and could be used to

Table 3. Design and results of response surface experiments.

Run	Factors			5-HMF /(mg/g)
	A	B	C	
1	0	0	0	17.8
2	0	-1	1	18
3	0	1	1	18.9
4	-1	-1	0	11.5
5	-1	0	1	13.9
6	1	0	-1	7.3
7	0	-1	-1	9.9
8	1	0	1	17
9	0	0	0	18.5
10	0	0	0	18.6
11	1	1	0	16.7
12	0	0	0	18.5
13	1	-1	0	15.1
14	-1	1	0	13.5
15	0	1	-1	11
16	0	0	0	18.6
17	-1	0	-1	3.6

Table 4. Regression results of RSM for the 5-HMF formation.

Parameter*	F Value	P-value Pr>F
Model	95.58	<0.0001
A-pre-treatment method	61.22	0.0001
B- fermentation temperature	10.23	0.051
C- fermentation humidity	420.39	<0.0001
A2	183.57	<0.0001
C2	61.80	<0.0001

*All parameters are as significant with the probability value set as <0.05.

predict and characterize the response value of 5-HMF. R^2adj = 0.9815 indicated that the fitting degree of the regression equation was very good. C. V. refers to the coefficient of variation of the equation, whose value was inversely proportional to the accuracy of the experiment. The larger the C.V. value was, the more unreliable the experimental result was, the worse the fitting effect of the equation was. C.V. % = 4.23 in the regression equation was within the acceptable range, so the experimental result had considerable accuracy. Therefore, the parameter regression model could be used to analyse the fermentation process of fermented garlic, and predict the 5-HMF formation.

Based on the analysis of flavour substances of black garlic, the effects of pre-treatment method, fermentation temperature and fermentation humidity on the 5-HMF formation were studied. Then the response surface experiment was carried out to optimize the fermentation process. Figure 5 shows the influence of the interaction among factors on the generation of 5-HMF. The results showed that the main factor affecting the quality of black garlic was the fermentation humidity, followed by the pre-treatment method. As shown in Figure 5b and c, the content of 5-HMF decreases with the reduction of fermentation humidity, and the decline trend is very steep. This may be because the dehydration reaction of the precursor of 5-HMF is inhibited under high humidity. In addition, the formation of 5-HMF can be inhibited by low-temperature freezing and high temperature boiling in Figure 5a and b. It can be seen from Figure 5a and c that compare with the influence of humidity and pre-treatment method on the formation of 5-HMF, the content of 5-HMF does not change significantly with the increase of temperature. In order to further determine the factor level combination of fermentation process of black garlic, the optimal fermentation conditions were manufactured by

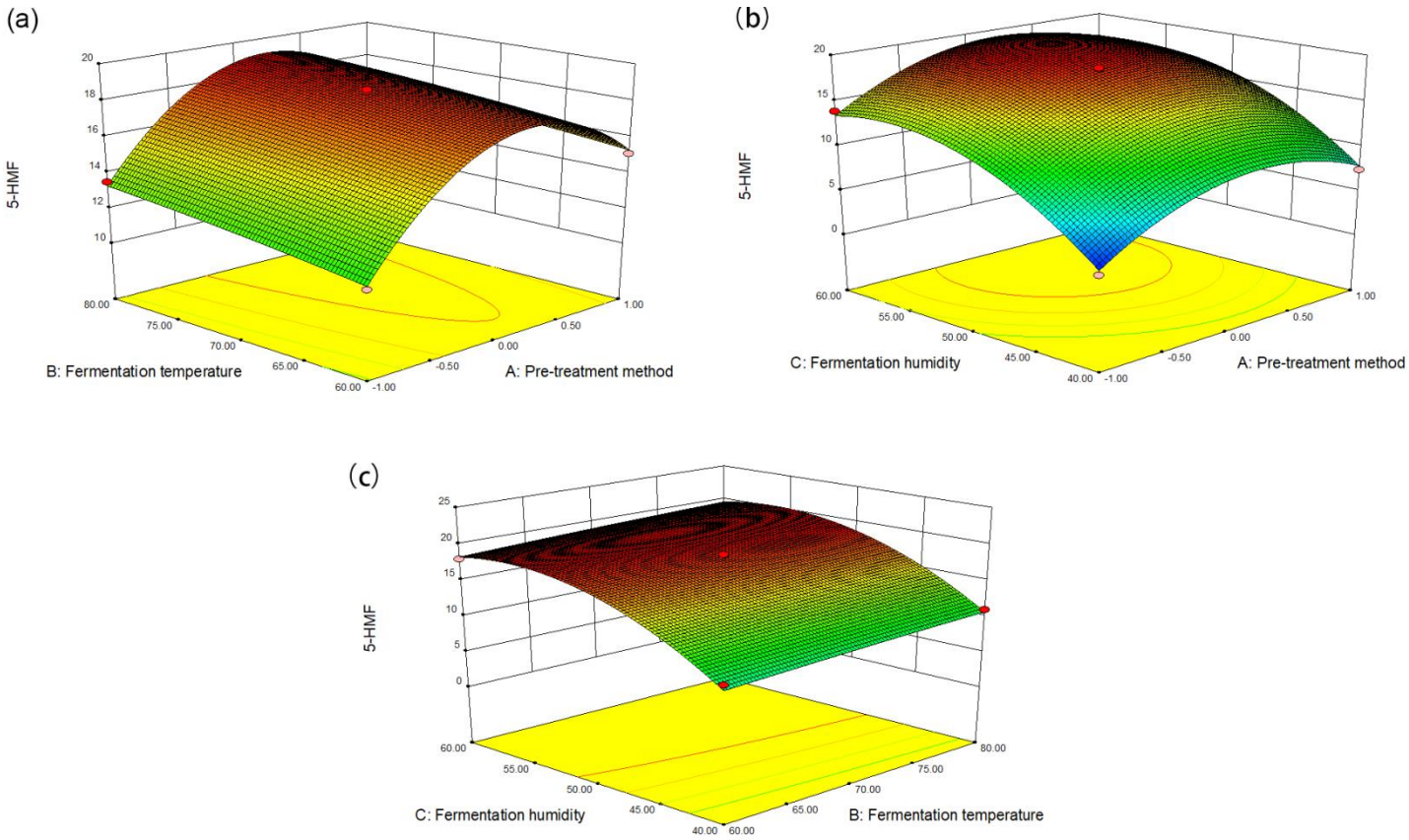

Figure 5. The optimization of fermentation process for the 5-HMF formation.

Response Surface Analysis that the pre-treatment method was low temperature freezing, fermentation temperature was 61.8°C, and fermentation humidity was 40.2%. The response predicted from the model was 3.6 mg/g. Repeated experiments were performed to verify the predicted optimum. The results from the means of three replications (the content of 5-HMF was 3.7 mg/g) were mainly coincident with the predicted value and the model was proven to be adequate. Meanwhile, the quality of black garlic produced under optimal conditions was determined with a high quality score of 19.

Conclusions

Through the accumulation of experience in production practice, it was determined that reducing sugars, acids, polyphenols, 5-HMF and water were key quality components of black garlic, and 5-HMF was the key aroma compounds to reflect the quality of black garlic. The content of reducing sugar, total acid, total phenol, 5-HMF and water content could be divided into four scoring indexes by data analysis of the content distribution of characteristic components of black garlic of different grades. Thus, a comprehensive quality scoring standard was established, which provided the theoretical basis for the standardized production of black garlic.

The effects of pre-treatment method, fermentation temperature and fermentation humidity on Maillard reaction were studied. On the basis of single factor experiment, response surface analysis was used to optimize fermentation conditions and improve the quality of black garlic. The results showed that the best pre-treatment method was low temperature freezing, the best fermentation temperature was 61.8°C, and the best fermentation humidity was 40.2%. The response predicted from the model was 3.6 mg/g. Repeated experiments were performed to verify the predicted optimum. The results from the means of three replications (the content of 5-HMF was 3.7 mg/g) were mainly coincident with the predicted value and the model was proven to be adequate. Meanwhile, the quality of black garlic produced under optimal conditions was determined with a high quality score of 19 by using the above proposed quality reference standard.

CONFLICT OF INTERESTS

The authors declare there are no conflicts of interest.

REFERENCES

Jiang X (2016). Study on quality standard of black garlic. (Master Thesis) Shandong Agricultural University, Shandong, China, 2016.

Kim J, Kang O, Gweon O (2013). Comparison of phenolic acids and flavonoids in black garlic at different thermal processing steps. Journal of Functional Foods 5:80-86.

Kim JS, Kang OJ, Gweon OC (2012). Comparison of phenolic acids and flavonoids in black garlic at different thermal processing steps. Journal of Functional Foods 5:80-86.

Kim S, Park S-L, Lee S, Lee S-Y, Ko S, Yoo M (2016). UPLC/ESI-MS/MS analysis of compositional changes for organosulfur compounds in garlic (*Allium sativum* L.) during fermentation. Food Chemistry 211:555-559.

Kimura S, Tung Y-C, Pan M-H, Su N-W, Lai Y-J, Cheng K-C (2017). Black garlic: A critical review of its production, bioactivity, and application. Journal of Food and Drug Analysis 25:62-70.

Lee CH, Chen YT, Hsieh HJ, Chen KT, Chen YA, Wu JT, Tsai MS, Lin JA, Hsieh CW (2020). Exploring epigallocatechin gallate impregnation to inhibit 5-hydroxymethylfurfural formation and the effect on antioxidant ability of black garlic. LWT 117:108628.

Liang TF, Wei FF, Lu Y, Kodani Y, Nakada M, Miyakawa T, Tanokura M (2015). Comprehensive NMR Analysis of Compositional Changes of Black Garlic during Thermal Processing. Journal of Agricultural and Food Chemistry 63:683-691.

Li NY, Lu XM, Pei HB, Qiao XG (2015). Effect of freezing pretreatment on the processing time and quality of black garlic. Journal of Food Process Engineering 38(4):329-335.

Li Y, Wang R, Li G, Gou P (2005). Purification and Properties of Alliinase from Fresh Garlic (*Allium sativum*). Chinese Bulletin of Botany 5:69-73.

Molina-Calle M, Priego-Capote F, Luque de Castro MD (2017). Headspace-GC-MS volatile profile of black garlic vs fresh garlic: Evolution along fermentation and behavior under heating. LWT – Food Science and Technology 80:98-105.

Nakagawa K, Maeda H, Yamaya Y, Tonosaki Y (2020). Maillard Reaction Intermediates and Related Phytochemicals in Black Garlic Determined by EPR and HPLC Analyses. Molecules 25:4578.

Qiu ZC, Zheng ZJ, Zhang B, Sun-Waterhouse, Qiao XG (2020). Formation, nutritional value, and enhancement of characteristic components in black garlic: A review for maximizing the goodness to humans. Food Science and Technology 19(2):801-834.

U. S. Department of Agriculture (USDA) (2018). Agricultural research service. FoodData Central (“Garlic, raw”). Washington, DC: USDA. Retrieved from https://fdc.nal.usda.gov/fdc-app. html#/food-details/169230/nutrients

Wang HS, Wu H, Yang SL, Zhou YM, Wang CR (2014). Quality characteristics of black garlic with different processing procedures. Modern Food Science and Technology 7:230-236.

Yang H, Zhang M, Ayipaxia R, Guo LJ, Wang L, Wang LT (2020). Optimization of fermentation technology for yogurt with donkey milk flavor by response surface methodology. China Condiment 39:147-151.

Zhang XY, Li NY, Lu XM, Liu PL, Qiao XG (2015). Effects of temperature on the quality of black garlic. Journal of the Science of Food and Agriculture 96:2366-2372.

Zhang YY, Song Y, Li QH (2012). A Review on Formation Mechanism, Determination and Safety Assessment of Furfural and 5-Hydroxymethylfurfural (HMF) in Foods. Food Science 33:275-280.

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