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Review

Application of molecular and biotechnological techniques in plant disease management: A review

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Plant diseases are a major challenge in crop production. They are caused by nematodes, bacteria, fungi, viruses as well as plant nutritional factors. Diseases interfere with the normal physiological and metabolic processes of plants. This results in various effects including wilting, stunting, yellowing and death of plant tissues and organs. Crop losses due to diseases manifest in form of reduced yield, poor quality produce, and reduced post-harvest storage. Past research has brought to the limelight the continuous capacity of pathogens to revert to new pathotypes and strains, some that break resistant varieties or are less sensitive to chemical control products. Currently, farmers are advised to combine several plant disease management practices, a strategy known as integrated plant disease management. Such strategies include crop rotation, use of disease free planting materials, field sanitation, and chemical methods as well as use of resistant varieties. However, some of these methods are expensive and substantially increase the cost of production. Development in molecular biology and biotechnology found application in plant disease management. This ranges from identification, diagnosis to control through gene transfer, mutation breeding and RNA interference, among others. In this paper, the current developments in the application of molecular techniques and biotechnology to manage plant diseases, outlining their possible future application and potential for enhanced plant disease management.

Key words: Phytopathogens, genetics, molecular biology, plant transformation, control options.

INTRODUCTION

Crop production traditionally depends on several inputs including certified planting materials, irrigation, fertilizers, and pesticides among others. However, recently, devastating cases of pathogen attacks have increased and are feared to worsen with the increasing variability in weather patterns and environmental conditions due to global climate change. Loses to farmers are significant, including low yields of reduced quality (FAO, 2017). Important examples include cassava mosaic and cassava brown streak virus (Legg et al., 2015), maize lethal

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necrosis outbreaks in Eastern and Southern Africa (Mahuku et al., 2015), and banana Xanthomonas wilt in Africa (Biruma et al., 2007; Kebede et al., 2017). There are also increased cases of crop damage by nematodes, for example potato cyst nematode (Globodera rostochiensis) in Kenya (Onkendi et al., 2014).

The impact of pathogens on agricultural crops has been wide. Pathogens release enzymes, growth regulators, toxins and other substances which manifests in the affected plants through a myriad of symptoms. The symptoms may be in form of destroyed vascular bundles, yellowing and drying of leaves, witling, and necrosis among others. These developments inhibit absorption and movement of water and minerals from the soil to plant tissues, leading to reduced photosynthesis and death of plants (De Werra et al., 2015; Prince et al., 2015). For the case of cassava brown streak virus, there is a

development of necrotic spots on the roots and rotting, which has a direct impact on the yields (Patil et al., 2015; Legg et al., 2015; Anjanappa et al., 2017). Maize lethal necrosis leads to stunted plants that could turn yellow and die causing 40% or higher loss of yields. The cyst nematodes in potato (*Solanum tuberosum*) growing areas attack tubers, which results in discoloration and sometimes rotting, which directly affect yields (Adams et al., 2014; Thorpe et al., 2014; Mahuku et al., 2015). In tomatoes, *Ralstonia solanacerum* attacks cause accumulation of bacterial mucilage and exudates in the vascular bundles that blocks flow of water and minerals to other parts of the plant (Sarkar and Chaudhuri, 2016). Furthermore, sometimes the bacteria act synergistically with *Fusarium* species as well as the root knot nematodes in an infection complex. In such a case the wilting is severe and impacts adversely on total yields (Lamichhane and Venturi, 2015). Some fungal pathogens lead to development of spots on leaves or fruits which substantially decrease their market value (Hayes et al., 2014).

The common plant disease management strategies can be categorized into cultural, mechanical, biological and chemical approaches. In cultural methods, there is maintenance of good hygiene at the field through removal and destruction of diseased plants, selection and use of clean planting materials and planting resistant crops (Katan, 2000, 2010; Mehta, 2014). Mechanical methods include heat treatments to kill pathogens in planting materials, in the soils borne pathogens (Bruez et al., 2017) and in reducing postharvest pathogens (Wisniewski et al., 2016). Chemicals are used widely in soil fumigation as well as in control of foliar pathogens and their vectors (Li et al., 2016; Gao et al., 2016). Biological methods involve use of viral, bacterial and fungal organisms as biopesticides in control of plant disease causing microorganisms (Tjamos et al., 2013; Mach, 2016).

Examples include bacteriophages (Balogh et al., 2003; Jones et al., 2007; Iriarte et al., 2007), baculoviruses

(Lincoln et al., 2002; Del Pozo and Lam, 2003). *Xanthomonas campestris* pv. *pruni* phage 1 was successfully tested against *Agrobacterium tumefaciens,* while *X. oryzae* phage reduced the incidence of leaf blight (Jones et al., 2007).

Integrated plant disease management combines several of the aforementioned methods to control particular plant diseases or the pathogen vectors (Fry, 1982). To complement the approaches described earlier, molecular tools have lately found wide applications in the study, diagnosis and management of plant diseases. This paper reviews the application of current molecular tools as well as some tissue cultures techniques in the management of plant diseases.

GENETIC ENGINEERING

Gene transfer

The initial work by Flor (1955) that gave rise to the gene-for-gene concept has been very instrumental in the study of resistance as well as spearheading development of disease resistant plants (Jones and Dangl, 2006). Genetically, this requires a virulence (Avr) gene in the parasite and a resistance gene (R-gene) in the host plant. Several R genes have been identified to date and characterization of the *Arabidopsis* resistance gene complement managed to provide better understanding of the r genes structure (Meyers, 2003). Identification of resistant plants initially provided sources for resistance genes; this has always been quickly followed by breeding efforts aimed at introgression of the resistance genes to cultivated crops. However, normal classical breeding faced many drawbacks including low success and carryover of unwanted traits to crops (Miklas et al., 2006; Fry, 1982).

Over time, new more precise molecular approaches came into play. These include proteomics, metabolomics, transcriptomics, plant tissue culture and genetic engineering. Genetic engineering involves gene transfer, gene silencing, mutation breeding, and regulation of transcriptional factors (Sankaran et al., 2010; Ocsoy et al., 2013; Mahlein, 2016). Gene transfer method of developing disease resistant plants has been used for several decades. Some of the earlier achievements include *Agrobacterium* mediated introduction of rice chitinase gene in strawberry (*Fragaria ananasa*), which led to resistance to powdery mildew, done using the CaMV 35S viral promoter. Expression of rice *chitinase*-3 gene in transgenic peanut through *Agrobacterium* mediated gene transfer was also found to be effective in conferring resistance against many soil borne fungal pathogens. This transformation involved *Agrobacterium tumefaciens* strain LB4404 having the binary vector (pB1333-EN4-RCG3) containing the chitinase (chit) and hygromycin resistance (hpt) gene as selectable marker

(Iqbal et al., 2012). Studies on chitinase genes have led to their wide utilization against many other fungal pathogens (Jabeen et al., 2015; Richa et al., 2016, 2017; Munir et al., 2016).

Embryogenic tissue transformation through particles bombardments using tungsten particles coated with DNA of the PRSV HA 5-1 coat protein gene was able to produce ringspot virus resistance in pawpaw. Polyethylene glycol (PEG) method was also successfully used to transfer Stilbene synthase gene to rice to increase resistance to blast caused by *Pyricularia oryzae* (Stark-Lorenzen et al., 1997).

CRISPR/Cas9 is a new technique in genome editing that has enabled manipulation of plants allowing study of resistance genes, and has been used in mutational breeding in rice (Zhou et al., 2014; Lu et al., 2017), tomato (Brooks et al., 2014) and tobacco (Gao et al., 2015). In *Arabidopsis*, CRISPR/cas9 was used to introduce mutation on eIF (iso) 4E locus enabling acquisition of resistance against Turnip mosaic virus (Pyott et al., 2016). CRISPR/cas9 was also used to modify eIF4E gene in cucumber thereby creating resistance to a number of viral diseases including cucumber yellowing virus, zucchini yellow virus and papaya ring spot virus (Chandrasekaran et al., 2016). The technique has also been used against fungal diseases (Wang et al., 2016). Currently, gene transfer is being widely applied in crop improvement techniques to introduce resistance mechanisms to plants (Vleeshouwers and Oliver, 2014).

RNA interference

RNA interference (RNAi) is a molecular technology that uses gene down regulation principle via transcriptional gene silencing (TGS) or posttranscriptional gene silencing. Three types of RNA silencing have been investigated in plants. These are cytoplasmic small interfering RNA (SiRNAs), micro RNA (miRNAs) in down regulating endogenous mRNA and DNA methylation-suppression of transcription. In these different RNA interference pathways, long double stranded RNA (dsRNA) precursors are cleaved by DICER enzyme (DCL2 or DCL3) into approximately short 21-nt length of SiRNAs and miRNAs (Bernstein et al., 20101). Once constructed, based on the fact that large and small RNA molecules are mobile between organisms especially in plant-pathogen interactions (Castel and Martienssen, 2013; Kim et al., 2014), SiRNAs and miRNAs will down regulate plant pathogen mRNA and chromatin modification. RNAi has been investigated as a powerful approach in developing disease-resistant crops. It has been used in combating plant fungi, for example *Sclerotinia sclerotiorum,* the causal agent of white mould, a devasting plant disease that causes up to 100% yield loss. RNAi approach has been reported to be more efficient against white mould as compared to conventional

methods. Transgenic tobacco plants were used to construct a hairpin RNA in order to down regulate Chsgene, the gene controlling chitin synthesis in the fungus. A reduction of 55.5 to 80% in disease severity was observed as compared to non-transgenic tobacco (Andrade et al., 2016).

Fusarium oxysporum is a soil-borne fungus responsible for significant economic damages in potato, bean, wheat and bananas, among other crops. Studies have shown that Fox can cause 30 to 70% yield losses in different host crops. Host-induced RNAi has been used in silencing the pathogenicity genes (FOW2, FRP1, and OPR) that allow *F. oxysporum* to counteract its host resistance mechanism (Hu et al., 2015). RNA silencing has also been used in protecting crops from viral infection such as tomato leaf curl virus, potato virus X (PVX) and citrus tristeza virus (CTV) (Soliman et al., 2008; Praveen et al., 2010; Soler et al., 2011). Despite being a powerful method for switching off expression of pathogen genes during infection, RNAi has some drawbacks to the plant as well as in the environment. RNA silencing could result in host genome modification which might interfere with gene flow between plants and their relatives leading to biodiversity reduction. In addition, RNAi construction is difficult for some plant species (Rodrigues et al., 2009).

Transcriptomics as an approach of managing plant diseases

Transcriptomics entails the study of RNA transcripts produced by the genome within a specific cell using high throughput approaches such as Illumina sequencing. Improved understanding of the cell genome has enabled various techniques such as genome editing which plays a vital role in plant disease elimination, besides improving plant immunity. Through transcriptomics, many disease resistance genes have been identified leading to significant breakthrough in the management of plant diseases (Horgan et al., 2011; Lowe et al., 2017).

Transcriptomics has been used successfully in the management of *Xanthomonas oryzae* on rice (Cheng et al., 2016). Genome editing technologies have been used successfully in enhancing plant resistance to phytopathogens (Andolfo et al., 2016). The advent of this technology has envisioned the use of RNA-sequencing for transcripts or genes expression profile in the management of various plant diseases (Prabha et al., 2013). Furthermore, manipulation of the key plant immunity modulators such as the R-genes can boost the generation of disease free plants. This technique has also improved understanding of the interaction of various diseases and the plant host such as *Phytophthora nicotianae* infecting *Nicotiana tabacum* (Yang et al., 2017). Understanding plant response to infections is important in the development of effective plant disease control measures. Investigation of gene expression profiles during viral infections would shed more light in

ascertaining significant components of the resistance alleyways (Yang et al., 2017).

Proteomics

Various proteins and their functions as well as their interaction in an organism can be studied through the mean of proteomics analysis (Zulkarnain et al., 2015). This can be useful in determining the pattern and the specificity of a particular protein released in plants when there is a pathogenic stress. Common techniques used in proteomics analysis are Two-dimensional Electrophoresis (2DE), Fluorescence 2D Difference Gel Electrophoresis (2D-DGE), Mass Spectrometry (MS) and Multidimensional Protein Identification Technology (MudPIT) also known as ''shotgun'' approach (Chandramouli and Qian, 2009).

About 1,500 proteins were identified in rice during bacterial leaf streak (BLS) infection with 23 up-regulated proteins that were potentially associated with BLS resistance in rice (Li et al., 2012). Brown root honey caused by *Monilinia laxa* proteins was investigated from apple and apricot, up to 800 proteins were expressed and around 10 proteins isolated from apple showed potential use in developing *M. laxa* host specific diagnostic marker (Bregar et al., 2012). In order to understand the host response mechanism against *Alternaria alternate* infection, both resistant and susceptible apple varieties were subjected to comparative proteomics analysis using Two-Dimension (2-DE) and Mass Spectrometry (MS). A total of 43 differentially expressed proteins were detected which included pathogenesis-related proteins beta-1,3 glucanase, mald 1 and ascorbate perioxidase. The pattern of mald1 in resistant, as well as in susceptible apple contributed to understanding the mechanism underlying *A. alternata* resistance (Zhang et al., 2015). Likewise, 2-DE and MS were used in identifying different proteins expressed during *Liberibacter asiaticus* (*Las*) infection on *Citrus*. The management of *L. asiaticus* disease commonly known as *Citrus* Huanglongbing (HBL) has been successful using heat treatment. Through comparative proteomics study, 107 Las/heat-induced proteins such as HSP70-like proteins, ribulose-1, 5-bisulphate and carboxylase were identified. They were up-regulated due to heat treatment, which gives an insight on the underlying heat-induced host defense mechanism (Nwugo et al., 2016). Proteomics as an approach in controlling plant disease is more efficient than conventional methods; however, it has some limitations in dynamic resolution for large-scale proteomes analysis as well as in quantifying proteomes (using Mass Spectrometry). Furthermore, separation, visualization and identification of hydrophobic proteins can be a challenge (Van Wijk, 2001).

Metabolomics

Plant-pathogen interaction could be better understood based on the identification and quantification of small molecules called metabolites (Rojas et al., 2014). A number of techniques have been used in the past in metabolomics analysis. To-date the commonly used are high performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS) as well as nuclear magnetic resonance spectroscopy (NMRS) (Kasture et al., 2012). Using GC-MS approach, Warth et al. (2015) showed that wheat metabolome is modified by deoxynivalenol (DOIN) secreted by *Fusarium graminearum* that causes Fusarium Head Blight (FBH) disease. Earlier, Levenfors et al. (2008) investigated biological management of snow pink mould (*Micrcodochium nivale*) on wheat and rye using *Pseudomonas brassicacearum* MA250. The study found a significant biocontrol effect of *P. brassicacearum* on *M. nivale.* Later, Anderson (2012) discovered that the biocontrol activity of *P. brassicacearum* on *M. nivale* was associated with the secondary metabolites Piliferolide A and SB0253514. Parker et al. (2009) had hinted on the possibility that metabolites control pathogenesis, when *Magnaporthe grisea* was observed to counteract rice, barley and *Brachypodium distachyon* responses by reprogramming its hosts through secretion of different patterns of metabolites.

Known for their devastating effect, *Botrytis cinerea* (*Bot*) and *Pseudomonas syringae* pv *tomato* (*Psd*) are two major pathogens affecting tomato production. Analysis of altered metabolites isolated from both *B. cinerea* and *P. syringae* infected tomatoes revealed that the host resistance is associated with metabolomics reprograming in the host allowing biochemical changes in tomatoes (Camañes et al., 2015). Melatonin-mediated innate immunity against host specific bacteria in *Arabidopsis* has been determined to be reinforced by sugars and glycerol increases.

Link between quorum sensing (bacterial communication system) and plant disease resistance

Quorum sensing (QS) is a cell-to-cell communication mechanism in bacteria allowing them to control their local population density and virulence factors (Bouayed et al., 2016). Through this mechanism small signaling molecules are secreted and detected by bacteria enabling them to assess their population. A number of signaling molecules are involved in QS. These include oligopeptides in Gram-positive bacteria and N-acylhomoserine lactones (AHL) in Gram-negative bacteria and some auto inducers (AI-2) in both Gram-positive and Gram-negative bacteria. AHL is the best characterized amongst QS molecules. Studies have shown the positive effect of AHL in priming induced resistance in plants against phytopathogens. It has been demonstrated that AHL-derived from *Serratia plymuthica* can induce systemic resistance in bean and tomato against *Pythium aphanidermatum* (Pang et al., 2009). Oxo-C14-HSL has been reported to induce resistance

against *P. syringae* in *Arabidopsis thaliana*. The induced resistance is due to accumulation of callose, phenolic compounds and lignification in plant cell wall (Schenk et al., 2014). Similarly, Oxo-C14-HSL derived from *Ensifer melioloti*, a rhizobium of root nodulation in legume, which has been found to fortify host response mechanism in plants. This has been reported for *Phytopthora infestans*, *Blumeria graminis* and *Puccinia graminis* resistance in tomato, barley and wheat, respectively. This Oxo-C14-HSL induced systemic resistance was later confirmed in *Arabidopsis* and barley against *B. graminis* and *Golovinomyces orontii* (Schikora et al., 2011).

TISSUE CULTURE AS AN APPROACH TO MANAGING PLANT DISEASES

Haberlandt (1969) published a paper which envisioned the idea of tissue culture procedures and provided a paradigm for many scientists to delve deeper into the aseptic production of plant cells, tissues and organs in culture (Akin-Idowu et al., 2009). In plant tissue culture, plant cells, tissues, and organs are propagated *in vitro* under aseptic conditions on artificial medium (Hussain et al., 2012). Plant tissue culture has gained popularity in the recent past, and it has been of great importance in plant disease elimination, large scale plant multiplication and plant improvement (Ogero et al., 2012) as well as in the production of secondary metabolites. Subsequently, the application of tissue culture in managing plant diseases is elucidated.

Meristem-tip culture and meristem heat therapy

Organized apex of the shoot from a selected donor plant can be subsequently cultured *in vitro* (Grout, 1990). The cultures are established from axillary buds or from shoot tips, after excision the explants are inoculated into a culture medium that allows the explant to propagate into shoot. The explant of meristem culture may either be the apical dome (apical meristem) or the apical dome plus a few leaf primordia. Studies have shown that larger explants are desirable as they are easier to dissect and have much higher survival growth rate than the smaller ones. The excised apical meristem tip often measures 0.1 mm in diameter and 0.25 to 3.0 mm in length and is done under sterile condition. The significant importance of using meristem tip cultures is that small explants are paramount for excluding devastating pathogens present in the donor plant (Grout, 1990). Besides, axillary shoot proliferation offers lower risks of genetic instability and is easily achievable in most plant species. This technique has been used to eliminate virus infection in sweet potato (Frison and Ng, 1981). Smith (2013) reported that meristem culture technique had made it possible to save many vegetative propagated plants from viruses. Earlier,

Ogero et al. (2012) demonstrated an optimized tissue culture approach for disease-free sweet potato seedlings production in Kenya. Furthermore, this technique can be optimized by combining with heat treatment prior to meristem culture, hence contributing vastly to production of healthy plants. Mwangangi et al. (2014) combined thermotherapy at 38°C with meristem tip culture to eliminate brown streak virus from infected cassava. Meristems excised from plants subjected to thermo-therapy had enhanced CBSV eradication as compared to the control resulting in 68.8% plant survival with 84% of the plants surviving being virus-free. These findings confirm previous reports (Acedo, 2006) and elimination of sweet potato fatherly mottle virus (Mashilo et al., 2013). Application of meristem culture combined with thermotherapy at 35°C is reported to increase the survival rate of *in vitro* explants (Manganaris et al., 2003; Mashilo et al., 2013). This is because larger tips can be obtained from heat-treated plants while ensuring virus-free plant production.

In some cases, it is problematic to eliminate viruses from meristem tip culture; hence, thermotherapy coupled with meristem culture has been proposed. Thermotherapy is applied before *in vitro* meristem-tip culture and has been shown to be effective against potato virus S (PVS) and PVX (López-Delgado et al., 2004). Chatenet et al. (2001) and Fitch et al. (2001) proved apical meristem culture to be effective in eliminating sugarcane yellow leaf virus (SCYLV). What is more, a combination of meristem culture, heat therapy, and cryotherapy has been used successfully in the elimination of various plant diseases. Thermotherapy coupled with meristem-tip culture has enabled the elimination of bean yellow mosaic virus (BYMV) from infected corms, hence leading to production of BYMV free plants (Sharifi Nezamabad et al., 2015).

In vitro shoot grafting and callus culture

In vitro shoot-tip grafting has also been applied successfully in the elimination of viruses in some woody plants. It entails grafting of apical meristem on young root stock seedling. According to Navarro (1992), this technique has been applied successfully in the elimination of approximately 16 diseases in citrus plants, including Psorosis (Navarro et al., 1980).

Calli is a group of unorganized proliferative cells produced by subjecting explants to suspension culture. During culturing, some cells may escape from viral infections due to the high rate of cell proliferation and attain viral resistance due to mutation. In callus culture derived from infected cells, it is evident that not all calli uniformly contain the viral infections. Studies showed that approximately 40% of calli derived from tobacco infected with tobacco mosaic virus (TMV) contained the virus (Hansen and Hildebrandt, 1966). The main reason for the escape from this devastating virus is the high rate of cell

proliferation hence the virus is unable to keep pace with the high rate of cell multiplication, and acquisition of resistance by some cells through mutagenesis (Warren et al., 1992).

Through somaclonal variation, an array of disease resistant plants has been developed. Out of 370 *Solanum lycopersicum* plants propagated from callus cultures, six showed enhanced resistance to TMV. Similarly, late blight *(P. infestans)* resistant potato plants and calli resistant to bacterial blight of rice have been developed. Different pathogens produce different secondary metabolites which can be used to screen different calli for disease resistance. Resistant calli can survive in the presence of toxins, hence generation of disease resistant plants. Through this technique, different disease resistant plants have been developed such as rice resistant to the brown spot pathogen *Helminthosporium oryzae* (Mwendo et al., 2017). Similarly, TMV resistant plants, *Helminthosporium maydlis* toxin resistant *Zea mays* plants and *Helminthosporium sacchari* resistant sugarcane have been generated. Besides, meristem callus culture has been used effectively in the eradication of PVX. Experiments on potato used culture media made up with culture filtrates of different *P. infestans* pathotypes to successfully isolate resistant lines. Embryo culture is the other technique used in tissue culture in embryo rescue in wide crosses, monoploid production and overcoming seed dormancy. It proved to be a very effective tool for transfer of *Alternaria* blight tolerance in oilseed brassicas (Yadav et al., 1991; Aneja and Agnihotri, 2016).

Somatic embryogenesis

Somatic embryogenesis refers to the *in vitro* development of embryo like structures from somatic cells rather than from combination of male and female gametes. According to D'onghia et al. (2001), somatic embryogenesis has been applied successfully in the management of devastating citrus psorosis virus (CPsV) from three different *Citrus* species, namely, Dweet tangor, Common Mandarin and sweet orange. Psorosis virus-free citrus can be propagated via heat therapy, and shoot grafting or combination of both techniques (Calavan et al., 1972; Navarro et al., 1980; D'onghia et al., 2001). However, enhanced competence during the sanitation procedure is necessary because virus eradication differs between isolates and hardly exceeded 70 to 80% (Roistacher, 1993). Somatic embryogenesis obtained by culture of style and stigma has been used successfully in the management of CPsV, hence a promising technique in the propagation of healthy citrus plants (D'onghia et al., 2001).

Protoplast fusion and somaclonal variation

The variability generated from *in vitro* cultured somatic cells, may be due to genetic, epigenetic or physiological causes. Somaclonal variation has been observed in economically important crop species such as wheat, rice, sugarcane, oats, potato, tobacco, among many other plant species with numerous traits, for example resistance to viruses, bacterial and fungal infections. This technique has been used for developing disease resistance in wheat and production of dihaploids through wheat × maize hybrids (Mehta and Angra, 2000) and also for resistance to *Verticillium dahliae* in potato (*S. tuberosum* var L.) plants regenerated from callus (Sebastiani et al., 1994). Protoplast fusion is a vital technique for the generation of hybrid plants among different incompatible species and incorporation of an alien genetic factor for pathogen resistance (Larkin and Scowcroft, 1981). In various cases, vital gene variability in the cultivated germplasm may be missing, and use of protoplast fusion can curb this. Hassan (2014) showed that protoplast fusion of two fungicide tolerant mutants of *Trichoderma harzianum* and *Trichoderma viride* enhances β-glucanase, chitinase and protease enzyme activity in fusant strains (fusion of two different species of fungus) as compared to the parental strains and they had a powerful antagonistic activity against grapevine pathogens *Macrophomina phaseolina, Pythium ultimum* and *Sclerotium rolfsii*. Plant fusion can also cause hypovirulence in other phytopathogenic fungi (Lee et al., 2011).

Haploid and polyploid plants

Haploid plants have been generated from anther and ovule culture. The production of homozygous lines within a span of a short period has paved the way for many types of research. Also, haploid plants are highly useful for research related to plant breeding and genetics. Furthermore, they provide convenient systems for induction of mutations and selection of plants with desired traits. Through these techniques, mutants that are resistant to various pathogens have been developed. For example haploids were used to produce melon with resistance to multiple virus diseases (Lotfi et al., 2003). Polyploidy usually occurs from one generation to another and it results from variation that alters the number of chromosomes in the cells. Different methods describe the origin of polyploidy but they mainly occur due to doubling of somatic cells in mitosis, non-reduction in meiosis yielding gametes that are unreduced, polyspermy and endo-replication (Bharadwaj, 2015). Due to increase in number of chromosomes of related gene dose in polyploids, the gene expression and some secondary metabolites production can be enhanced, thus, boosting host plant resistance mechanisms. According to Van (1975), *Lolium*, an autotetraploid has extra structural carbohydrate and good resistance to diseases than the diploids due to relationship changes in dose of genes, silencing of gene and secondary metabolites expression.

By applying polyploidy, one can produce allopolyploids from parent plants having multiple endogenic chemicals of protection and secondary metabolites which usually provide all metabolites and biocatalysts of the two fused parent plants, thus, successfully promoting the resistance to pest characteristic (resistance thus is more in horizontal form). This strategy can also strengthen tolerance to specific stresses of the environment (Bharadwaj, 2015).

FUTURE PROSPECTS OF APPLYING MOLECULAR TECHNIQUES IN PLANT DISEASE MANAGEMENT

Pathogens have the potential to develop resistance against the mechanisms employed to manage them. The use of only one type of R genes is quite a temporary disease solution. For example, the *Brassica* resistance mediated in Rim1 collapsed in 5 years of use (Sprague et al., 2006). As done in *S. tuberosum* (Kim et al., 2012; Vossen et al., 2014), integrating several R genes may be the way out to make sure that whenever mutations occur in the pathogen to surpass one of the R genes, additional sources of resistance will persist. R genes have to be prolonged by studying the complex of resistance of the evolution of R gene. Grzeskowiak et al. (2014) studied the mechanism of resistance in tomato focusing on Pto/Fen/Prf resistance complex. Some studies have shown that studying non-host resistance (NHR) may lead to and devise mechanisms of resistance that are long lasting and independent of recognition of R protein. According to Singh et al. (2013), non-host resistance emerges when the whole species of a plant is pathogen resistance and compared to resistance mediated by R gene, it is more persistent, thus being a new method for enhancement of crops.

There are two promising tools to exploit for genome editing; the first one is the system of nuclease (Christian et al., 2010; Bogdanove and Voytas, 2011; Schornack et al., 2013) which uses effectors of TAL from *Xanthomonas* species a pathogenic plant to bind DNA regions that are short in a way that is specific to sequence a method described by Boch et al. (2009). The second method is referred to as modulate gene expression and the system of CRISPR which is a technique that enables creation points of gene mutations in absence of placing additional unwanted DNA that is foreign (Belhaj et al., 2013).

Various techniques have emerged that permit hasty cloning and gathering of standard constructs. For instance, fusion of USER that uses cloning based on excision of uracil (Geu-Flores et al., 2007) and golden gate with its associated organization like golden braid (Engler et al., 2008; Sarrion-Perdigones et al., 2013). Tool kits of molecular studies are also available and permit cloning to be prompt and adjustable as discussed by Engler et al. (2014) and Binder et al. (2014). Such techniques may be utilized both in favor of the prevailing immunity mediated by R gene through assembling of R genes in cassettes of resistance and in recent constructed biology advances. Constructed biology methods have thrilling possibility of forming inductive resistance of diseases in plants. According to Hou et al. (2012), one can direct constructed promoters that are sensible to stress in *Arabidopsis*. This upcoming technology may aid in dealing with larger problems related to crops inclusive of genome of polyploids (Galletta and Maas, 1990). As described by Wang et al. (2014), three homoalleles of the susceptible gene of powdery mildew Mlo was fruitfully mutated from wheat that was hexaploid for prevention of growth of pathogen.

CONCLUSION

In this work, how plant diseases are being managed through application of molecular techniques was discussed. However, plants are still being affected by diseases thus, there is need for sustained innovation in this area of science to identify more effective strategies. The use of ecologically safe and environmental friendly methods of protecting crops from diseases is gaining importance. Among the various methods of control of plant diseases, resistance of host is still the method of preference despite the fact that shortfall of persistence has been a repeated limitation. Advancement in transfer of genes systems in crops is perhaps the most difficult form of research in plants. Presently, the preferred two methods are the biolistic-mediated and *Agrobacterium*-mediated DNA delivery systems. The evolution of nanoparticles for delivery of DNA cells of plants is coming up and the probabilities of incorporating the success of *Agrobacterium* and biolistic mediated systems is looming. The use of *Agrobacterium* in transforming plants will continue to have consideration since one avoids the step of tissue culture during regeneration of plants. Genomics has proven to be a potential tool in plant disease management either by targeting virulence of pathogen or by genetic manipulation of the host plant. The most important advantage of utilizing transcriptomics techniques is the capability of being able to carry out detailed studies at transcriptional level of interactions between host plant-pathogens interplay. Some of the other molecular methods are associated with limitations for example. Polymerase chain reaction (PCR) can only be used to find sequences that are known in some details due to primer requirement, but is still a useful tool in crop disease management. Tissue culture on the other hand can be associated with problems in maintaining uniformity and stability of clonally propagated plants and can lead to loss of morphogenetic capacity. Luckily, new advancements will arrive through combination of the available techniques that will enable gene transfer that is accessible and easy to apply plant species in large numbers.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

In vitro inhibitory potentials of aqueous and ethanol extracts of *Hyptis suaveolens* on fungi associated with postharvest spoilage of *Brassica oleracea*

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The inhibitory potential of *Hyptis suaveolens* leaf and root extracts on fungi associated with postharvest spoilage of cabbage (*Brassica oleracea* var. *capitata*) was examined. Collection of diseased cabbage was done in sterile sample bags. Isolation of fungi was carried out by inoculating small sections of diseased tissues excised from the cabbages unto Petri-dishes containing Acidified Potato Dextrose Agar (APDA). The isolated fungi were inoculated in triplicates unto Petri-dishes impregnated with 20, 40, 60, 80 and 100% concentrations of leaf and root extracts. Fungi inoculated on PDA alone and PDA impregnated with extraction solvents, served as controls. Diametric growths of the fungi were measured 24 hourly for 10 days after inoculation. Isolated fungi associated with postharvest spoilage in *B. oleracea* were *Aspergillus niger* and *Rhizopus nigricans*. Ethanol root extracts were highly effective as inhibitors of fungal growth. Ethanol leaf and aqueous root extracts only effectively inhibited *A. niger* growth. Aqueous leaf extracts of *H. suaveolens* irrespective of concentration did not inhibit growth of either of the fungal species. Generally, inhibition of fungal species growth decreased based on duration of exposure; effects were in the order: Day 5 effects > day 7 effects > day 10 effects. Ethanol and aqueous leaf and root extracts of *H. suaveolens* possess fungitoxic properties that might be effective as phytofungicides against fungi responsible for postharvest bio-deterioration of *B. oleracea*. Better understanding of the bioactive components of these natural extracts and more research into how they can be obtained in large quantities and packaged in a form that can be attractive to farmers is needed.

Key words: Cabbage spoilage, *Aspergillus niger*, *Rhizopus nigricans*, growth inhibition, concentration dependent activity, food security, bio-deterioration.

INTRODUCTION

Cabbage (*Brassica oleracea* var. capitata) is a highly nutritive vegetable consumed globally as a major component of salad, shawarma, coleslaw etc. The phytonutrients and antioxidants of cabbage aids in the

the prevention of a number of human diseases such as breast cancer, prostate cancer, vascular inflammatory diseases and high blood pressure (National Cancer Institute, 2012; Higdon et al., 2017, Joo et al., 2018). Although cabbage has tremendous nutritional and medicinal benefits to the growth, development and health of humans, it has been reported to be susceptible to quite a number of diseases caused by pathogens such as fungi and bacteria (Weinberger and Lumpkin, 2005; Omokore et al., 2008, 2009; Mochiah et al., 2011). Such diseased cabbages when consumed by humans are capable of affecting human immune system negatively, leaving the individuals with a deteriorated health condition. In addition, the presence of pathogens on cabbages precisely reduces their nutritional and market value.

Fungal disease constitutes a menace in vegetable production, and many pathogens have been reported to be associated with vegetable crops in the field as well as at storage and processing stages (Salau and Shehu, 2015). Cabbage can be attacked by pathogens before, during or after cultivation (Kurtzman et al., 1987). Postharvest diseases in cabbage are a result of packing, storage, transport and marketing conditions (Hung et al., 2004). However, it is quite possible for latent infections during cultivation to manifest after harvest, thereby causing bio-deterioration (Barnes and Shaw, 2002).

Till date, the most common method of disease control in cabbage is the chemical control measure. This is expensive and continues to be hazardous to man and the environment. Despite the wide usage and application of chemicals in plant disease control, postharvest diseases are still prevalent, causing huge losses via deterioration. Therefore, attention has drifted towards development of suitable alternative plant disease control measures; one of which is the use of botanicals. Hence, this research was conducted to determine the efficacy of ethanol and aqueous leaf and root extracts of *Hyptis suaveolens* on fungal pathogens associated with postharvest cabbage spoilage.

H. suaveolens (L.) Poit. (Family: Lamiaceae) is a common weed that is native to tropical America. However, the plant is presently considered a worldwide weed (Chukwujekwu et al., 2005). It is an annual herb found in dense clumps occupying roadsides, rail tracks, wastelands, watercourses, pastures and open forests where the soil is well drained (Carlos et al., 2012; Sharma et al., 2013). It can form dense thickets in all areas of growth. *H. suaveolens* (pignut) is an obnoxious weed that is distributed throughout the tropics and subtropics (Rajarajan et al., 2014). As reported by Sharma et al. (2013), *H. suaveolens* is widespread in West and Central Africa, Australia (northern territory and Queensland), China, Indonesia, Papua New Guinea, Solomon Islands, French Polynesia, Federated States of Micronesia (Chuuk and Yap Islands), Niue Islands, Guam and the Hawaiian Islands in the USA. According to Abdullahi et al. (2003), *H. suaveolens* may be found in abandoned farmlands in West Africa especially in Northern Nigeria.

MATERIALS AND METHODS

Collection of plant samples

Diseased and healthy cabbage samples were obtained from major vendors in Ojoo, Sango, Bodija and Agbowo in Ibadan, Oyo State, Nigeria. *H. suaveolens* samples were collected from Morondiya Distance Learning Centre along Ibadan - Ilorin highway, Nigeria. The plants were identified and authenticated at the herbarium, Department of Botany, University of Ibadan, Nigeria.

Preparation of culture media

The employed nutrient media was the Potato Dextrose Agar (PDA). It was prepared following standard procedure (Difco^{TM} & BBL^{TM} Manual, 2009; Remel, 2010); by thoroughly mixing 39 g of PDA with 1000 ml of distilled water in a conical flask. The resultant mixture was autoclaved at 103 KNM^{-2} pressure and 121°C for 15 min, after which it was allowed to cool; thereafter it was acidified using lactic acid (100 drops per 1000 ml) to prevent bacterial growth. The resultant Acidified Potato Dextrose Agar (APDA) was poured into sterilized Petri-dishes and allowed to gel and solidify. This was used for initial isolation and sub-culturing of the fungi.

Isolation of fungal pathogens

Fungi responsible for spoilage in cabbage were isolated from the infected cabbage samples. Diseased tissues were excised from the periphery of infected cabbage using sterilized scalpel. The diseased tissues were surface-sterilized by placing them in 80% ethanol for 2 min after which they were immediately rinsed in two changes of sterile distilled water (Amadi et al., 2013). The sterilized diseased tissues were then plated unto APDA with the aid of inoculation needles. The inoculated APDA plates were incubated at room temperature (28 ± 2°C) and observations were made daily for emergence of culture (Babu et al., 2008). The mycelia of the resulting fungi were sub-cultured unto APDA plates and incubated for 7 days. Several sub-culturing unto APDA plates was done until pure cultures were obtained. Thereafter, agar slants were prepared and used to preserve fungal isolates until they were needed.

Identification of fungal isolates

The isolated fungi were identified based on mycelia growth patterns and microscopic examinations (Jonathan et al., 2013). Slides of pure cultures of the fungal isolates were prepared for microscopic observation and identification. Culture and morphological characteristics of the isolates were observed and noted and formed part of the criteria used for identification (Barnett and Hunter, 1987; Domsch et al., 1993). Detailed morphological characteristics of the fungi such as hyphae (septation), reproductive structure (sporangia/conidia) in chain or single; the type of spore, etc. were observed and recorded (Amadi et al., 2013).

Pathogenicity test

Pathogenicity test was carried out according to Koch's postulate. Six healthy cabbages were surface-sterilized using 80% ethanol and inoculated with test fungi (Amadi et al., 2013). Sterile cork

borers were used to remove cylindrical discs (3 mm diameter) from the healthy cabbages. Mycelia plugs (3 mm diameter) were excised from 7 days old pure cultures of the fungal isolates using cork borers and plugged into the pores made in the cabbages. However, some of the cabbage were inoculated with sterile APDA discs instead and these served as the control. After inoculation, the cabbage discs were replaced and the points of inoculation sealed with Vaseline to prevent contamination. The inoculated cabbage were incubated at room temperature (28 ± 2°C) in the laboratory. These cabbage were examined for appearance of disease symptoms after 48 h and subsequently on daily basis for 7 days. Re-isolation of fungal pathogens unto PDA plates was done from inoculated cabbage that showed disease symptoms. The characteristics of the resultant fungal isolates were compared with that of the original cultures of the fungal pathogens in order to confirm they were the same. Likewise, the fungal isolates were re-inoculated into healthy fruits for confirmation as the implicated pathogens.

Preparation of plant extracts of *H. suaveolens*

Two types of extracts were employed in this research (aqueous and ethanol extracts). The extracts were prepared using leaves and roots of the plant according to the method described by Babu et al. (2008), Alo et al. (2012) and Rajarajan et al. (2014). Fresh samples of *H. suaveolens* were harvested and thoroughly washed using tap water and rinsed with distilled water. These washed samples were then taken to the laboratory where mature healthy non-infected leaves and roots were harvested and dried at room temperature for one week. The dried leaves and roots were then pulverized into fine powder.

Prior to use, the powdered samples were preserved in air tight bottles. In conical flasks, 150 g of each powder were soaked in 750 ml of each extraction solvents (water and ethanol), while stirring vigorously was performed with a glass rod for proper extraction after which the flasks were covered with rubber corks. The mixtures were allowed to stand for 48 h at room temperature with occasional shaking and then filtered through a double layered muslin cloth and Whatman filter paper (No. 1) into separate clean conical flasks (Rahman et al., 2009). The filtrates were concentrated by evaporation to dryness in an evaporating dish (Arikpo et al., 2013). Crude extracts obtained were stored in glass bottles at 4°C prior to use (Akueshi et al., 2002).

Preparation of extract concentrations

The used extract concentrations were 20, 40, 60, 80 and 100%. These extract concentrations were prepared by serial dilutions using the method adopted by Mahesh and Satish (2008), Ivoke et al. (2009) and Ademe et al. (2013) which involves reconstituting the crude extracts obtained in their respective extraction solvents. For instance, 80 ml of 100% extract was diluted with 20 ml of the respective extraction solvent to obtain an 80% extract concentration; 60 ml of 100% extract was diluted in 40 ml of the respective solvent to obtain 60% extract concentration, and so on.

Application of leaf and root extracts

The method used for testing fungitoxic properties of plant extracts was a modification of the poisoned food technique (Nene and Thapliyal, 1993; Suleiman and Ogundana, 2010). Different concentrations of leaf and root extracts (1 ml each) were placed on sterile Petri-dishes, molten APDA medium was added and the Petri-dishes were swirled gently to permit even distribution of the plant extracts. However, 1 ml of the respective extraction solvent was added in place of plant extract in some plates and in some others, only APDA was added. These served as Control 2 and Control 1, respectively. After the APDA solidified, mycelia plugs (5 mm diameter) of fungi, taken from the edge of 5-days old cultures were put in the center of the APDA (Umesh, 2013). The inoculated plates were incubated at room temperature for 10 days. However, the effect of the extracts on radial growth of fungal isolates was examined daily and the radial growth (cm) of each fungus was measured for 10 days consecutively after inoculation at an interval of 24 h (Babu et al., 2008).The experiment was setup in triplicates and laid out in a completely randomized design (CRD).

Evaluation of effects of extracts on growth of fungal isolates

The antifungal activity of the extracts was evaluated by measuring the inhibition zones against the tested fungi. Since the experiment was carried out in triplicates, the mean and % inhibition of mycelia growth were determined. Growth inhibition (%) was calculated using the following equation (Odebode, 2006):

$$\text{Growth inhibition (\%)} = \frac{R_1 - R_2}{R_1} \times 100$$

R_1 = radial growth of the pathogen in control medium. R_2 = radial growth of the pathogen in the treated/test medium.

Statistical analysis

Inhibitory effects of extract concentrations on fungal growth were compared using one-way Analysis of Variance (ANOVA) coupled with Least Square Difference (LSD) *post hoc* multiple pairwise comparisons. Difference in fungal growth inhibition between ethanol and aqueous extracts were compared using Student *t*-Test. Level of significance was set at $p < 0.05$. Statistical analysis was conducted using SPSS® version 20.0 (IBM Corp., Armonk, USA). Charts were prepared in Microsoft Office Excel® (Microsoft Inc., Redmond, USA).

RESULTS

Growth inhibition of *A. niger* and *R. nigricans* by extracts

The isolated fungi associated with postharvest cabbage deterioration were *Aspergillus niger* and *Rhizopus nigricans* (Figure 1(ai and iii). Ethanol and aqueous extracts of *H. suaveolens* were potent against *A. niger* and *R. nigricans* isolates from the cabbage. Ethanol root extracts were highly effective as growth inhibitors of both isolates. Ethanol leaf extract of the plant was only highly effective against *A. niger*, but weakly effective against *R. nigricans*. Aqueous root extracts of *H. suaveolens* were similarly highly potent against *A. niger*, but was only weakly inhibitory to *R. nigricans* growth. Aqueous leaf extracts of *H. suaveolens* irrespective of concentration did not inhibit growth of either of the fungal isolates. The inhibitory activities of the different extract concentrations on *A. niger* and *R. nigricans* are presented in Tables 1

Table 1. Growth inhibition of *Aspergillus niger* by ethanol and aqueous extractsof *Hyptis suaveolens*.

Concentration (%)	Leaf				Root			
	Ethanol	**Aqueous**	***MD (SE)**	***p*- value**	**Ethanol**	**Aqueous**	***MD (SE)**	***p*-value**
Day 5								
Control 1	0.00 ± 8.21	0.00 ± 4.72			0.00 ± 5.87	0.00 ± 16.18		
Control 2	55.20 ± 6.20‡	14.61 ± 1.86*			-10.14 ± 6.15	-15.82 ± 2.21		
20	62.87 ± 5.89‡	-3.97 ± 2.51	66.84 (6.40)	< 0.0001	65.57 ± 9.47‡	54.24 ± 0.85‡	11.33 (9.51)	0.299
40	63.12 ± 1.62‡	0.42 ± 1.30	67.70 (2.08)	< 0.0001	71.23 ± 1.55‡	54.80 ± 2.26‡	16.42 (2.77)	**0.004**
60	65.59 ± 4.29‡	2.30 ± 4.89	63.30 (6.50)	< 0.0001	67.45 ± 8.65‡	50.28 ± 2.99‡	17.17 (9.15)	0.134
80	68.32 ± 2.79‡	8.77 ± 7.97	59.55 (8.45)	0.002	62.74 ± 6.95‡	49.44 ± 3.33‡	13.30 (7.71)	0.159
100	70.54 ± 2.36‡	10.23 ± 6.35	60.31 (6.77)	0.001	71.23 ± 0.47‡	48.02 ± 2.69‡	23.20 (2.74)	**0.011**
Day 7								
Control 1	0.00 ± 2.78	0.00 ± 1.59			0.00 ± 1.96	0.00 ± 6.61		
Control 2	39.05 ± 3.79‡	7.17 ± 2.79			-6.84 ± 2.14	-1.74 ± 2.27		
20	46.49 ± 6.14‡	-1.59 ± 0.00	48.08 (6.14)	0.01	45.30 ± 6.37‡	58.39 ± 1.33‡	-13.09 (6.51)	0.115
40	45.66 ± 2.69‡	-1.59 ± 000	47.25 (2.69)	< 0.0001	56.84 ± 1.50‡	55.56 ± 2.10‡	1.28 (2.58)	0.645
60	46.28 ± 2.54‡	1.79 ± 3.39	44.49 (4.23)	< 0.0001	56.20 ± 5.65‡	55.56 ± 2.85‡	0.64 (6.33)	0.924
80	54.55 ± 3.76‡	0.40 ± 1.99	54.15(4.25)	< 0.0001	46.58 ± 10.80‡	57.52 ± 0.75‡	-10.94 (10.83)	0.418
100	55.17 ± 2.16‡	2.39 ± 3.98	52.77 (4.53)	< 0.0001	63.68 ± 3.19‡	52.72 ± 3.30‡	10.95 (4.59)	0.075
Day 10								
Control 1	0.00 ± 0.00	0.00 ± 0.00			0.00 ± 0.00	0.00 ± 0.59		
Control 2	13.33 ± 6.74	0.00 ± 0.00			0.00 ± 0.00	-0.20 ± 0.39		
20	21.57 ± 6.71†	0.00 ± 0.00	21.57 (6.71)	0.032	29.80 ± 7.13‡	52.66 ± 1.71‡	-22.86 (7.34)	**0.036**
40	16.27 ± 3.04*	0.00 ± 0.00	16.27 (3.04)	0.033	41.57 ± 3.33‡	47.14 ± 2.52‡	-5.57 (4.18)	0.253
60	22.94 ± 0.90†	0.39 ± 0.39	22.55 (0.98)	< 0.0001	45.83 ± 5.47‡	47.93 ± 1.23‡	-2.05 (5.60)	0.733
80	27.84 ± 6.68†	0.00 ± 0.00	27.84 (6.68)	0.053	35.88 ± 7.65‡	51.08 ± 0.86‡	-15.20 (7.70)	0.184
100	33.92 ± 3.79‡	0.00 ± 0.00	33.92 (3.79)	0.012	46.08 ± 3.98‡	43.20 ± 0.90‡	2.88 (4.08)	0.519

*MD, Mean difference derived by subtracting % aqueous extract inhibition from that of ethanol extract. Values as mean ± standard error (SE). For each of days 5, 7 and 10 and per ethanol/aqueous extracts, % inhibition is significantly higher than control 1 at *p* < 0.05, †*p* < 0.01, ‡*p* < 0.001. Values in column '*p*-value' represent probability level from comparison of inhibitory performance of ethanol and aqueous leaf and root extracts using Student T-test; *p* < 0.05 (significant) are in bold font. Control 1, 0% extract; Control 2, 100% extraction solvent.

and 2, respectively. Generally, inhibition of growth of the fungal species decreased based on duration of exposure; effects were in the order: Day 5 effects > day 7 effects > day 10 effects. Growth inhibition of *A. niger* by 20, 40, 60, 80 and 100% ethanol leaf and root extracts of *H. suaveolens* were significantly different from control 1 (*p* < 0.0001). *A. niger* growth inhibition by 20, 40, 60, 80 and 100% aqueous extract of *H. suaveolens* was significantly different from control 1 (*p* < 0.0001). Compared to aqueous leaf extracts, all concentrations of ethanol leaf extracts used were significantly more inhibiting on *A. niger* growth at days 5, 7 and 10 (*p* < 0.05) (Table 1). Growth inhibition of *A. niger* by ethanol and

**Table 2.** Growth inhibition of *Rhizopus nigricans* by ethanol and aqueous extracts of *Hyptis suaveolens*.

Concentration (%)	Leaf				Root			
	Ethanol	Aqueous	*MD (SE)	*p*-value	Ethanol	Aqueous	*MD (SE)	*p*-value
Day 5								
Control 1	0.00 ± 9.44	0.00 ± 9.44			0.00 ± 0.00	0.00 ± 0.00		
Control 2	38.41 ± 8.59*	13.09 ± 22.53			86.67 ± 3.60‡	11.18 ± 11.18		
20	56.44 ± 8.29†	-9.44 ± 0.00	65.88 (8.29)	**0.015**	93.92 ± 0.90‡	29.61 ± 4.52*	64.31 (4.55)	**0.004**
40	42.49 ± 11.5*	-9.44 ± 0.00	51.93 (11.15)	**0.010**	93.53 ± 4.81‡	9.80 ± 5.96	83.73 (7.66)	**<0.0001**
60	65.88 ± 17.59†	6.65 ± 16.09	59.23 (23.84)	0.068	94.51 ± 0.71‡	13.92 ± 7.82	80.59 (7.85)	**0.001**
80	53.86 ± 9.77†	22.53 ± 19.82	31.33 (22.09)	0.229	96.27 ± 2.21‡	37.06 ± 5.34*	59.22 (5.78)	**0.001**
100	60.52 ± 4.85‡	10.09 ± 19.53	50.43 (20.12)	0.115	98.43 ± 1.57‡	49.31 ± 18.97†	49.02 (19.04)	0.122
Day 7								
Control 1	0.00 ± 0.59	0.00 ± 0.59			0.00 ± 0.00	0.00 ± 0.00		
Control 2	1.18 ± 1.78	10.65 ± 11.24			54.90 ± 7.07‡	5.49 ± 5.49		
20	15.19 ± 8.15	-0.59 ± 0.00	15.78 (8.15)	0.193	67.65 ± 7.40‡	29.61 ± 4.52*	38.04 (8.67)	**0.012**
40	7.50 ± 4.08	-0.59 ± 0.00	8.09 (4.08)	0.186	78.43 ± 8.82‡	9.80 ± 5.96	68.63 (10.65)	**0.003**
60	39.25 ± 27.75*	8.28 ± 8.88	30.97 (29.14)	0.348	73.33 ± 4.30‡	13.92 ± 7.82	59.41 (8.93)	**0.003**
80	24.46 ± 4.45	20.51 ± 14.36	3.94 (15.03)	0.806	81.18 ± 6.77‡	36.67 ± 5.14†	44.51 (8.50)	**0.006**
100	33.33 ± 3.17	14.40 ± 14.99	18.93 (15.32)	0.333	94.31 ± 2.89‡	49.41 ± 18.97‡	44.90 (19.19)	0.139
Day 10								
Control 1	0.00 ± 0.00	0.00 ± 0.00			0.00 ± 0.00	0.00 ± 0.00		
Control 2	0.00 ± 0.00	11.18 ± 11.18			18.63 ± 6.43	5.49 ± 5.49		
20	3.92 ± 2.59	0.00 ± 0.00	3.92 (2.59)	0.270	36.86 ± 9.03†	29.61 ± 4.52*	7.25 (10.10)	0.512
40	0.00 ± 0.00	-21.57 ± 21.54	21.57 (21.54)	0.423	59.80 ± 15.03‡	9.80 ± 5.96	50.00 (16.17)	**0.036**
60	31.37 ± 31.37	8.82 ± 8.82	22.55 (32.58)	0.529	48.63 ± 11.40†	13.92 ± 7.82	34.71 (13.82)	0.066
80	1.96 ± 0.98	14.31 ± 14.31	-12.35 (14.35)	0.479	58.82 ± 12.25‡	36.67 ± 5.14†	22.16 (13.28)	0.171
100	1.96 ± 1.96	14.90 ± 14.90	-12.44 (15.03)	0.477	83.73 ± 9.34‡	49.41 ± 18.97‡	34.31 (21.15)	0.180

*MD, Mean difference derived by subtracting % aqueous extract inhibition from that of ethanol extract. Values as mean ± standard error (SE). For each of days 5, 7 and 10 and per ethanol/aqueous extracts, % inhibition is significantly higher than control 1 at *$p < 0.05$, $^{†}p < 0.01$, $^{‡}p < 0.001$. Values in column '*p*-value' represent probability level from comparison of inhibitory performance of ethanol and aqueous leaf and root extracts using Student T-test; $p < 0.05$ (significant) are in bold font. Control 1, 0% extract; Control 2, 100% extraction solvent.

aqueous roots were similar except for 40 and 100% concentrations on day 5, and 100% concentration on day 10. Duration and % growth inhibition of *R. nigricans* by ethanol leaf extracts were low compared to inhibition of *A. niger* (Table 2). Ethanol leaf extracts and control 2 (ethanol solvent only) significantly reduced *R. nigricans* growth on day 5 ($p < 0.05$); by days 7 and 10, the activity had ceased except for 60% concentration. The solvent ethanol appeared to assist inhibition of *R. nigricans* by *H. suaveolens* on day 5. Aqueous leaf extract was also unable to inhibit *R.*
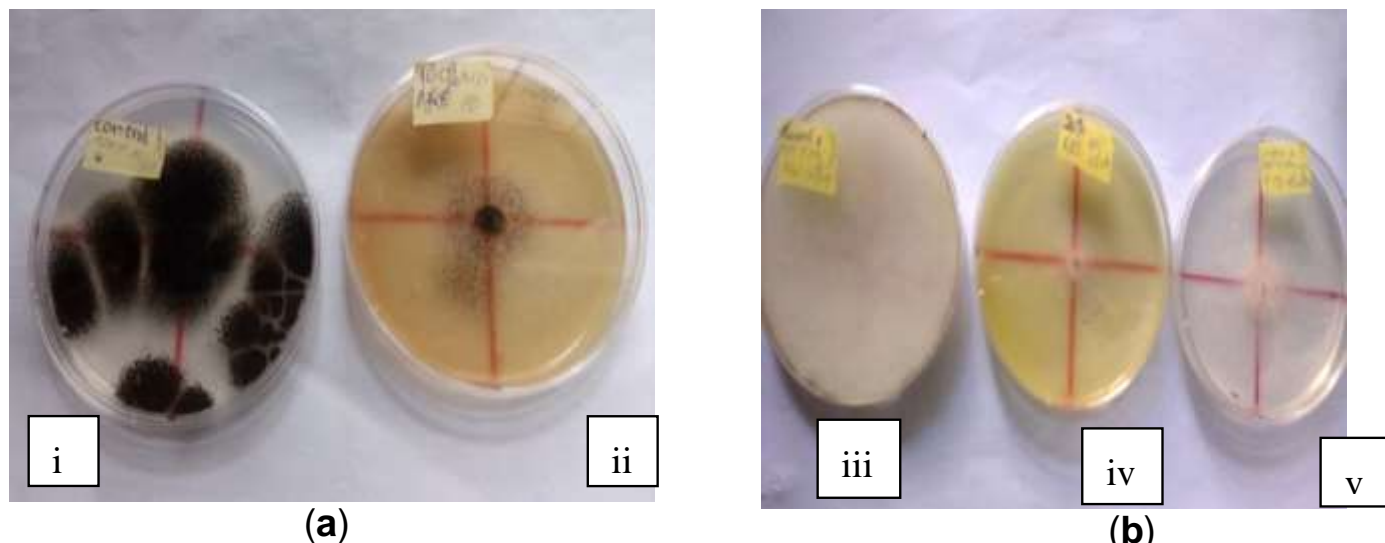
Figure 1. Fungal isolates indicating growth on day 7 in some of the groups. (a) *Aspergillus niger* growth in control 1 (i), and 100% aqueous root extract (ii); (b) *Rhizopius nigricans* growth in control 1(iii), 20% ethanol root extract (iv), and control 2 (v).
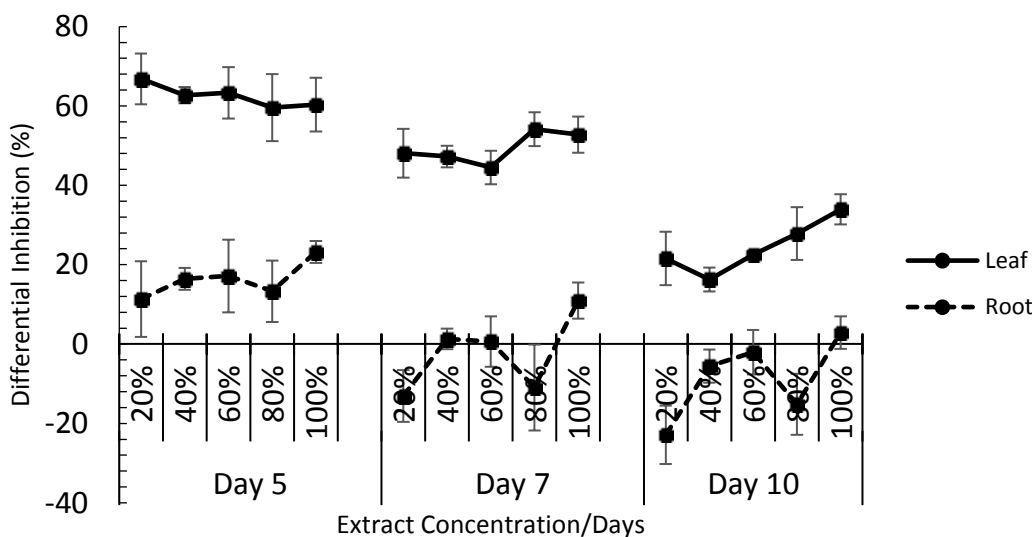
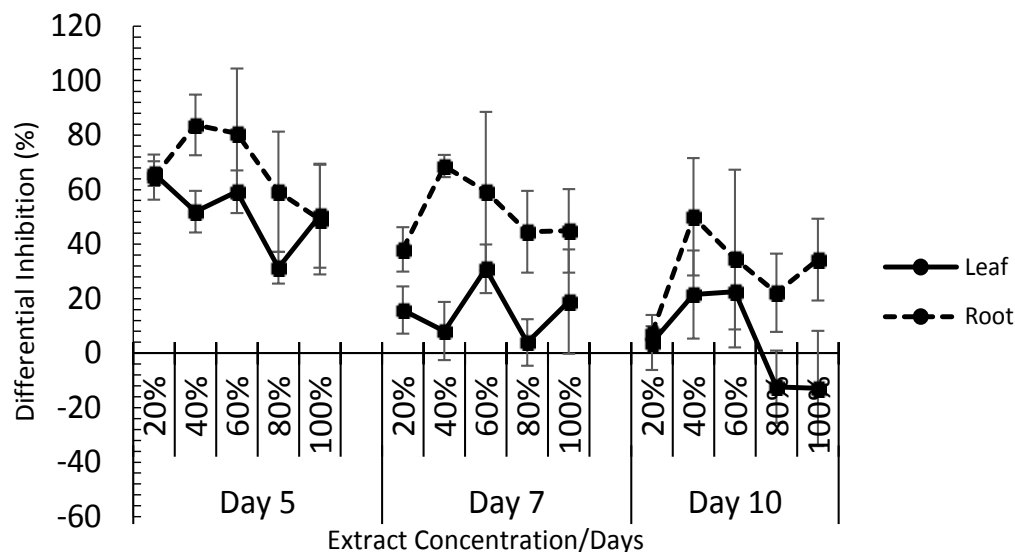**Figure 2.** Differential growth inhibition of *A. niger* by ethanol and aqueous leaf and root extracts of *H. suaveolens*. Graph WAS plotted from difference in inhibitory effect between ethanol and aqueous leaf extracts (━━●━━) and ethanol and aqueous root extracts (··●··). Mean differential growth inhibition is derived by subtracting % growth inhibitionof aqueous extracts from that of ethanol extracts.

nigricans growth at all used concentrations; all detected inhibitory activities were not different from control 1 (p > 0.05). Ethanol root extract of *H. suaveolens* had a very high inhibitory effect on *R. nigricans*; the inhibitory activity was concentration and duration of exposure dependent. The two highest concentrations (80 and 100%) of the extracts inhibited *R. nigricans* growth most effectively, though activities decreased on day 10 compared to day 5, but both concentrations retained a very significantly higher activity compared to control 1 (p < 0.0001).

Aqueous root extract also showed a duration dependent effect against *R. nigricans* growth, but the activities were generally lower than ethanol root extracts. Aqueous root extracts concentrations of 20, 80 and 100% retained same level of significantly higher inhibitory activity when compared to control 1 against *R. nigricans* on days 5, 7 and 10 (p < 0.05).

Figures 2 and 3 show the difference between growth inhibitions caused by ethanol leaf and aqueous leaf extracts, and between ethanol root and aqueous root

Figure 3. Differential growth inhibition of *R. nigricans* by ethanol and aqueous leaf and root extracts of *H. suaveolens*. Graph was plotted from difference in inhibitory effect between ethanol and aqueous leaf extracts (━●━) and ethanol and aqueous root extracts (▪▪●▪▪). Mean differential growth inhibition is derived by subtracting % growth inhibition of aqueous extracts from that of ethanol extracts.

extracts on *A. niger* and *R. nigricans*. In Figure 2, differential growth inhibition of *A. niger* was observed. Ethanol leaf extract showed over 60% greater performance than aqueous leaf extract on day 5; though the difference decreased by days 7 and 10 as an indicative of generally observed decline in inhibitory potency of the extract as days progressed. Despite the decline in inhibitory activities of all concentrations of ethanol leaf extract against *A. niger*, it retained a positive differential inhibition compared to aqueous leaf extract which was completely ineffective against the fungus. Ethanol and aqueous root extracts had low differential inhibitory activities; both were similarly very potent against *A. niger*. On day 5, ethanol root extract had higher inhibitory effect against *A. niger*; this was only retained by 100% concentration by days 7 and 10. This indicates that aqueous root extract retained potency against *A. niger* than ethanol root extract for the duration of the study.

The observed pattern for leaf and root extracts of *H. suaveolens* against *A. niger* was the reversed for *R. nigricans*: Slightly higher differential performance occurred in roots extracts against *R. nigricans* than differential performance of leaf extracts (Figure 3). This is unlike what occurs for *A. niger*, where leaf extracts had much higher differential performance than root extracts. This reverse action is an indicative of poor performance of ethanol and aqueous leaf extracts as growth inhibitors of *R. nigricans*. Only on day 5, ethanol leaf extract was inhibitive to *R. nigricans* growth; by days 7 and 10, the inhibitory activity was completely lost attaining same level

as aqueous leaf extract. The higher root differential inhibition compared to leaf is also indicative of very high growth inhibitory effect of ethanol root extract on *R. nigricans* and relatively low inhibitory effect of some concentrations of aqueous root extract.

Performance of only solvent against extracts

The outcomes of experimental setups to evaluate the fungi growth inhibitory performance of only the extraction solvent against different concentrations of the extracts on day 7 post-treatment are shown as Figures 4 and 5. This distinguishes the additional inhibitory activity of the extracts where inhibition is observed. If inhibition is due to solvent only or extracts, this helps clarification. Generally, all concentrations of leaf and root ethanol extracts inhibited growth of *A. niger* and *R. nigricans* compared to ethanol only. The 20, 40, 60 and 80% concentrations of ethanol only (that is, the respective quantities serially diluted with water) had some inhibitive activities against *A. niger*; this activity was highest at 80% concentration (Figure 4). But their activities were much lower when compared to ethanol leaf and root extracts of *H. suaveolens*. At 100% ethanol concentration, inhibition of *A. niger* growth was abrogated. The pattern of growth inhibition *R. nigricans* by concentrations of ethanol only compared to the leaf and root extracts of same solvent was similar to that of *A. niger*. Though growth inhibition of *R. nigricans* by 60 and 80% ethanol only was similar to corresponding 40 and 20% leaf extracts, r espectively
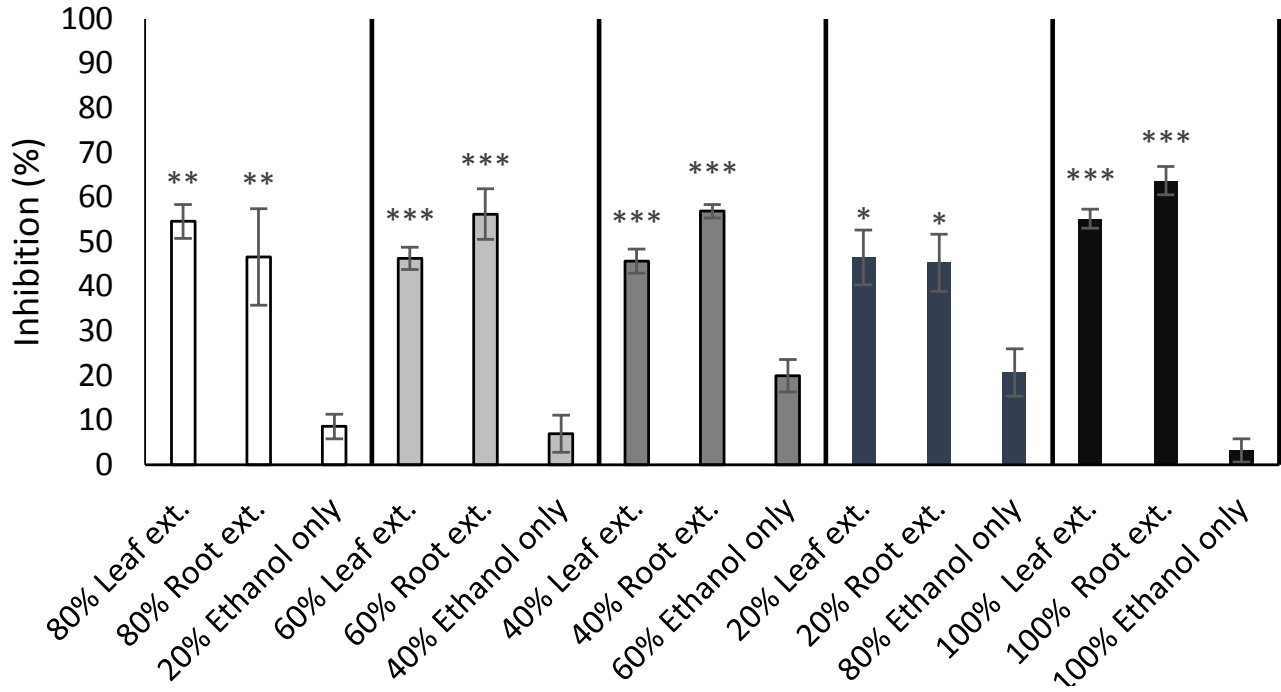
Figure 4. Growth inhibition of *A. niger* by ethanol extracts of *H. suaveolens* compared to solvent only at the end of day 7. *, ** and *: Significantly higher than corresponding solvent only at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.
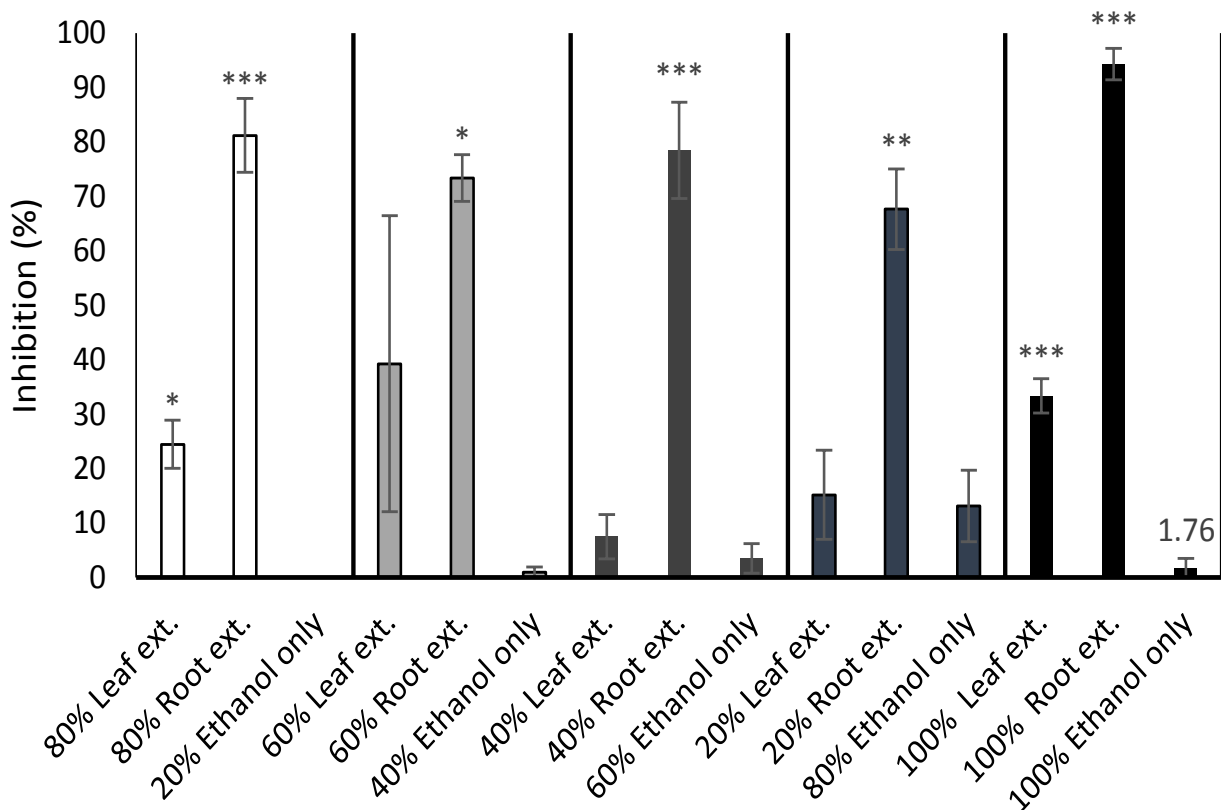
Figure 5. Growth inhibition of *R. nigricans* byethanol leaf and root extracts of *H. suaveolens* compared to ethanol only at the end of day 7. *, ** and *: Significantly higher than corresponding solvent only at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

(Figure 5).

DISCUSSION

The obtained results revealed that *A. niger* and *R. nigricans* are the most common pathogens responsible for postharvest cabbage spoilage in Ojoo, Sango, Bodija and Agbowo in Ibadan, Oyo State, Nigeria, and probably other parts of the globe. This is in line with the submission of Junghare et al. (2014) which reported the close association of *Aspergillus* sp. and *Rhizopus* sp. with vegetable spoilage. The results also showed that leaf and root extracts from *H. suaveolens* can be used effectively to inhibit the growth of fungi associated with postharvest bio-deterioration of cabbage. Thus, *H. suaveolens* possesses antifungal properties as reported by Okonogi et al. (2005) and Sharma et al. (2013).

Antifungal activities observed in the present study corroborates the works of Parichad and Krittaporn (1999) and Ahmad et al. (2013) who found that leaf and root extracts of *H. suaveolens* possess antimicrobial properties. However, the growth inhibition caused by the root extracts was significantly higher than that caused by leaf extracts in the present study. Similar findings were noted by Olofsdotter et al. (2002) and Zhang and Fu (2010) who suggested that root extracts exudes higher amount of the bioactive compounds than the leaves and fruits.

A. niger was more sensitive to the inhibitory effect of the leaf and root extracts of *H. suaveolens*. Sharma et al. (2013) had made a similar observation, where it was suggested that various extracts from *H. suaveolens* showed better antifungal activity against *A. niger* when compared with other fungi. However, the effectiveness of ethanol and aqueous root extracts in inhibiting the growth of the two pathogens differed significantly from each other. This supports the findings of Enyiukwu et al. (2013) who emphasized the influence of extraction solvents on the solubility of the active ingredient(s) in plant extracts. The inhibitory effect exerted by 80 and 100% extract concentrations on mycelia growth of *A. niger* and *R. nigricans* were higher than that caused by other concentrations. This agrees with the report of Babu et al. (2008) who observed higher inhibition of fungal growth at higher concentrations of plant extracts. Duration of exposure to extracts had impact on the mycelia growth of isolated pathogens. This is in accordance with the report of Sobowale et al. (2010) which suggested that there is a relationship between duration of contact and growth inhibition of fungal pathogens.

Conclusion

Ethanol and aqueous leaf and root extracts of *H. suaveolens* possess fungitoxic properties that might be effective as phytofungicides against fungi responsible for

postharvest bio-deterioration of *B. oleracea* and possibly other vegetables. Better understanding of the chemical components of these natural extracts and more researchinto how they can be obtained in large quantities and packaged in a form that can be attractive to farmers is needed. This might also to some extent, solve the problem of chemical pollution and poisoning arising from the use of chemicals in disease management.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Technological properties and sugar tolerance performance of palm wine yeasts isolated from parts of Nsukka, Nigeria

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The ability of yeasts to survive and produce significant ethanol in high sugar medium and high ethanol environment is essential for the use of such yeasts in industrial and edible ethanol production. Four *Saccharomyces* spp. strains ($s_1^t, n_1^t, u_1^t, k_1^t$) isolated from palm wine and an industrial strain were studied in high glucose medium for ethanol production and ability to survive in high sugar medium. *Saccharomyces cerevisiae* s_1^t produced remarkable cell concentration relative to other isolates and the industrial strain, IR-2 in 16, 24 and 36% (w/v glucose) fermentation broth. *S. cerevisiae* s_1^t survived well with good biomass yields of 2.21 and 6.74 fold in 24 and 36% w/v glucose broth, respectively. Ethanol fermentation at glucose concentration of 40% (w/v) produced 42.45 g ethanol concentration (P), 0.387g L^{-1}h^{-1} volumetric productivity (Qp) and a yield (Yps) of 0.329 gg^{-1}. The sugar tolerance property was observed in a fermentation broth with an initial pH of 5.8. Additionally, *S. cerevisiae* s_1^t strain was adaptive to 10% ethanol in 24% glucose solution. The yield obtained and properties exhibited by this isolate compares outstandingly with published data for a range of industrially important isolates; thus, this isolate could be used to produce bioethanol in industrially sustainable processes.

Key words: *Saccharomyces,* bioethanol, osmotic stress, ethanol tolerance, sugar tolerance, indigenous yeast.

INTRODUCTION

Ethyl alcohol (ethanol, bio-ethanol) is a primary metabolite of yeast produced by fermentation of sugar. Yeast is used for the fermentation of simple sugar containing substrate and polysaccharide that can be depolymerized to fermentable sugars (Rajasekaran et al., 2008). Yeast is a small-cell fungus that ferments sugars and reproduced

by budding (Walker, 2009).

The ability of yeast to thrive (ferment) in high sugar medium is one among other attributes required to qualify it for use in industrial ethanol production (Ogbonna, 2013). To ethanol producers, fermentation of high sugar substrate offers economic advantage in the production cost and yield that will be beneficial to the energy balance (Sanchez and Cardona, 2008; Puligundla et al., 2011). As known, the theoretical ethanol yield is 0.51 g in every 1 g of glucose (Bai et al., 2008). Thus, the higher the sugar concentration, the higher the ethanol yields.

However, successful fermentation of high sugar substrate is dependent on the yeast ability to withstand increased osmotic stress and to tolerate high ethanol concentration (Nuanpeng et al., 2011). Yeast cells exceed their normal sugar tolerance limit at more than 24 g/100 mL dissolved solids and thus limit the implementation of fermentation at elevated sugar concentration (Puligundla et al., 2011). The search for this sugar tolerance property in yeast strain has taken a center stage in ethanol research. Screenings for this property (fermenting power) have been done on a large number of strains isolated from grapes, fermenting grape musts and wines (Vaughan-Martini and Martini, 1998). So far, sugar tolerance trait tested and greater than 30% (w/v) has been identified in very few studies with hardly a study from Nigeria (Taing and Fumio, 1997; Scree et al., 2000, Erasmus et al., 2003; Bechem et al., 2007; Laopaiboon et al., 2009; Elizabeth et al., 2014).

Furthermore, bio-ethanol is currently the dominant renewable biofuel used in the transport sector (Sanchez and Cardona, 2008). It has already been introduced on a large scale in various countries such as Brazil and the US, and increasingly in European countries, and is now predominantly produced from sucrose-containing material such as cane molasses and starchy material (mostly grains). Also, at present, all beverage ethanol is made by fermentation (Sanchez and Cardona, 2008). Industrial ethanol is mainly manufactured by fermentation, but some are produced from ethylene by the petrochemical industry (Rajasekaran et al., 2008).

The main challenges in ethanol production at elevated sugar concentration is an effective industrial yeast which is expected to meet the criteria of sugar and ethanol tolerance, ability to thrive in concentrated solution, high overall volumetric productivity and high final ethanol (Slade, 2009). To achieve this target, organism must be able to grow in the inhibitory environment of high concentrations of sugar and other compounds, including ethanol (U.S. DOE, 2006). To enhance ethanol production, screening of palm wine yeast for sugar tolerance attribute is necessary. The aim of the present study was to evaluate the sugar tolerance performance of yeast strain (*Saccharomyces* genus) found in palm wine collected from Nsukka area of Enugu State, Nigeria and to evaluate the suitability of these yeast strains in ethanol production at elevated glucose concentration.

MATERIALS AND METHODS

Collection of sample

The palm wine samples used in this research study was from oil palm (*Elaeis guinensis*), purchased from palm wine tappers (inflorescence and stem tapping) in Nsukka areas (Opi, Ogurute, Udenu and Obukpa communities) of Enugu State. The palm wine was dispensed from the tappers container into a sterile sample bottle and transported immediately in an ice pack to the laboratory for analysis.

Yeast strain, media and culture conditions

Glucose peptone yeast agar (GPY) comprising of 8 g of glucose, 1 g of peptone, 1 g of yeast extract and 1.5 g of agar in 100 mL of sterile distilled water was used as growth medium in the culture of yeast cells from palm wine (Yarrow, 1998; Kurtzman et al., 2011). A ten-fold serial dilution was performed for the palm wine. An aliquot of 0.1 mL of 10^{-4}, 10^{-5} and 10^{-6} dilutions of the palm wine samples were cultured using spread plate technique and incubated at 27±2°C.

Single colonies were selected from GPY plate and purified by successive sub-culturing on GPY agar plates using streaking technique. They were preserved on slants of GPY media in the refrigerator at 4°C till needed.

Standardization of inoculums

Yeast strains selected as potential starter culture were standardized to obtain a uniform cell concentration to be used in subsequent experiment according to the methods of Moonja et al. (2003) and Zheng et al. (2012).

Phenotypic characterization of alcohol producing yeast strains

Twenty eight (28) yeast isolate and a typed strain *S. cerevisiae*-IR-2 (Acession no: DF 396938.1) were screened for ability to produce alcohol according to the method of Brooks (2008). Four alcohol producing strains were obtained, and identified according to the method of Yarrow (1998), Qureshi et al. (2007) and Kurtzman et al. (2011) as *Saccharomyces* s_1^t, *Saccharomyces* n_1^t, *Saccharomyces* u_1^t and *Saccharomyces* k_1^t. These four isolates and the type strain IR-2 were further screened for glucose tolerance.

Glucose tolerance test

Fermentation was carried out using borosilicate glass test tube of 15 mL capacity containing 12 mL of GPY broth at glucose concentrations of 240 and 360 g/L. Cells were inoculated at an initial $O.D_{620}$ of 0.18 from cell suspension in ringer solution. At 3 h interval, a glass test tube was withdrawn and analyzed. The following analyzes were performed at each time interval: biomass determination, reducing sugar concentration and pH.

Genotypic characterization of yeast strain

Based on growth performance, isolate s_1^t was considered suitable for ethanol production studies at more than 360 g/L (w/v) glucose.

At this stage, the identity of the isolate was confirmed molecularly by sequencing the rDNA internal transcribed spacer region (ITS) using the method of Fietto et al. (2004).

Adaptation of yeast cells for combined glucose and ethanol tolerance

S. cerevisiae s_1^t strain was selected and prepared to cope with harsh environmental condition by adapting it to ethanol tolerance in high glucose solution to obtain a starter culture and ensured its viability at ethanol production at 400 g glucose/L. This was achieved in a stepwise manner of culturing the isolate at 24% w/v glucose, and then transferred to 36% w/v glucose and finally 40% w/v glucose. The yeast cells were harvested by centrifuging the culture at 4000 rpm for 5 min and suspended in ringer solution (Moonjai et al., 2003).

The strain was further adapted to ethanol tolerance at three different concentrations of ethanol (5, 10 and 15% v/v) in 240 g/L glucose solution using modified method of John and Watmore (1999). Samples were taken to analyze their viability using pour plate technique.

Ethanol production at 40% (w/v) initial glucose concentration

S. cerevisiae s s_1^t cells were grown in synthetic medium containing (per litre): 400 g of glucose, 3 g of yeast extract, 5 g of peptone, 2.6 g of $(NH_4)_2SO_4$, 2.72 g of KH_2PO_4 and 0.2 g of $Zn(NO_3)_2$. The pH was adjusted to 5.2 using citric buffer (0.04 M) containing (per litre): 1.5 g of citric acid and 6.0 g of sodium citrate (Moonjai et al., 2003). Cells were inoculated at an initial O.D$_{620}$ of 0.18 which correspond to 6.6×10^9 cells/mL. The fermentation was carried out at 27±2°C under static condition in duplicates for 120 h. At 12 h interval, a glass test tube was withdrawn and analyzed for biomass, reducing sugar concentration and ethanol concentration.

Analytical methods

Measurement of cell concentration

The change in biomass was estimated via optical density reading of the sample using colorimeter at 620 nm (Digital colorimeter, Model 312E, EI products, India) and compared with a standard graph of optical density of the yeast cell versus cell concentration.

Glucose concentration measurement

The cell free extract obtained by centrifugation of the fermentation broth at 4000 rpm for 5 min was analyzed for total residual sugars by dinitrosalicylic acid method (Miller, 1959).

Measurement of pH

The changes in pH were measured using a digital pH meter (Hanna Instrument- H198107, pHep pH Tester, Italy).

Measurement of ethanol concentration

The ethanol concentration was estimated by iodine/thiosulphate method (A.O.A.C, 1980). The number of moles of thiosulphate titre volume was used to estimate the concentration of ethanol. The EBAS stoichiometry calculator software downloaded from www.titrations.info/iodometric-titrations was used in calculating thiosulphate solution concentration.

Mathematical estimation

Change in biomass ($Y_{x/s}$)

The change in biomass ($Y_{x/s}$) was calculated as the actual viable cells produced and expressed as grams per gram glucose utilized (g g^{-1} glucose). The actual viable cell obtained in cells/ml was converted to grams based on the thumbs rule that one gram dry weight of yeast equates approximately 4.87×10^{10} cells (Russell, 2003).

Ethanol yield (Y_{ps})

The ethanol yield (Yps) was calculated as the actual ethanol produced and expressed as g ethanol per g glucose utilized (g g^{-1}).

Volumetric productivity (Q_p) and yield efficiency (E_y)

The volumetric ethanol productivity (Qp) and the percentage of conversion efficiency or yield efficiency (Ey) were calculated by the following equations (Laopaiboon et al., 2008):

$$Q_P = \frac{p}{t} \ and E_Y = Y_P \times \frac{100}{0.51}$$

Where, P is the actual ethanol concentration produced (g L^{-1}), t is the fermentation time (h) giving the highest ethanol concentration and 0.51 is the theoretical yield of ethanol on glucose.

Statistical analysis

One way analysis of variance (ANOVA) comparison was performed using stata version 12 statistical software package. SPSS version 20 statistical software was used for graphical illustrations.

RESULTS

Screening/characterization of yeast strains

In the screening of 28 yeast isolates and a type strain (*S. cerevisiae* IR-2) for fermentative ability, 21 isolates were observed to be capable of gas production, while 7 isolates produced no gas in Durham's tube (Table 1). Four alcohol productive strains, one from each sample location and the type strain IR-2, were selected. The five selected yeast isolates s_1^t, k_1^t, n_1^t, u_1^t and IR-2 were identified to belong to the genus *Saccharomyces*. However, *Saccharomyces* spp. n_1^t and u_1^t yielded low biomass at 16% (w/v, glucose) and were thus screened out.

Growth studies of yeast isolates during glucose tolerance

The *S. cerevisiae* s_1^t growth response pattern in 24 and

Table 1. Screening results of yeast strains.

Column 1	Column 2	Degree of gas production	Degree of gas production
S/N	Isolate	24hours	48 hours
1.	n_1^t	+++++	++++
2.	n_2^t	+++	++++
3.	n_3^t	++	++++
4.	n_4^d	++++	++++
5.	n_5^d	++	++++
6.	n_6^d	-	-
7.	n_7^d	++++	++++
8.	n_8^d	+++	++++
9.	s_1^t	++++	++++
10.	s_2^t	++++	++++
11.	s_3^t	++++	++++
12.	s_4^t	++++	++++
13.	s_5^d	++++	++++
14.	s_6^d	-	-
15.	s_7^d	++++	++++
16.	u_1^t	++++	++++
17.	u_2^t	+++	+++
18.	u_3^t	++++	++++
19.	u_4^t	-	+++
20.	u_5^t	-	+
21.	u_6^d	++++	++++
22.	u_7^d	-	+++
23.	k_1^t	++++	++++
24.	k_2^t	-	+++
25.	k_3^t	++	++++
26.	k_4^t	-	-
27.	k_5^t	++++	++++
28.	k_6^d	++++	++++
29.	IR-2	++++	++++

+++++: Very high gas production; ++++: high gas production; +++; moderate gas production; - no gas production; n^t: top palm wine from Ogurute; n^d: down palm wine from Ogurute; s^t: top palm wine from Opi; s^d down palm wine from Opi; u^t: top palm wine from Udenu; u^d: down palm wine from Udenu; k^t: top palm wine from Obukpa; k^d: down palm wine from Obukpa.
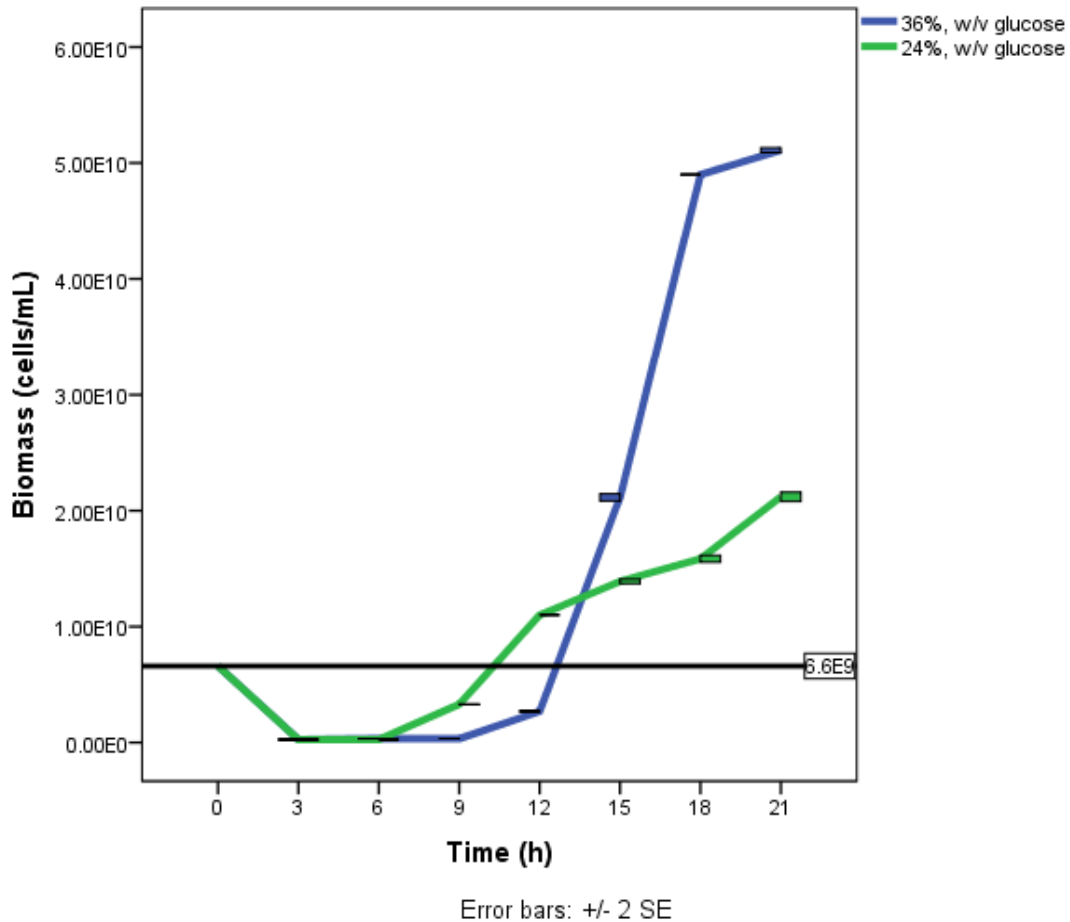
Figure 1. Response pattern of *S. cerevisiae* s_1^t strain in GPY medium.

36% w/v, glucose GPY medium during 21 h fermentation studies showed glucose tolerance qualities (Figure 1). At 24% (w/v glucose), the cell concentration increased 2.21-fold at the end of the fermentation (2.12×10^{10} cells/mL compared with 6.6×10^9 cells/mL initial concentration), while at 36%, the cell concentration increased 6.74-fold (5.11×10^{10} cells/mL when compared with 6.6×10^9 cells/mL initial concentration). However, at 36% glucose concentration, the lag phase of s_1^t strain last longer than at 24% glucose concentration. In contrast, k_1^t strain and IR-2 strain were unable to thrive at both concentrations of glucose tested (Figures 2 and 3). In the light of the growth response, k_1^t was not used in further studies.

S. cerevisiae s_1^t **glucose consumption rate and change in biomass during 21 h fermentation**

The *S. cerevisiae* s_1^t strain utilized 42.91% glucose in 24% w/v, glucose with a corresponding biomass yield of 0.003 gg^{-1} (0.435 g when compared with 0.136 g initial biomass) (Figure 4). A residual sugar of 57.09% was *cerevisiae* s_1^t strain utilized 29.2% glucose with a corresponding biomass yield of 0.009 gg^{-1} (1.049 g when compared with 0.136 g initial). A residual sugar of 70.8% was observed.

pH profile of medium during 21 h fermentation

Figure 5 shows the pH profile of *S. cerevisiae* s_1^t strain during fermentation. The initial pH of the fermentation broth was 5.8. It was observed that in 24% (w/v) sugar, the pH dropped to 4.8 as compared to 5.2 at 36% (w/v) sugar. At both concentrations, the pH of the broth slightly increased within 6 h before decreasing until the end of fermentation period. Similarly, Figure 6 shows the pH profiles of IR-2 strain. In 24% (w/v, glucose concentration), a slight increase in the pH from initial Ph of 5.8 was observed at 6 h before decreasing to the initial pH of 5.8 at the end of the fermentation study.
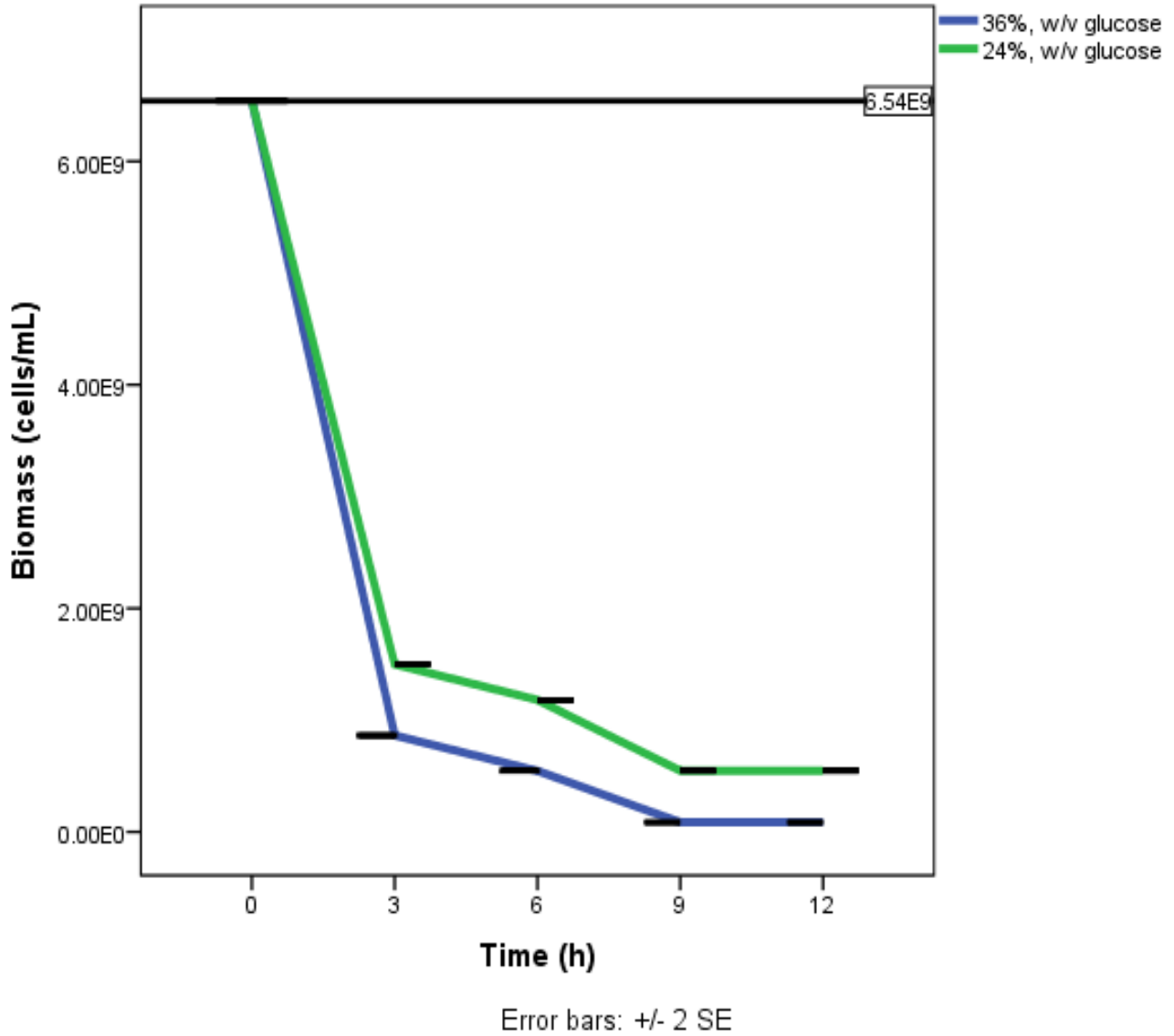
Figure 2. Response pattern of *Saccharomyces* sp. k_1^t strain in GPY medium.

Combined glucose and ethanol tolerance of S. cerevisiae s_1^t strain

The s_1^t strain was cultured at 24% w/v glucose supplemented with different concentrations of ethanol (5, 10 and 15% v/v). Figure 7 shows the growth response pattern at the different concentrations of ethanol. The total cell concentration produced at 0, 10 and 15% v/v were 6.15 ×10^9, 1.11×10^10 and 4.15 × 10^9 CFU/mL, respectively. These values were significantly lower than biomass at 5% v/v (2.30 × 10^10 CFU/mL) (P<0.05), but there was no significant difference in cell concentration at 0 (that is, when no ethanol was supplemented) and 15%

v/v.

Ethanol production of S. cerevisiae s_1^t strain

Ethanol fermentation studies with *S. cerevisiae* s_1^t strain at glucose concentration of 40% (w/v) produced 46.45 g/L ethanol and productivity of 0.387 Lh^-1 at 120 h fermentation period (Figure 8). The *S. cerevisiae* s_1^t strain utilized up to 32.25% glucose (Figure 9). Under this anaerobic condition, the cell concentration increased 10 fold (7.16 × 10^10 cells/mL when compared with 6.6 × 10^9
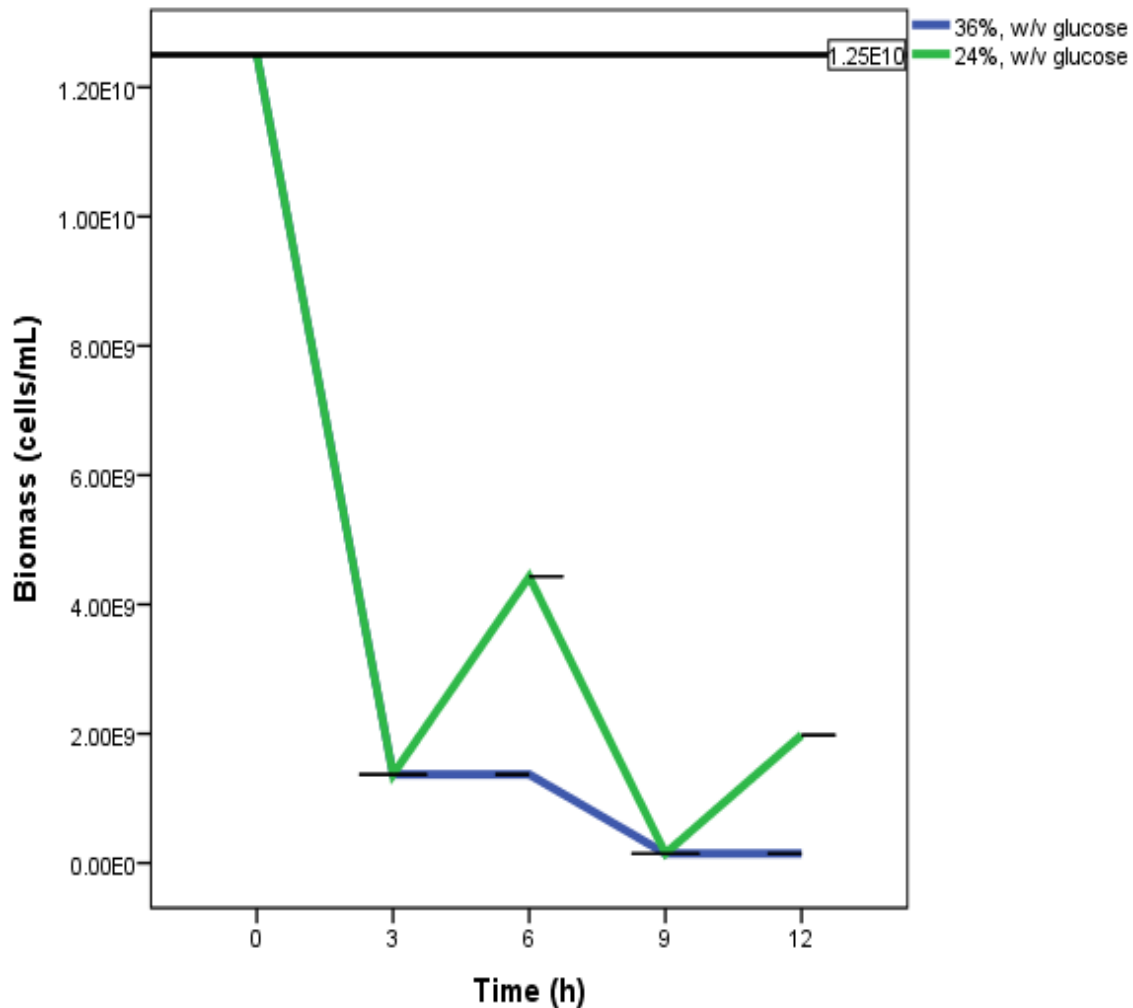
Figure 3. Response pattern of *S. cerevisiae*-IR-2 strain in GPY medium.

cells/mL initial concentration) (Figure 10) and an ethanol yield of 0.329 gg^{-1} was observed.

Genotypic characterization of *S. cerevisiae* s_1^t strain

The yeast isolate was confirmed with partial 18S rDNA sequencing. The phylogenetic relationship of this isolates is shown in Figure 11. *S.cerevisiae* s_1^t is closely related to *S. cerevisiae* AD115 with 100% similarity.

DISCUSSION

Glucose tolerance

Out of the 28 palm wine yeast isolates assessed for the ability to ferment sugar to ethanol, 21 were positive.

These findings suggest that most yeast of palm wine were likely to have sugar fermentative tendency. Glucose at 24 and 36% w/v inhibited the growth rate of *S. cerevisiae* k_1^t and IR-2. Hence, it was needless to continue the fermentation experiment for 21 h run. In addition, there was no observed evidence of fermentation such as gas evolution; rather the cells died. According to Puligundla et al. (2011), some yeast fermentative ability and viability are severely compromised under high osmo-stress conditions. Similarly, Bonin and Skwira (2008) identified that high initial glucose-containing medium with sugar concentration of 200 to 300 g/L results in significant decrease of fermentation efficiency and yeast viability. However, high growth rate and fermentation rate was observed with the *S. cerevisiae* s_1^t strain. The strain had a biomass yield of 0.003 gg^{-1} in 24% (w/v) glucose concentrations which increased 2-fold in 36% (w/v)
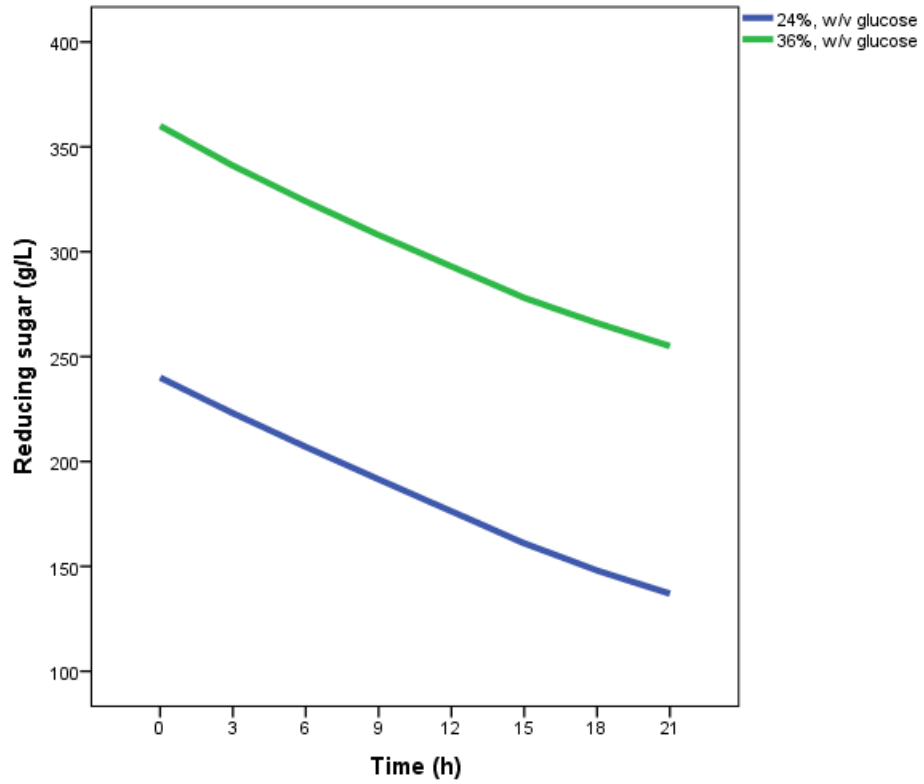
Figure 4. Glucose consumption pattern of S. cerevisiae strain in GPY medium.

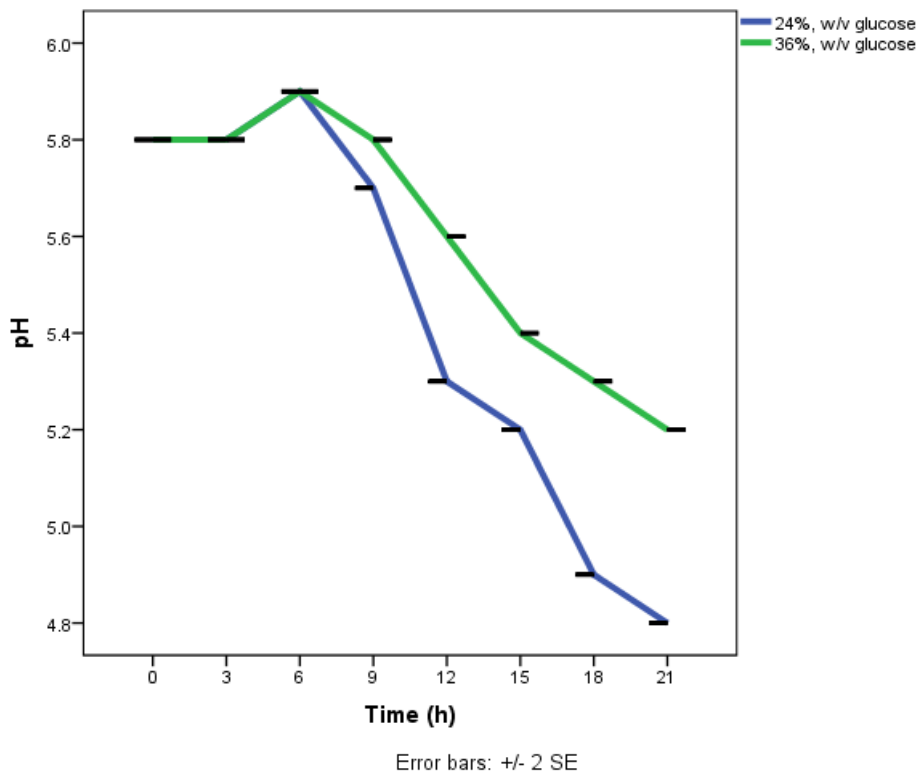
Error bars: +/- 2 SE

Figure 5. pH variation of S. cerevisiae strain in GPY medium.
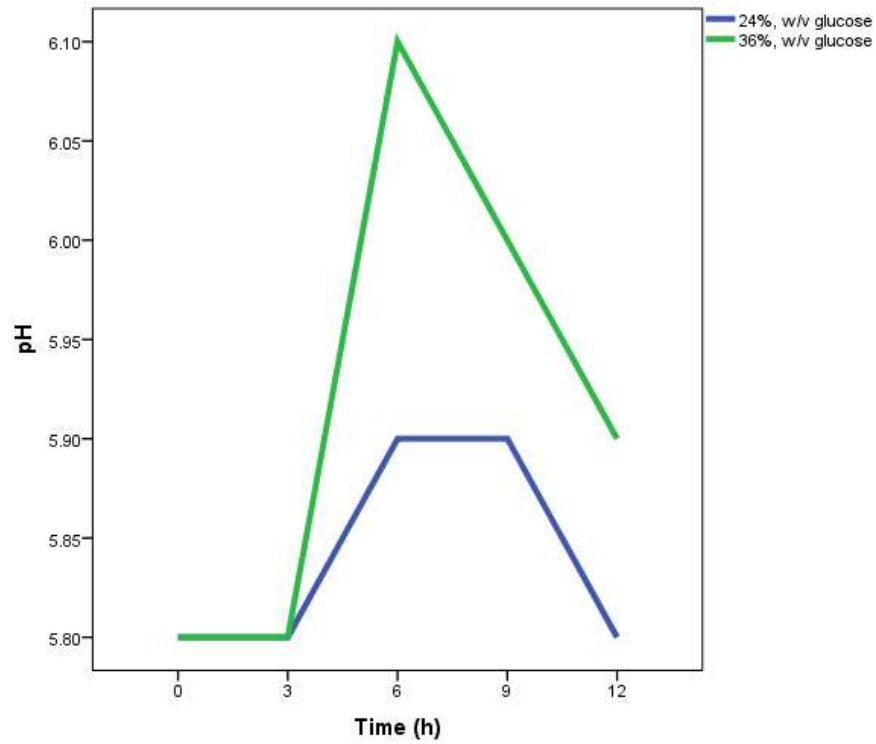
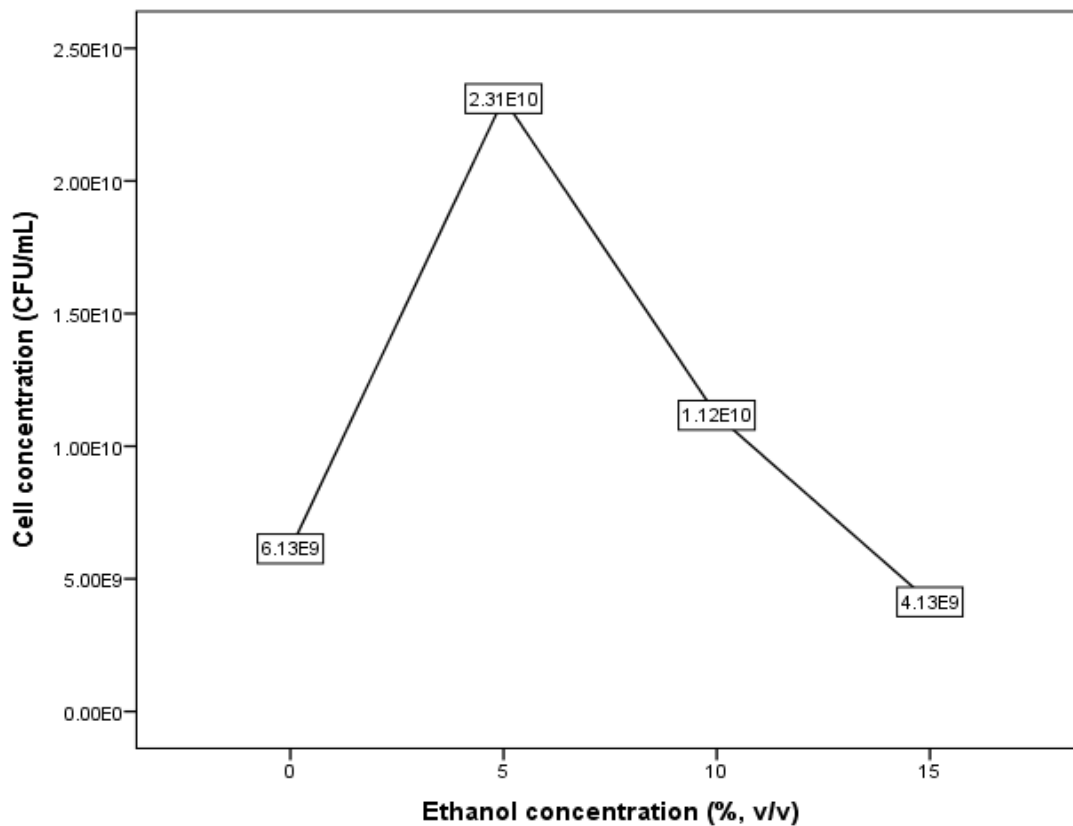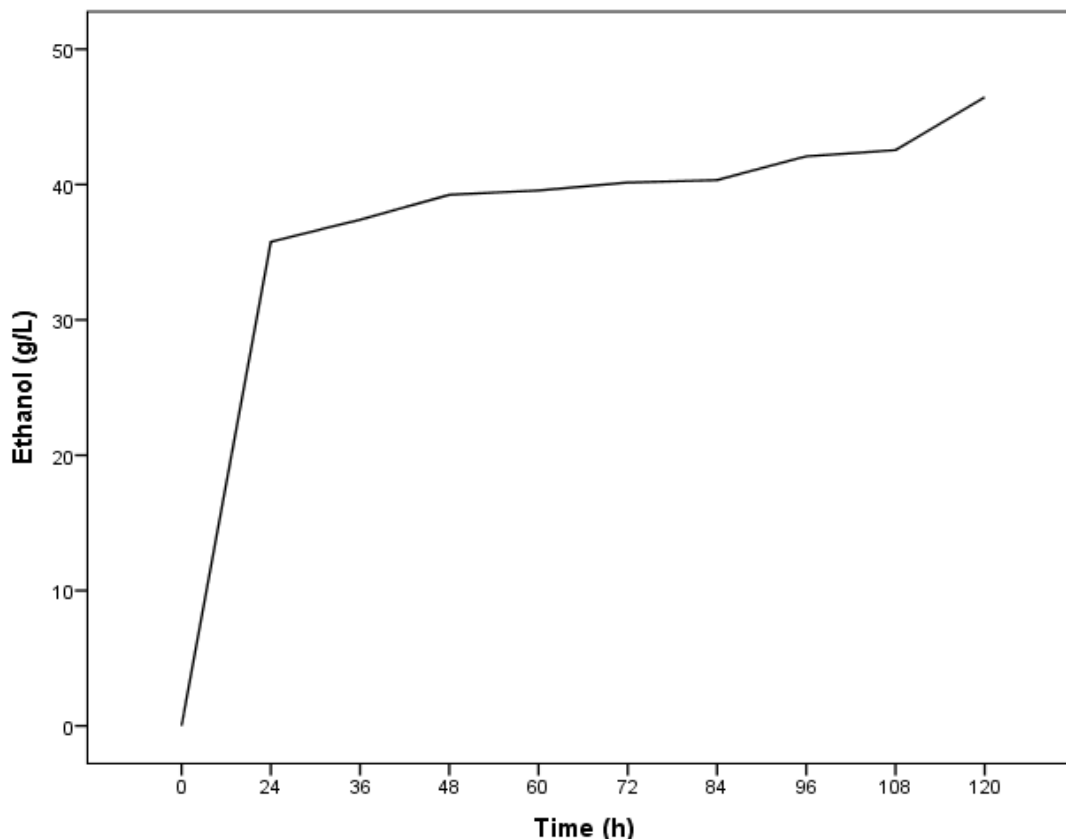**Figure 6.** pH variation of *S. cerevisiae*-IR-2strain in GPY medium.

Figure 7. Growth response of S. cerevisiae strain during ethanol adaptation.

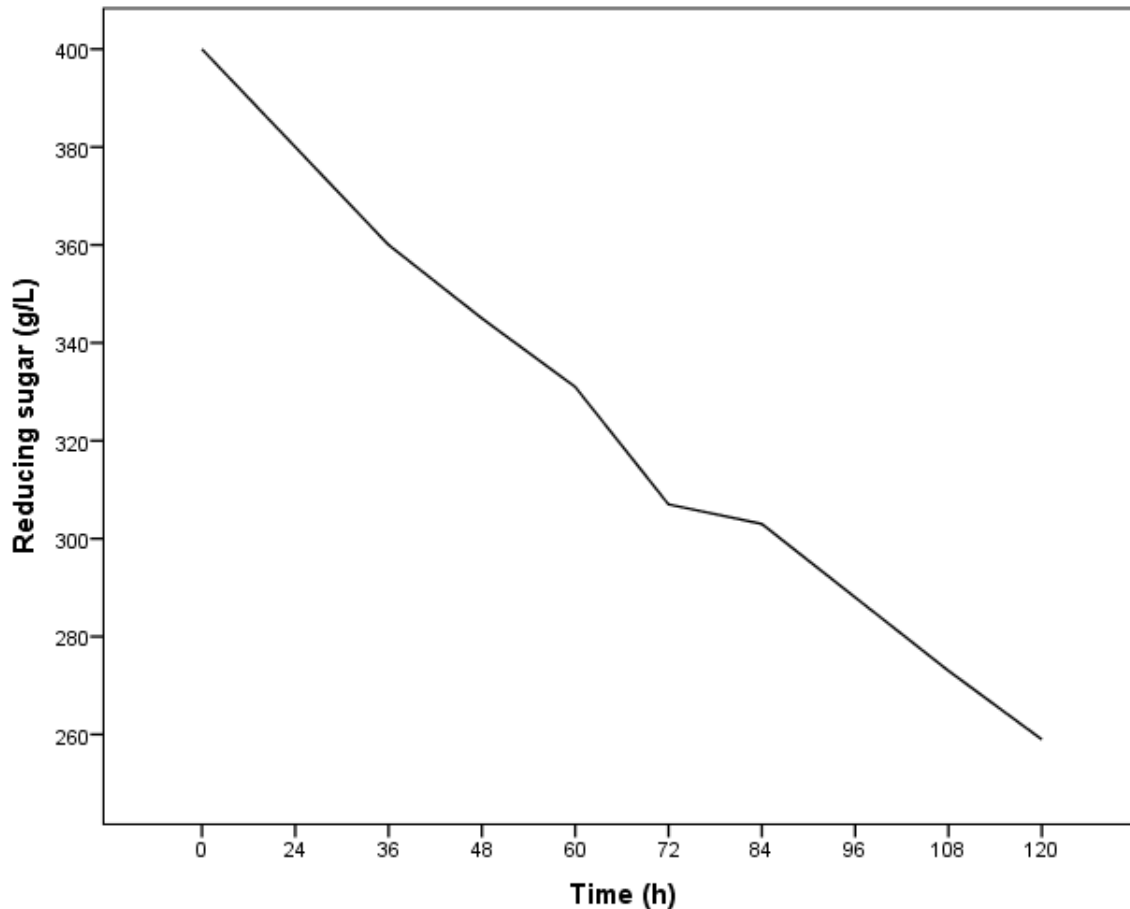**Figure 8.** Ethanol productivity of S. cerevisiae   strain in 40% (w/v) glucose ethanol production medium.

glucose concentration (0.003 as compared to 0.009 g g^{-1}). This physiological character is uncommon and has been reported in very few studies. Puria et al. (2009) reported on a yeast strain which was adaptive to 20 and 25% w/v glucose concentration. In Japan, 23 yeast strains were identified with the ability to grow on 50% w/v glucose and all but two strains grew on 60% w/v glucose medium (Taing and Fumio, 1997). In Cameroun, Bechem et al. (2007) found that 20% of the yeast strain from palm wine grew on 40% sucrose solution. Scree et al. (2000), reported on four *S. cerevisiae* from soil sample. They observed that all isolates were able to tolerate up to 350 g/L glucose. In a similar finding, Erasmus et al. (2003) observed yeast tolerance and growth in low water activity (40% w/v sugars) with a maximum specific growth rate of 0.023 h^{-1}. In Asia, Laopaiboon et al. (2009) observed yeast tolerance up to a concentration of 320 g/L. In Mexico, Elizabeth et al. (2014) assessed the osmotolerance properties in yeast strain in glucose media to be as high as 40% and their findings detected fermentative ability. To some yeast producers, sugar tolerance is a characteristic that varies the most between regions (Lallemand, 1996). Similarly, in the current research, finding supports these high sugar tolerance possibilities in yeast as the *S. cerevisiae* s_1^t strain held

up well under the 360 g/L glucose stress. However, at 36% glucose concentration, the lag phase of *S. cerevisiae* s_1^t strain lasted longer than at 24% glucose concentration. The difference may be due to the lowering of the water activity at higher glucose concentration. As known, sugar tolerance ability of yeast cells is critical in excess of glucose (>20% w/v). This is because as water concentration is lowered below the optimum level, the length of the lag phase increases and the growth rate decreases (Jay, 2005). Similarly, Osho (2005) identified that at increased sugar concentration of 20 to 25%, some strains of *Saccharomyces* species had prolonged lag phase of 12 h.

Combined glucose and ethanol tolerance

In this study, the result shows that the *S. cerevisiae* s_1^t strain was adaptive to 10% ethanol in 24% (w/v) glucose solution. From this observation, ethanol play a crucial substrate role in yeast propagation. The biomass yield improved significantly with supplementation at 5% ethanol, though yield tilted downwards at 10% ethanol concentration but it was statistically clear that the

Figure 9. Glucose consumption pattern of S. cerevisiae strain in 40% (w/v) glucose ethanol production medium.

biomass yield was still greater than glucose solution without ethanol supplementation (0%). However, at 15% (v/v) ethanol viability decreased significantly. This result suggest the possibility S. cerevisiae s_1^t strain to thrive at high ethanol and glucose concentrations. Strobel and Lynn (2004) reported that an adapted strain of *Clostridium thermocellium* tolerated an ethanol concentration of 6 (wt/v) while the wild-type did not tolerate beyond 1.5 (wt/v) ethanol.

Ethanol production

Studies on high substrate fermentation have confirmed the fact that higher substrate concentration results in higher ethanol concentrations (Laopaiboon et al., 2008, 2009). A study by Laopaiboon et al. (2008) showed that at 240 g/L glucose concentration, the ethanol concentration produced with *S. cerevisiae* TISTR 5048 was 99.58 g/L and at 280, it was 99.42 g/L, at 320, it was 97.01 g/L while *S. cerevisiae* strain NP01 produced ethanol concentration of 101.95 g/L at 240, 104.680 g/L

at 280 and 104.68 g/L at 320. In the current study, the *S. cerevisiae* s_1^t yeast strain produced 46.45 g/L of ethanol at 400 g glucose/L with a productivity and ethanol yield of 0.387 gL^{-1} h^{-1} and 0.329 g g^{-1}, respectively, after five days cultivation. The result of ethanol fermentation in the current study with *S. cerevisiae* s_1^t strain affirms past findings on sugar tolerance and ethanol concentration of yeast cells at elevated sugar concentration.

Reducing sugar utilization by *S. cerevisiae* s_1^t

The glucose utilization rate appears slow as the sugar concentration increased. As known, ethanol inhibits cell growth, and also represses glucose transport (Salmon, 1989). In the current study, utilization of 42.91% was observed at 21 h fermentation in 24% (w/v) glucose concentration while utilization of 29.2% was observed at the same incubation time at 36% glucose initial concentration. In the course of 120 h ethanol fermentation, utilization of 32.25% was observed at 40%
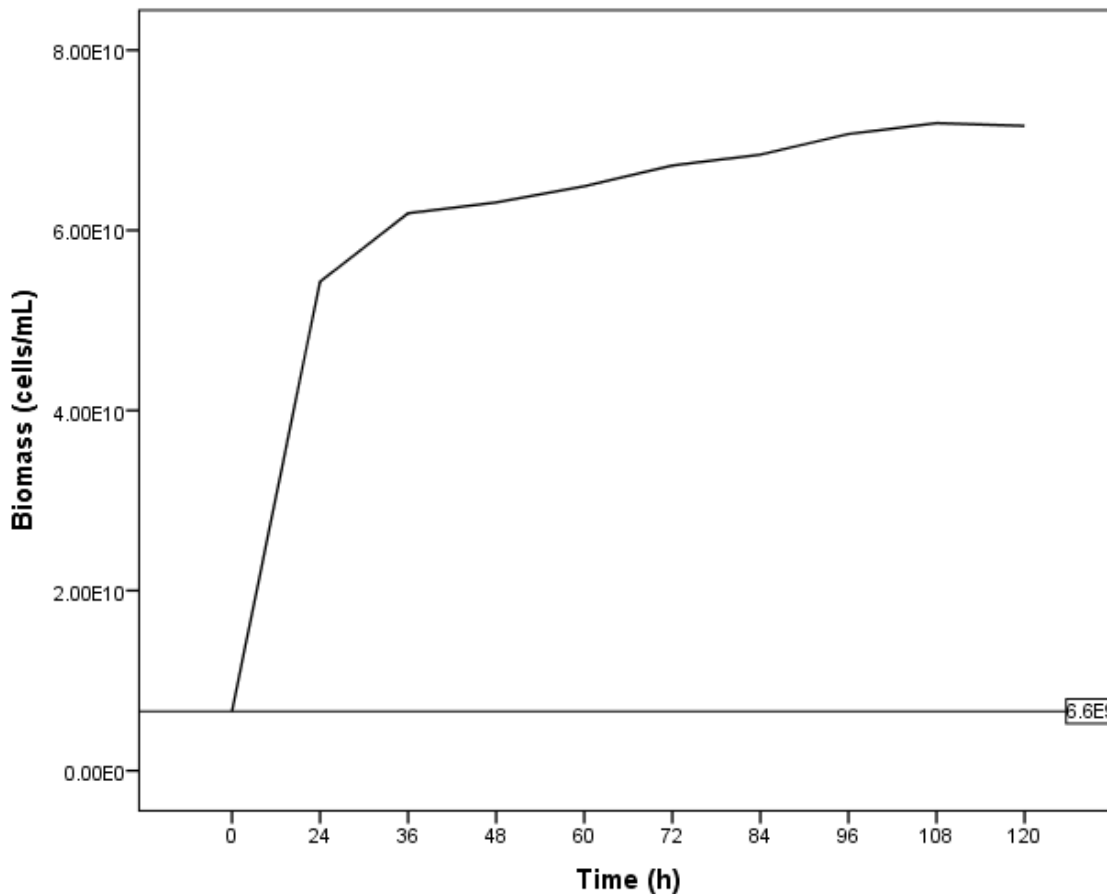
Figure 10. Growth extent of S. cerevisiae   strain in 40% (w/v) glucose ethanol production medium.
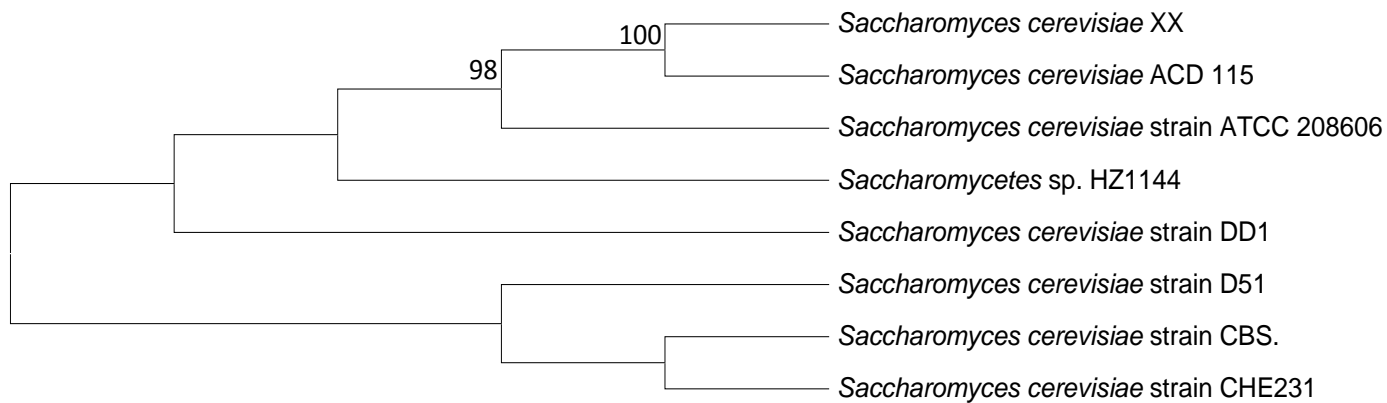
Figure 11. The phylogenetic tree of *S. cerevisiae* SCPW17. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA5.

(w/v) glucose initial concentration. This trend suggests a repressed glucose transport as the sugar concentration increased. In addition, the volumetric ethanol productivity with *S. cerevisiae* s_1^t strain (0.387 gL^{-1}h^{-1}) was lower than the expected for high substrate medium (2-5 gl^{-1}h^{-1}) (USDOE, 2012). These suggest the possibility that the isolate may not be a good ethanol producer despite is sugar tolerant ability. As reported by Jay (2005) and Puligundla et al. (2011), *Saccharomyces rouxii* grew well

in high sugar media with a water activity of 0.6 but its ethanol production levels was relatively low. Nonetheless, in this current research, the *S. cerevisiae* s_1^t strain apparent ability to cope with the high initial glucose concentration was a good physiological trait. As also reported by Basso et al (2011), yeast strain that can ferment substrate with high productivity or at least cope with high substrate concentration even operating at normal ethanol titres is required in ethanol production.

Moreover, the *S. cerevisiae* s_1^t strain can come handy in high-sugar fermented food products for which sugar tolerant yeast could be employed during processing. For instance, a food product processed from fermentation of high sugar vegetables have been reported in Japan (Taing and Fumio, 1997). It could also be used in the fermentation of high fructose corn syrup (HFCS) and in alcoholic beverage production. In addition, one technology used in increasing brewing capacity is to ferment 18 g extract per 100 g liquid (18°P) to produce beers rather than the 12°P fermentation (Huuskoneni et al., 2010; Puligundla et al., 2011) thus requiring a yeast strain such as *S. cerevisiae* s_1^t that tolerate high gravities.

Conclusions

The results obtained from the study have shown that isolate *S. cerevisiae* s_1^t strain is tolerant to high glucose-containing medium. The isolate was tolerant to concentration of glucose higher than 24% (w/v), which is a physiological character highly considered in yeast utilized as fermentation starters in ethanol industry. Based on these findings, the *S. cerevisiae* s_1^t strain proved to be a good choice for industrial ethanol production. Finally, the *S. cerevisiae* s_1^t strain sugar tolerance trait is of interest in some food industries which may be exploited. For maximum accumulation of ethanol by the *S. cerevisiae* s_1^t strain, further studies may be undertaken on metabolic engineering of the isolate.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

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Genetic variability, heritability and expected genetic advance of yield and yield related traits in common bean genotypes (*Phaseolus vulgaris* L.) at Abaya and Yabello, Southern Ethiopia

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This experiment was conducted to evaluate 36 common bean genotypes including seven released varieties to generate information on the extent of genetic variability, heritability and expected genetic advance of yield and yield related traits. The field experiment was conducted in 2015 at two locations (Abaya and Yabello) and genotypes were planted in triple lattice design. Data were collected on yield and important agronomic traits. The estimated genotypic (GCV) and phenotypic (PCV) coefficient of variations ranged from 4.82 to 9.85% and 7.03 to 12.93%, respectively for combined analyses. The PCV values were relatively greater than GCV in magnitude for all traits, of which the magnitude of the differences were large for grain yield, seeds number per plant and number of primary branches, but was relatively low for plant height and number of seeds per pod. Broad sense heritability ranged from 18.29 to 58.6%, and genetic advance as percentage of mean ranged from 4.25 to 14.42%. Only plant height and seed number per pod had moderate heritability coupled with relatively high genetic advance values.

Key words: Genotypic coefficient of variation, phenotypic coefficient of variation, broad sense heritability, genetic advance as percentage of mean.

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.), also called field bean, dry bean, french bean, kidney bean etc. is one of the most important legume crops in the world (Karasu and Oz, 2010). Common bean is a diploid ($2n = 2x = 22$) and predominantly self-crossing species although 3% or more out crossing rate has also been observed (Ibarra-

Perez et al., 1997). It is the world's second most important pulse after soybean (Parades et al., 2009) and is regarded as "Grain of hope" as it is an important component of subsistence agriculture and feeds about 300 million people in tropics and 100 million people in Africa alone (Sofi et al., 2011). Common bean is among

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the major pulses grown in the lowland to mid-altitude sub-humid agro-ecologies of Ethiopia (Teshale et al., 2006). It is a crop of rich protein and minerals such as iron and zinc in the diet, and has short maturity period of about three months, thus it is available for family consumption during the period when other crops are immature (Teshale et al., 2006).

Variability is the occurrence of differences among individuals due to differences in their genetic composition and/or the environment in which they are raised (Allard, 1960; Falconer and Mackay, 1996). For effective selection, information on nature and magnitude of variation in populations are necessary (Yagdi, 2009). The choice of promising genotypes from diverse genetic base, and their subsequent utilization for hybridization is one of the strategies for improving the productivity of common beans (Mulugeta et al., 2013). Genetic variability studies have been conducted in Ethiopia by considerable number of researchers on common bean (Daniel et al., 2015; Alamayehu, 2010; Barecha, 2015; Kassaye, 2006; Boru, 2014). Most of the studies on common bean variability were conducted in other parts of the country not in Borena zone (southern Ethiopia) where moisture stress is a major crop production problem and the agriculture production is dominated by pastoralist and agro-pastoralist. Common bean is a major pulse crop grown by Agro pastoralist and Farmers of the study area. Moreover, information is lacking on the potential of common bean genotypes in southern Ethiopia in general and Abaya, and Yabello district of Borana zone in particular. Hence the present study was undertaken to estimate the extent of genetic variability, heritability and genetic advance in common bean genotypes for yield and yield related traits.

MATERIALS AND METHODS

The experiment was conducted at Yabello and Abaya during 2015 cropping season. The experimental areas are located in the Southern part of the Ethiopia in the Oromia Regional State. The detail description of the study areas are listed in the Table 1.

Experimental materials

For this study, 36 common bean genotypes were obtained from Melkasa Agriculture Research Centre (MARC) and evaluated for genetic variability, heritability and genetic advance for yield and yield related traits. Among the tested genotypes, seven are varieties released in the different years and for different areas. Description of the new common bean entries and released varieties are presented in Appendix Tables 1 and 2.

Experimental design and managements

The experiment was laid out in 6 × 6 triple lattice design. Each entry was planted in a plot having 6 rows of 4 m length. Four rows were harvested and two border rows were left to exclude border effect. The row and plant spacing was kept at 40 and 10 cm, respectively. Individual plot size was 2.4 m × 4 m = 9.6 m^2 and 1 and 1.5 m between replication and sub block, respectively. Fertilizer was

applied as nationally recommended for the crop at the rates of 46 kg P_2O_5 and 18 kg N /ha (100 kg/ha DAP) at the time of planting. All other agronomic managements were applied uniformly in all experimental plots as per national recommendation for the crop.

Data collection

The following data were collected during the experiment time both from the whole plot, net plot and sampled plants by random selection method from the middle of four rows of each plot.

Data recorded on plant basis

Plant height at harvest (cm)

Height of five randomly taken plants during harvest period from each experimental plot was measured in centimetre from the ground level to top of the plants and the average height was recorded.

Number of primary branches

Number of productive branches extending from the main stem was recorded from five randomly selected plants and average branch number was taken.

Pod length (cm)

The length of five randomly selected pods from each of the five randomly selected plants was measured at harvesting and the average was used.

Number of pods per plant

This was recorded as average total number of pods of five randomly selected plants from each experimental plot at harvest.

Number of seeds per pod

This was recorded as average total number of seeds of five randomly selected plants from each experimental plot divided by total number of pod of the same plants at harvest.

Seeds per plant

Average number of seeds counted from five randomly selected plants.

Data collection on plot basis

Days to flowering

This was recorded as numbers of days from the date of emergence to the date on which about 50% of the plants in each plot produce flowers.

Days to maturity

This is the number of days from planting to the date when 90% of the morphological observation of the plant turned to yellow straw colour.

Table 1. Description of the study area.

Variable	Locations	
	Yabello	Abaya
Distance from Addis Ababa (km)	561	365
Soil type	Sandy	Sandy clay loam
Altitude (m.a.s.l.)	1631	1442
Latitude	02°88'006"N	06°43'520"N
Longitude	038°14'761"E	038°25'425"E
Annual temperature (°C)		
Minimum	14.5	12.6
Maximum	26.3	29.9
Annual rainfall (mm)		
Minimum	400	500
Maximum	700	1100

Stand count at harvest

This was recorded by counting the total number of plants from the four middle rows of each plot at harvest.

Grain yield (g/plot)

Grain yield in grams obtained from the central four harvestable rows of each plot was harvested, threshed and weighted using sensitive balance and then adjusted to 10% moisture content.

Grain yield (ton/ha)

Grain yield obtained from each plot was used to estimate grain yield (tons) per hectare.

Thousand seed weight (g)

The weight in grams of 1000 seed was randomly taken from each experimental plot using sensitive balance and adjusted to 10% moisture content.

Data analysis

Analysis of variance

Analysis of variance (ANOVA) was computed for grain yield and other traits as per the methods described by Gomez and Gomez (1984) using SAS computer software (Version 9) for triple lattice design. Comparison of treatment means was made using Duncan Multiple Range test (DMRT) at 5% level of significance test. Location wise analyses were performed and error variances were subjected to F-test for homogeneity test of variances. Variables with homogeneous error variances were directly used for combined analyses, while those with heterogeneous error variances were analysed in individual locations. The combined analysis was based on mixed model (fixed genotype and random environment).

Individual locations ANOVA were computed using the following mathematical model:

$$Yijl = \mu + rj + gi + P\,l(j) + \varepsilon ijl$$

Where, Yij = the observed value of the trait Y for the ith genotype in j^{th} replication; μ= the general mean of trait Y; rj = the effect of jth replication; gi= the effect of ith genotypes and $pl(j)$ =block within replicate effect, and εijl = the experimental error associated with the trait y for the ith genotype in lth block with in replication and j^{th} replication.

Combined ANOVA model:

$$Yijk = \mu + gi + Rl(j) + Ej + GEij + Bk(j) + \varepsilon ijk$$

Where, $Yijk$ = observed value of genotype i in block $_k$ of location j; μ = grand mean; Gi = effect of genotype I; Ej = environment or location effect; Rl (j) = effect of replication I within j^{th} environment; $GEij$ = the interaction effect of genotype $_i$ with location/environment j; $Bk(j)$ = effect of block k within environment; εijk = random error or residual effect of genotype i in block k of location j.

Estimation of variance components

Phenotypic and genotypic variances were computed as per the methods suggested by Burton and Devane (1953).

Individual location:

$\sigma^2 p = \sigma^2 g + \sigma^2 e$

$$\delta^2 g = \frac{(MSg - MSe)}{r}$$

$\sigma^2 e = MSse$

Combined over location:

$\sigma^2 p = \sigma^2 g + \sigma^2 gl/r + \sigma^2 e/rl$

$$\delta^2 g = \frac{(MSg - MSgl)}{rl}$$

$\sigma^2 e = MSse$

Where, MSg and MSe = mean squares for genotypes and error respectively. σ^2_g = genotypic variances; σ^2_e = environmental (error) variance; σ^2_P = phenotypic variance; $\sigma^2 gl$ =variance due to genotype by environment interaction; r = number of replications. The genotypic and phenotypic coefficients of variation were computed according to the formulae of Singh and Chaundary (1979):

Table 2. Variability, Heritability and genetic advance of 10 yield and yield related traits in 36 common bean genotypes tested at Yabello in 2015 cropping season.

Trait	GV	PV	EV	H^2 (%)	GCV (%)	PCV (%)	GA	GAM (5%)
FD	2.69	6.34	3.650	42.43	3.73	5.73	2.20	5.01
MD	6.89	9.02	2.128	76.40	3.22	3.69	4.73	5.81
PH (cm)	178.3	230.03	51.78	77.49	21.78	24.74	24.25	39.55
NPB	0.08	0.28	0.194	29.96	7.79	14.22	0.33	8.79
PL (cm)	0.56	0.76	0.200	73.49	8.56	9.99	1.32	15.15
PPP	1.83	3.80	1.970	48.18	7.41	10.68	1.94	10.61
SPP	0.32	0.42	0.099	76.56	15.84	18.10	1.03	28.59
SPNT	74.55	100.21	25.66	74.39	13.32	15.44	15.36	23.70
TSW (g)	297.1	456.56	159.5	65.06	7.52	9.32	28.68	12.51
GY (t/ha)	0.15	0.20	0.053	73.49	13.92	16.24	0.68	24.62

FD, Days to flowering; MD, days to maturity; EV, environmental variance; GA, genetic advance; GAM, genetic advance as percent of mean at 5% selection intensity; GCV (%), genotypic coefficient of variation in percent; GY(t/ha), Grain yield per hectare in ton; GV, genotypic variance; H2 (%), heritability in broad sense in percent; NPB= number of primary branch; PH= plant height in centimetre; PPP, pod per plant; PCV (%), phenotypic coefficient of variation in percent; PV, phenotypic variance; SPNT= seeds per plant; SPP, seeds per pod; TSW, thousand seed weight in gram.

Genotypic coefficient of variation (GCV) = (σg/grand mean) × 100
Phenotypic coefficient of variation (PCV) = (σph/grand mean) × 100

Where, σg and σph are genotypic and phenotypic standard deviations, respectively.

Estimate of heritability in broad sense

Broad sense heritability values were estimated based on the formula suggested by Falconer and Mackay (1996) as follows:

Heritability in broad sense $H^2 = \dfrac{\sigma 2g}{\sigma 2p}$ *100

σ^2_g =genotypic variances; $\sigma^2 p$ = phenotypic variances.

Estimation of expected genetic advance

The genetic advance for selection intensity (k) at 5% was estimated by the following formula (Johnson et al., 1955; Allard, 1960):

EGA = k * σph * H^2

Where, EGA represents the expected genetic advance under selection; σph is the phenotypic standard deviation; H^2 is heritability in broad sense and k is selection differential (k = 2.06 at 5% selection intensity).
   The genetic advance as percent of population mean was estimated following the procedure of Johnson et al. (1955).

Genetic advance as percent of population mean (GAM) = (EGA/grand mean) * 100

RESULTS AND DISCUSION

Variance component, heritability and genetic advance

Phenotypic and genotypic variations

Estimated phenotypic and genotypic variances:

Phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV) of the 10 studied traits are presented in Tables 3 and 4. Genotypic coefficient of variation (GCV) ranged from 3.22 (days to maturity) to 21.78% (plant height) at Yabello (Table 2) and 3.34 (days to maturity) to 16.27% (grain yield /ha) at Abaya (Table 3). The phenotypic coefficient of variation (PCV) ranged from 3.69 (days to maturity) to 18.10% (seed number per pod) at Yabello and 4.7 (days to maturity) to 20.01 (grain yield /ha) at Abaya (Table 3). On the basis of combined analysis over locations, the genotypic coefficient of variation (GCV) ranged from 4.6 (thousand seed weight) to 9.86% (seeds per pod), while phenotypic coefficient of variation (PCV) ranged from 6.91 (number pods per plant) to 12.93% (seeds per pod) (Table 4). In all variances analyses, phenotypic coefficient of variation was higher than genotypic coefficient of variation. However, relatively the larger magnitude of difference between the two was observed in grain yield ha^{-1}, seeds per plant and thousand seed weight. This indicates the higher influence of environmental factors than genetic factors in the expression of these traits. In these traits, selection based on the phenotype performance may not be appropriate. Nechifor et al. (2011) and Amir et al. (2015) suggested that larger difference between GCV and PCV is due to larger influence of environment on that trait. In common bean, a wide range of GCV and PCV values were reported (Nechifor et al., 2011; Alemayehu, 2010; Amir et al., 2015; Ahmed and Kamaluddin, 2013). According to Deshmukh et al. (1986), and Sivasubramanian and Madhavamenon (1973) genotypic and phenotypic coefficient of variations can be categorized as low (<10%), medium (10-20%) and high (>20%). In this study, both GCV and PCV were low for days to flowering, days to maturity and pod length (Tables 2 and 3), number of primary branches, number pods per plant, and thousand seed weight. Moderate

Table 3. Variability, Heritability and genetic advance of 10 yield and yield related traits in 36 common bean genotypes tested at Abaya in 2015 cropping season.

Trait	GV	PV	EV	H² (%)	GCV (%)	PCV (%)	GA	GAM (5%)
FD	7.55	18.83	11.28	40.10	6.75	10.66	3.59	8.81
MD	6.98	13.83	6.85	50.48	3.34	4.70	3.87	4.89
PH (cm)	61.77	87.99	26.22	70.20	7.76	9.26	13.59	13.41
NPB	0.11	0.30	0.19	35.96	10.05	16.76	0.40	12.43
PL (cm)	0.23	0.91	0.68	25.74	5.62	11.07	0.51	5.88
PPP	1.98	3.96	1.97	50.16	7.86	11.10	2.06	11.48
SPP	0.22	0.34	0.12	64.36	14.02	17.48	0.77	23.20
SPNT	49.64	78.37	28.73	63.34	11.97	15.04	11.57	19.66
TSW (g)	483.84	644.57	160.73	75.06	10.23	11.81	39.32	18.29
GY (t/h)	0.15	0.23	0.05279	66.13	16.27	20.01	0.65	27.30

FD, Days to flowering; MD, days to maturity; EV, environmental variance; GA, genetic advance; GAM, genetic advance as percent of mean at 5% selection intensity; GCV (%), genotypic coefficient of variation in percent; GY(t/ha), Grain yield per hectare in ton; GV, genotypic variance; H2 (%), heritability in broad sense in percent; NPB= number of primary branch; PH= plant height in centimetre; PPP, pod per plant; PCV (%), phenotypic coefficient of variation in percent; PV, phenotypic variance; SPNT= seeds per plant; SPP, seeds per pod; TSW, thousand seed weight in gram.

Table 4. Variability components of seven yield and yield related traits in 36 common bean genotypes Combined across two locations in 2015 cropping season.

Traits	GV	PV	GLV	EV	H² (%)	GCV (%)	PCV (%)	GA	GAM
PH (cm)	55.07	65.08	93.99	38.23	58.60	9.13	11.92	11.7	14.41
NPB	0.04	0.04	0.09	0.19	44.19	5.84	8.78	0.28	8.01
PPP	0.79	0.83	1.56	2.15	50.35	4.90	6.91	1.30	7.17
SPP	0.12	0.12	0.20	0.13	58.12	9.86	12.93	0.53	15.50
SPNT	14.49	41.28	39.84	28.24	36.38	6.16	10.21	4.74	7.66
TSW (g)	104.6	225.0	244.12	162.3	42.83	4.60	7.03	13.81	6.22
GY (t/ha)	0.02	0.11	0.08	0.07	18.29	4.82	11.26	0.11	4.25

EV, Environmental variance; GA, genetic advance; GAM, genetic advance as percent of mean at 5% selection intensity; GCV (%), genotypic coefficient of variation in percent; GY(t/ha), Grain yield per hectare in ton; GV, genotypic variance; H2 (%), heritability in broad sense in percent; NPB= number of primary branch; PH= plant height in centimetre; PPP, pod per plant; PCV (%), phenotypic coefficient of variation in percent; PV, phenotypic variance; SPNT= seeds per plant; SPP, seeds per pod; TSW, thousand seed weight in gram.

PCV and low GCV was observed for seed per pod, plant height, and yield ha⁻¹ (Table 4). The relative difference between GCV and PCV was small in plant height, number of seed per pods and pod number per plant. This suggested that the chance of improving these traits through selection is high. Ahmed and Kamaluddin (2013), Roy et al. (2006), Raffi and Nath (2004), and Nechifor et al. (2011) also reported similar results in common bean.

The variance components for grain yield showed variation for locations and combined analyses. Moderate GCV (16.27%) and high PCV (20.01%) at Abaya (Table 3) were as medium GCV (13.92%) and PCV (16.24%) was observed at Yabello (Table 2). Very low GCV (4.82%) and moderate PCV (11.26%) values were observed for this trait in combined combined analysis of variance across location (Table 4). The relative difference between genotypic coefficient of variation (GCV) and phenotypic coefficient of variation (PCV) was large for grain yield/ha (57.24%). This is the indications of the greater influence of environmental factors on these traits.

In agreement with this finding, Nechifor et al. (2011) reported the presence of relatively large difference between GCV and PCV for grain yield in common bean which attributed to environmental factors.

Heritability and genetic advance

Estimated heritability in broad sense ranged from 29.96% for number of primary branches to 77.49% for plant height at Yabello; and from 25.74% for pod length to 75.06% for thousand seed weight at Abaya. The calculated genetic advance as percent of mean at 5% selection intensity ranged from 5.01% for flowering date to 39.55% for plant height at Yabello (Table 2) and 4.89% for days to maturity to 27.30% for grain yield at Abaya (Table 3).

On the basis of combined analysis over the two locations, the estimated heritability values ranged from 18.29% for grain yield ha⁻¹ to 58.6% for plant height. The

calculated genetic advance as percentage of mean ranged from 4.25% for grain ha^{-1} to 15.51% for seeds number per pod. As demonstrated by Robinson et al. (1949), heritability can be categorized as low (0-30%), moderate (30-60%) and high (60% and above). Johnson et al. (1955) suggested genetic advance as percent of mean can be categorized as low (0-10%), moderate (10-20%) and high (20% and above). In the present study, moderate heritability coupled with moderate genetic advance as percent of mean was observed for plant height and number of seeds per pod suggesting that selection based on the phenotype performance of genotypes for these traits might improve the performance of the progenies. In agreement the result of this study, Nechifor et al. (2011) and Alemayehu (2010) reported moderate estimates of heritability and genetic advance for seeds number per pod in common bean. On the other hand, both values of heritability and genetic advance were low for grain yield/ha. This suggested the low heritability of trait is due to the influence of environment that limits the scope of improvement by selection. Dursun (2007) also reported low broad-sense heritability values for grain yield.

Conclusions

The estimated genetic (GCV) and phenotypic (PCV) values were low to medium for most of the traits. The PCV values were relatively greater than GCV in magnitude for all characters under study. However, the difference between genotypic coefficient of variation and phenotypic coefficient of variation was relatively large in magnitude for grain yield, seed number per plant and number of primary branches indicating the higher influence of environmental factors than genetic factors in the expression of these traits. The difference between phenotypic coefficient of variation and genotypic coefficient of variation was low for plant height and seed per pod indicating that the observed variations for these traits were mostly due to genetic factors but environment also played a role in the expression of these traits. Broad sense heritability ranged from 18.29% in grain yield to 58.6% in plant height. The genetic advance as percentage of means in combined analysis ranged from 4.25% for grain yield to 15.42% for seeds number per pod. Moderate values of heritability coupled with relatively high genetic advance as percentage of means was observed for plant height and seed number per pod. Therefore, selection based on the phenotypic performance of genotypes could increase the mean performance of the selected progenies.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Appendix Table 1. Description of the new common bean entries and released varieties.

S/N	Genotype	Pedigree	Source
1	ALB58	SER 16 x G35346	CIAT
2	ALB36	SER 16 x G35346	CIAT
3	ALB25	SER 16 x G35346	CIAT
4	ALB61	SER 16 x G35346	CIAT
5	ALB167	SER 16 x G35346	CIAT
6	ALB163	SER 16 x G35346	CIAT
7	ALB212	SER 16 x G35346	CIAT
8	ALB204	SER 16 x G35346	CIAT
9	ALB145	SER 16 x G35346	CIAT
10	ALB133	SER 16 x G35346	CIAT
11	ALB151	SER 16 x G35346	CIAT
12	ALB149	SER 16 x G35346	CIAT
13	ALB179	SER 16 x G35346	CIAT
14	ALB209	SER 16 x G35346	CIAT
15	ALB207	SER 16 x G35346	CIAT
16	G21212	-	CIAT
17	BFS 27	SBCZ16257-33/-MC-2P-MQ-1D-MC	CIAT
18	BFS 320	-	CIAT
19	BFS 34	SBCF16231-002/-MC-8P-MQ-4D-MC	CIAT
20	BFS 24	SBCZ16253-040/-MC-23P-MQ-6D-MC	CIAT
21	BFS 55	SBCZ16234-004/-MC-1P-MQ-12D-MC	CIAT
22	BFS 35	SBCF16231-002/-MC-8P-MQ-5D-MC	CIAT
23	BFS 10	SBCZ16245-01/-MC-4P-MQ-2D-MC	CIAT
24	BFS 30	SBCZ16257-33/-MC-2P-MQ-5D-MC	CIAT
25	BFS 39	SBCF16231-005/-MC-2P-MQ-5D-MC	CIAT
26	BFS 18	SBCZ16253-040/-MC-12P-MQ-9D-MC	CIAT
27	SX b 412	BM14524-16/-MQ-MQ-25C-MC-MC-2	CIAT
28	BFS 23	SBCZ16253-040/-MC-23P-MQ-5D-MC	CIAT
29	BFS 33	SBCF16231-002/-MC-8P-MQ-3D-MC	CIAT

Appendix Table 2. List of released varieties used as a check.

S/N	Variety	Year of release	Yield ton /ha		Recommended altitude (masl)	Days to maturity	Breeding center
			On station	Farmers field			
30	NASIR	2003	2.3	2.03	1200-1800	86-88	MARC
31	ROBA-1	1990	2.0-2.4	1.9-2.1	1400-1800	75-95	MARC
32	Awash 1	1989	2.0-2.4	1.8-2.1	1400-1800	90	MARC
33	Awash Melka	1999	2.5	2.0-2.3	1400-1900	88-95	MARC
34	Awash 2	2013	2.8-3.1	1.8-2.2	1300-1700	85-90	MARC
35	Mexican-142	1973	2.1	1.3	1400-1800	95-100	MARC
36	Chorie	2006	2.3	1.9	1300-1950	87-109	MARC

Source: MARC = Malkassa Agriculture Research Centre.

Related Journals:

African Journal of
Biochemistry Research

Journal of
Microbiology and Antimicrobials

African Journal of
Microbiology Research

Journal of
Biophysics and Structural Biology

International Journal of
**Biotechnology and Molecular
Biology Research**

Journal of
Bioinformatics and Sequence Analysis

African Journal of
Biochemistry Research

Journal of
Microbiology and Antimicrobials

African Journal of
Microbiology Research