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Review

Nanotechnology in sustainable agriculture: Present concerns and future aspects

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Nanotechnology is a promising field of interdisciplinary research. It opens up a wide array of opportunities in various fields like medicine, pharmaceuticals, electronics and agriculture. The potential uses and benefits of nanotechnology are enormous. The current global population is nearly 7 */billion with 50% living in Asia. A large proportion of those living in developing countries face daily food shortages as a result of environmental impacts or political instability, while in the developed world there is surplus of food. For developing countries, the drive is to develop drought and pest resistant crops, which also maximize yield. The potential of nanotechnology to revolutionise the health care, textile, materials, information and communication technology, and energy sectors has been well publicized. The application of nanotechnology to agriculture and food industries is also getting attention nowadays. Investments in agriculture and food nanotechnologies carry increasing weight because their potential benefits range from improved food quality and safety to reduced agricultural inputs and improved processing and nutrition. While most investment is made primarily in developed countries, research advancements provide glimpses of potential applications in agricultural, food, and water safety that could have significant impacts on rural populations in developing countries. This review is concentrated on modern strategies used for the management of water, pesticides, limitations in the use of chemical pesticides and potential of nano-materials in sustainable agriculture management as modern approaches of nanotechnology.

Key words: Agriculture, nanotechnology, nanofertilizer, nanoencapsulation, nanoherbicides.

INTRODUCTION

Nanotechnology is a novel scientific approach that involves the use of materials and equipment capable of manipulating physical as well as chemical properties of a substance at molecular levels. On the other hand, bio-technology involves using the knowledge and techniques of biology to manipulate molecular, genetic and cellular processes to develop products and services and is used in diverse fields from medicine to agriculture (Fakruddin et al., 2012). Agriculture is the backbone of developing countries, with more than 60% of the population depending on it for their livelihood (Brock et al., 2011).

Nanotechnology has the potential to revolutionize the agricultural and food industry with novel tools for the molecular management of diseases, rapid disease

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Abbreviations: CEA, Controlled Environment Agriculture; **GPS,** global positioning system; **IPM,** Integrated Pest Management; **CHIP,** Chicken and Hen Infection Program; **PDA,** potato dextrose agar; **PHSNs,** porous hollow silica nanoparticles; **DDT,** dichorodiphenyltrichloroethane.

detection, enhancing the ability of plants to absorb nutrients, among others. On the other hand, nanobiotechnology can improve our understanding of the biology of various crops and thus can potentially enhance yields or nutritional values, as well as developing improved systems for monitoring environmental conditions and enhancing the ability of plants to absorb nutrients or pesticides (Tarafdar et al., 2013).

Agricultural and food systems security, disease management delivery systems, new techniques for molecular and cellular biology, new materials for pathogen detection and protection of the environment are examples of the important links of nanotechnology to the science and engineering of agriculture and food systems (Welch and Graham, 1999; Suman et al., 2010). There are new challenges in this sector including a growing demand for healthy, food safety, an increasing risk of disease and threats to agricultural production from changing weather patterns (Biswal et al., 2012).

Nanobiotechnology operates at the same level with virus or disease infecting particle, and thus holds the potential for primordial detection and eradication. It also holds out the possibility that smart sensors and delivery systems will help the agricultural industry combat viruses and other crop pathogens. Long before the symptoms develop, the integrated sensing, monitoring and controlling system could detect the presence of disease and notify the farmer and activate bioactive systems such as drugs, pesticides, nutrients, probiotics, nutraceuticals and implantable cell bioreactors. In the near future, nanostructured catalysts will be available which will increase the efficiency of pesticides and herbicides, allowing lower doses to be used. Nanotechnology will also protect the environment indirectly through the use of alternative (renewable) energy supplies, and filters or catalysts to reduce pollution and clean-up existing pollutants in soil and water. In the agricultural sector, nanotech research and development is likely to aid and frame the next level of expansion of genetically modified crops, animal production inputs, chemical pesticides and precision farming techniques (Prasad et al., 2012a).

Changes in agricultural technology have been a major factor shaping modern agriculture. Among the latest line of technological innovations, nanotechnology occupies a prominent position in transforming agriculture and food production. The development of nano-devices and nanomaterials could open up novel applications in plant biotechnology and agriculture. Currently, the main thrust of research in nanotechnology focuses on applications in the field of electronics, energy, medicine and life sciences (Scrinis and Lyons, 2007), as agriculture is not considered as potent industry. While nano-chemical pesticides are already in use, other applications are still in their early stages, and it may take many years before they are commercialized or reach the common man. These applications are largely intended to address some of the limitations and challenges facing large scale, chemical and capital intensive farming systems. This includes the fine tuning and more precise microma-nagement of soils; the more efficient and targeted use of inputs, new toxin formulations for pest control, new crop and animal traits, and the diversification and differentiation of farming practices and products within the context of large scale and highly uniform systems of production. Nanotechnology will leave no field untouched by its ground breaking scientific innovations. The agri-cultural industry is no exception. So far, the use of nanotechnology in agriculture has been mostly theo-retical, but it has begun and will continue to have a significant impact in the main areas of food industry, development of new functional materials, product development and design of methods and instrumentation for food safety and bio-security. The effects on society as a whole will be dramatic (Prasad et al., 2012a). This review is focused on modern strategies used for the management of water, pesticides, sensors, fertilizers, limitations in the use of chemical pesticides and potential of nanomaterials in sustainable agriculture management as modern approaches of nanotechnology.

OVERVIEW OF NANOTECHNOLOGY APPLICATIONS IN AGRICULTURE

Nanotechnology and agricultural production developments

In the near future, nanostructured catalysts will be available which will increase the efficiency of pesticides and herbicides, allowing lower doses to be used. An agricultural system widely used in the USA, Europe and Japan, which efficiently utilises modern technology for crop management is called Controlled Environment Agriculture (CEA). CEA is an advanced and intensive form of hydroponically based agriculture. Plants are grown within a controlled environment so that agricultural practices can be optimized. The computerized system monitors and regulates localised environments such as fields of crops and irrigated water. CEA technology provides an excellent platform for the introduction of nanotechnology to agriculture. Nanotechnological devices for CEA that provide “scouting” capabilities could tremendously improve the grower’s ability to determine the best time to harvest the crop, the vitality of crop, and food security issues, such as microbial or chemical contamination.

Nanosensors for monitoring soil conditions and plant growth hormone

Nanotechnology delivery systems for nutrients and plant hormones

The proficient use of agricultural natural assets like water, nutrients and chemicals during farming as nanosensors is

user friendly. It makes use of nanomaterials and global positioning systems with satellite imaging of fields and might make farmers to detect crop pests or facts of stress such as drought. Nanosensors disseminated in the field are able to sense the existence of plant viruses and the level of soil nutrients (Ingale and Chaudhari, 2013). They also minimize fertilizer consumption and environmental pollution. Nano-encapsulated slow- release fertilizers have been widely used (DeRosa et al., 2010). To check the quality of agricultural manufacture, nanobarcodes and nanoprocessing could be used. Li et al. (2005) used the idea of grocery barcodes for economical, proficient, rapid effortless decoding and recognition of diseases. They created nanobarcodes that may tag perhaps multiple pathogens in a farm, which may simply be detected using any fluorescent based tools. Nanotechnocrates are capable of studying plant's regulation of hormones such as auxin, which is accountable for root growth and seedling organization. Nanosensors that react with auxin have been developed. This is a step forward in auxin research, as it helps scientists to know how plant roots acclimatize to their environment, particularly to marginal soils (McLamore et al., 2010).

Nanobiosensors

Sensors are sophisticated instruments which respond to physico-chemical and biological aspects and transfer that response into a signal or output that can be used by humans (NNCO, 2009). They allow the detection of contaminants such as microbes, pests, nutrient content and plant stress due to drought, temperature, insect or pathogen pressure, or lack of nutrients. Nanosensors have the potential to allow farmers to utilize inputs more efficiently by indicating the nutrient or water status of crop plants over fine spatial and temporal scales. This makes the farmers to apply nutrients, water, or crop protection (insecticide, fungicide or herbicide) only where necessary. One of the major roles of nanotechnology enabled devices is to increase the use of autonomous sensors linked to a global positioning system (GPS) system for real time monitoring. These nanosensors could be distributed throughout the field where they can monitor soil conditions and crop growth. Nanoparticles or nanosurfaces can be engineered to trigger an electrical or chemical signal in the presence of a contaminant such as bacterium. Ultimately, precision farming, with the help of smart sensors leads to enhanced productivity in agriculture by providing accurate information, thus helping farmers to make better decisions.

Nanotechnology in irrigation water filtration

The emerging technologies that will benefit farmers all over world, especially in developing countries include several nanomaterials which are considered economically effective in purification of irrigation water. Nano-enabled water treatment techniques based on membranes filters derived from carbon nanotubes, nanoporous ceramics, and magnetic nanoparticles inspite using chemicals and UV light are common in traditional water treatment (Hillie and Hlophe, 2007). Filters made from carbon nanotube could be employed in removing contaminants and toxicants from potable water. Carbon nanotube fused mesh that can remove water-borne pathogens, heavy metals like lead, uranium and arsenic has been suggested by researchers. Employing nanoceram filter with positive charge can trap bacteria and viruses with negative charge. This sophisticated filtering machine removes microbial endotoxins, genetic materials, pathogenic viruses, and micro-sized particles (Argonide, 2005).

Magnetic nanoparticles for filtration

At very low magnetic field gradients, the use of magnetic nanoparticles and magnetic separations is now possible. Nanocrystals, such as monodisperse magnetite (Fe_3O_4) have a strong and irreversible interaction with arsenic while retaining their magnetic properties (Yavuz et al., 2006).

A simple handheld magnet can be used to remove nanocrystals and arsenic from water. Such a treatment could be used for irrigation water filtration process.

Detoxification or remediation of harmful pollutants

Using synthetic clay nanomineral does not require expensive laboratory equipment for arsenic removal. The water to be filtered is percolated through a column of hydrotalcite (synthetic clay mineral). Gilman (2006) suggests that this technology can be coupled with leaching through porous pots or filter candles, the technology available in many developing countries to filter organisms from drinking water. Zinc oxide nanoparticles can be used to remove arsenic using a point-of-source purification device. Nanoscale zero-valent iron is the most widely used the set of nanomaterials that could be deployed to remediate pollutants in soil or groundwater. Other nanomaterials that could be used in remediation include nanoscale zeolites, metal oxides, carbon nanotubes and fibers, enzymes, various noble metals (mainly as bimetallic nanoparticles) and titanium dioxide.

Nanoparticle filters can be used to remove organic particles and pesticides (for example, dichorodiphenyl-trichloroethane (DDT), endosulfan, malathion and chlorpyrifos) from water. A variety of nanoparticle filters have been used in remediation of waste sites in developed countries (Karn et al., 2009).

Nanocapsules for efficient delivery of pesticides, fertilizers and other agrochemicals

Nanoencapsulation is a process through which chemicals like insecticides are slowly but efficiently released to a particular host plant for insect pest control. Nanoencapsulation with nanoparticles in the form of pesticides allows for proper absorption of the chemicals into the plants (Scrinis and Lyons, 2007). This process can also deliver DNA and other desired chemicals into plant tissues for protection of host plants against insect pests (Torney, 2009). Release mechanisms of nanoencapsulation include diffusion, dissolution, biodegradation and osmotic pressure with specific pH (Ding and Shah, 2009; Vidhyalakshmi et al., 2009). Nanoencapsulation is currently the most promising technology for protection of host plants against insect pests. Now, most leading chemical companies focus on formulation of nanoscale pesticides for delivery into the target host tissue through nanoencapsulation.

Fertilizer plays a pivotal role in agriculture production (35 to 40%). To enhance nutrient use efficiency and overcome the chronic problem of eutrophication, nanofertilizer might be a best alternative. Nanofertilizers are synthesized in order to regulate the release of nutrients depending on the requirements of the crops, and it is also reported that nanofertilizers are more efficient than ordinary fertilizer (Liu et al., 2006a). Nanofertilizers could be used to reduce nitrogen loss due to leaching, emissions, and long-term incorporation by soil microorganisms. They could allow selective release linked to time or environmental condition. Slow controlled release fertilizers may also improve soil by decreasing toxic effects associated with fertilizer over- application (Suman et al., 2010).

Technologies such as encapsulation and controlled release methods have revolutionised the use of pesticides and herbicides. Pesticides inside nanoparticles are being developed that can be timely released or have release linked to an environmental trigger. Combined with a smart delivery system, herbicide could be applied only when necessary, resulting in greater production of crops and less injury to agricultural workers. Many companies make formulations which contain nanoparticles within the size ranges of 100-250 nm; they are able to dissolve in water more effectively than existing ones (thus increasing their activity). Other companies employ suspensions of nanoscale particles (nanoemulsions) which can be either water or oil-based and contain uniform suspensions of pesticidal or herbicidal nanoparticles in the range of 200-400 nm. These can be easily incorporated in various media such as gels, creams, liquids among others, and have multiple applications for preventative measures, treatment or preservation of harvested products.

Syngenta, world's largest agrochemical corporation, is using nanoemulsions in its pesticide products. One of its successful growth regulating products is the Primo MAXX® plant growth regulator, which if applied prior to the onset of stress such as heat, drought, disease or traffic can strengthen the physical structure of turf grass and allow it to withstand ongoing stresses throughout the growing season. Another encapsulated product from Syngenta delivers a broad control spectrum on primary and secondary insect pests of cotton, rice, peanuts and soybeans. Marketed under the name Karate® ZEON this is a quick release microencapsulated product containing the active compound lambda-cyhalothrin (a synthetic insecticide based on the structure of natural pyrethrins) which breaks open on contact with leaves. In contrast, the encapsulated product "gutbuster" only breaks open to release its contents when it comes into contact with alkaline environments, such as the stomach of certain insects.

Nano based smart drug-delivery systems

Smart delivery systems can detect and treat an animal infection or nutrient deficiency and provide timed-release drugs or micronutrients. The use of pesticides increased in the second half of the 20th century with DDT becoming one of the most effective and widespread throughout the world. However, many of these pesticides, including DDT were later found to be highly toxic and persistent, affecting human and animal health and as a result whole ecosystems. As a consequence, they were banned. To maintain crop yields, Integrated Pest Management (IPM) systems, which mix traditional methods of crop rotation with biological pest control methods, are becoming popular and implemented in many countries, such as Tunisia and India.

In the future, nanoscale devices with novel properties could be used to make agricultural systems smart. For example, devices could be used to identify plant health issues before these become visible to the farmer. Such devices may be capable of responding to different situations by taking appropriate remedial action. If not, they will alert the farmer to the problem. In this way, smart devices will act as both a preventive and an early warning system. Such devices could be used to deliver chemicals in a controlled and targeted manner in the same way as nanomedicine has implications for drug delivery in humans.

Zeolites for water retention

These are naturally occurring crystalline aluminum silicates. Zeolite assists water infiltration and retention in the soil due to its very porous properties and the capillary suction it exerts. Acting as a natural wetting agent, it is an excellent amendment for non wetting sands and assists water distribution through soils.

This can improve significantly the water retention of

sandy soils and increase porosity in clay soils. Improving water-retention capacity of soils could result in increased crop production in areas prone to drought. With subsequent applications, zeolite will further improve soil's ability to retain nutrients and produce improved yields.

Nanocoatings and nanofeed additives

Self-sanitizing photocatalyst coating used in poultry houses with nano-titanium dioxide (TiO_2) could be used to oxidize and destroy bacteria in the presence of light and humidity. Poultry feed having nanoparticles that binds pathogenic bacteria could help in decreasing food-borne pathogens. The unique photocatalytic properties of the nano TiO_2 are activated when the coating is exposed to natural or UV light. In the presence of light and humidity, TiO_2 oxidizes and destroys bacteria. Once coated, the surface remains self-sanitizing as long as there is enough light to activate the photocatalytic effect. The coating is approved by the Canadian Food Inspection Agency. In Denmark, the Chicken and Hen Infection Program (CHIP) involves self-cleaning and disinfection nanocoatings (Clemants, 2009). The nanoscale smooth surface makes more effective disinfection and cleaning. Ultimately, Danish researchers are also working on coatings incorporating nanosilver which does not need UV light for activation. The ions from nanosilver avert the progress of biofilms (Clemants, 2009). Surface-modified hydrophobic as well as lipophilic nanosilica can be significantly used as new drugs for healing nuclear polyhedrosis virus (BmNPV), a major problem in silkworm industry. *Bombyx mori* (Silk worm) has clearly shown that nanoparticles could stimulate more fibroin protein production which can assist in the future in producing carbon nano tube (Bhattacharyya et al., 2008; Bhattacharyya, 2009). Modified nanoclays (montmorillonite nanocomposite) can ameliorate the harmful effects of aflatoxin on poultry (Shi et al., 2006). Research on nanoparticles and insect control should be geared toward introduction of faster and eco-friendly pesticides in the future (Bhattacharyya et al., 2010).

Nanoherbicides

The easiest way to eliminate weeds is to destroy their seed banks in the soil and prevent them from germinating when weather and soil conditions become favourable for their growth. Being very small, nanoherbicides will be able to blend with the soil, eradicate weeds in an eco-friendly way without leaving any toxic residues, and prevent the growth of weed species that have become resistant to conventional herbicides. Weeds survive and spread through underground structures such as tubers and deep roots. Ploughing infected fields while removing weeds by hand can make these unwanted plants spread to uninfected areas. Whether the nano application is due to a nanosized active ingredient or the creation of a nanosized formulation through the use of an adjuvant, the use of nano application is same. If the active ingredient is combined with a smart delivery system, herbicide will be applied only when necessary according to the conditions of the agriculture field. Lower agricultural yields are obtained in soils contaminated with weeds and weed seeds. Improvements in the efficacy of herbicides through the use of nanotechnology could result in more crop production without causing any harmful effects to agricultural workers who are supposed to physically remove weeds if no application of herbicides is practised.

Nanotechnology in organic farming

Organic farming has been a long-desired goal to increase productivity (that is, crop yields) with low input (that is, fertilizers, pesticides, herbicides among others) through monitoring environmental variables and applying targeted action. Organic farming makes use of computers, GPS systems, and remote sensing devices to measure highly localized environmental conditions, thus determining whether crops are growing at maximum efficiency or precisely identifying the nature and location of problems. By using centralised data to determine soil conditions and plant development, seeding, fertilizer, chemical and water use can be fine-tuned to lower production costs and potentially increase production all benefiting the farmer. Precision farming can also help to reduce agricultural waste and thus keep environmental pollution to a minimum.

Nanoparticles and plant disease control

Some of the nano particles that have entered into the arena of controlling plant diseases are nanoforms of carbon, silver, silica and alumino-silicates. At such a situation, nanotechnology has astonished scientific community because at nano-level, material shows different properties. The use of nano size silver particles as antimicrobial agents has become more common as technology advances, making their production more economical. Since silver displays different modes of inhibitory action to microorganisms (Young, 2009), it may be used for controlling various plant pathogens in a relatively safer way compared to commercially used fungicides. Silver is known to affect many biochemical processes in the microorganisms including the changes in routine functions and plasma membrane (Pal et al., 2007). The silver nanoparticles also prevent the expression of ATP production associated proteins (Yamanka et al., 2005). In a nutshell, the precise mechanism of bio molecules inhibition is yet to be understood.

Thus, use of nanoparticles has been considered an alternate and effective approach which is eco-friendly and cost effective for the control of pathogenic microbes (Kumar and Yadav, 2009; Prasad et al., 2011; Swamy and Prasad, 2012; Prasad and Swamy, 2013). These nanoparticles have a great potential in the management of plant diseases compared to synthetic fungicides (Park et al., 2006). Zinc oxide (ZnO) and magnesium oxide (MgO) nanoparticles are effective antibacterial and anti-odour agents (Shah and Towkeer, 2010). The increased ease in dispensability, optical transparency and smoothness make ZnO and MgO nanostructures an attractive antibacterial ingredient in many products. Both have also been proposed as an anti-microbial preservative for wood or food products (Aruoja et al., 2009; Huang et al., 2005; Sharma et al., 2009). Properly functionalized nanocapsules provide better penetration through cuticle and allow slow and controlled release of active ingredients on reaching the target weed. The use of such nano-biopesticide is more acceptable since they are safe for plants and cause less environmental pollution in comparison to conventional chemical pesticides (Barik et al., 2008).

Nanosilver is the most studied and utilized nano particle for bio-system. It has long been known to have strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities (Swamy and Prasad, 2012; Prasad et al., 2012b; Prasad and Swamy, 2013). Silver nanoparticles, which have high surface area and high fraction of surface atoms, have high antimicrobial effect compared to the bulk (Suman et al., 2010). Fungicidal properties of nano-size silver colloidal solution are used as an agent for antifungal treatment of various plant pathogens; the most significant inhibition of plant pathogenic fungi was observed on potato dextrose agar (PDA) and 100 ppm of AgNPs (Kim et al., 2012).

Nanoparticles as pesticides

Nanoparticles are also effective against insects and pests. Nanoparticles can be used in the preparation of new formulations like pesticides, insecticides and insect repellants (Barik et al., 2008; Gajbhiye et al., 2009). Torney (2009) reviewed that nanotechnology has promising applications in nanoparticle gene mediated DNA transfer. It can be used to deliver DNA and other desired chemicals into plant tissues for protection of host plants against insect pests. Porous hollow silica nanoparticles (PHSNs) loaded with validamycin (pesticide) can be used as efficient delivery system of water-soluble pesticide for its controlled release. Such controlled release behaviour of PHSNs makes it a promising carrier in agriculture, especially for pesticide controlled delivery whose immediate as well as prolonged release is needed for plants (Liu et al., 2006b). According to Wang et al. (2007), oil in water (nano-emulsions) was useful for the formulations of pesticides and these could be effective against the various insect pests in agriculture. Similarly, essential oil-loaded solid lipid nanoparticles were also useful for the formulations of nano-pesticides (Liu et al., 2006b). Nanosilica, a silica product, can be effectively used as a nanopesticide.

Barik et al. (2008) reviewed the use of nano-silica as nano-insecticide. The mechanism of control of insect pest using nano-silica is based on the fact that insect pests used a variety of cuticular lipids for protecting their water barrier and thereby prevent death from desiccation. But here, the nanosili caparticles when applied on plant surface, cause death by physical means of insects by being absorbed into the cuticular lipids. Modified surface charged hydrophobic nano-silica (~3-5 nm) could be successfully implemented to manage a variety of ectoparasites of animals and agricultural insect pests (Ulriches et al., 2005). The insecticidal activity of polyethylene glycol-coated nanoparticles loaded with garlic essential oil against adult *Tribolium castaneum* insect was found in stored products.

It has been observed that the control efficacy against adult *T. castaneum* was about 80%; presumably due to the slow and persistent release of the active components from the nanoparticles (Yang et al., 2009). The applications of diverse kind of nanoparticles viz. silver nanoparticles, aluminium oxide, zinc oxide and titanium dioxide in the management of rice weevil and grasserie disease in silk worm (*B. mori*) are caused by *Sitophilus oryzae* and baculovirus BmNPV (*B. mori* nuclear polyhedrosis virus, respectively (Goswami et al., 2010). Teodoro et al. (2010) studied the insecticidal activity of nanostructured alumina against two insect pests viz. *S. oryzae* L. and *Rhyzopertha dominica* (F.), which are major insect pests in stored food supplies throughout the world. Significant mortality was observed after 3 days of continuous exposure to nanostructured alumina-treated wheat. Therefore, compared to commercially available insecti-cides, inorganic nanostructured alumina may provide a cheap and reliable alternative for control of insect pests, and such studies may expand the frontiers for nanoparticle-based technologies in pest management.

POTENTIAL BENEFITS OF NANOTECHNOLOGY APPLICATIONS

Currently the research and development pipeline has the potential to make agriculture more efficient, increase yields and product quality, and thereby increasing nutriational benefits. Developed countries are using or testing nanosensors and nanoagricultural chemicals, nanoparticles for soil cleaning and nanopore filters, nanoceramic devices, and nanoparticles. An increasing number of applications are expected for food and agriculture uses, including nanosensors, potentially capable of detecting chemical contaminants, viruses, and bacteria; nano-

delivery systems, which could precisely deliver drugs or micronutrients at the right time and to the right part of the body; as well as nanocoatings and films, nanoparticles, and quantum dots (Bouwmeester et al., 2009).

There are several reports on the great potential of agricultural and food nanotechnology in developing countries. Promising nanotechnology applications address low use efficiency of agricultural production inputs and stress of drought and high soil temperature. Nanoscale agrichemical formulations can increase efficiency use and decrease environmental losses. Nanoporous materials capable of storing water and slowly releasing it during times of water scarcity could also increase yields and save water.

Researchers have shown that applying nanotechnology to reduce the effects of aflatoxin (a fungal toxin) increases the weight of food animals. The potential for nanotechnology in agriculture continues to grow (Shi et al., 2006).

Barik et al. (2008) opined that more ambitious uses of nanoparticles are bio-remediation of contaminated environments, biocides and antifungals on textiles. Photocatalysis in agriculture is another direction in which nanomaterials can play an important role. Different nanostructures of titanium dioxide (TiO_2) and zinc oxide (ZnO) have been widely studied as photocatalysts (Ullah and Datta, 2008). Chemicals presented in pesticides are transformed in relatively harmless molecules such as CO_2, N_2 and H_2O.

Under progress is also the removal of pesticides and herbicides on plants and the soil through photocatalysis. Carbamate pesticides used in a variety of field crops are completely mineralized in the presence of ZnO and TiO_2, dichloropyrifos being an example of an often used pesticide.

Apart from nanoparticles, there are reports on the use of nanotubes and nanostructures thin films for degrading pesticides.

Nanotechnology for crop biotechnology

Nanocapsules can facilitate successful incursion of herbicides through cuticles and tissues, allowing slow and regular discharge of the active substances. This can act as 'magic bullets', containing herbicides, chemicals origins which target exacting plant parts to liberate their substance (Pérez-de-Luque and Rubiales 2009). Torney et al. (2007) exploited a 3 nm mesoporous silica nanoparticle in delivering DNA and chemicals into isolated plant cells. Mesoporous silica nanoparticles are chemically coated and act as containers for the genes delivered into the plants; they trigger the plant to take the particles through the cell walls, where the genes are put in and activated in a clear-cut and controlled way, without any toxic side effects. This technique firstly has been applied to establish DNA fruitfully to tobacco and corn plants.

Nanotechology and societal stigma

The effects of exposure to engineered nanoparticles may be different from the effects caused by naturally occurring nanoparticles. Engineered nanoparticles may be better to evade the body's defences because of their size or protective coatings. Moreover, the health and environmental risks raised due to the exposure to engineered nanoparticles need further study.

Up-coming nanotechnologies in the agricultural field seem quite interesting and promising. However, the probable risks in using nanoparticles in agriculture are no diverse than those in any other business. Through the rapid distribution of nanoparticles to food products, whether it is in the food itself or part of the packaging, nanoparticles will virtually come in direct or indirect contact with everyone. The probability could be that "the merger of nanotech and biotech may cast unknown consequences on soil, health, biodiversity and the environment. Since there is no standardization for the use and testing of nanotechnology, products incorporating the nanomaterials are being produced without check. The ability for these materials to infiltrate the human body is well known, but there is really no information on the effects that they may have. While there is no evidence of harm to people or the environment at this stage, nanotechnology is a new and evolving area of study that could cause a great deal of harm due to its still ambiguous chemical properties. With the current application and advancements soon to come, nanotechnology will have a great impact on the direction that agriculture will take. Scientists are blazing a trail for a new technology and looking at every possible avenue to improve upon current methods in every possible field. In the field of agriculture, there are still many possibilities to explore and a great deal of potential with up-coming products and techniques. Therefore, extensive studies are required to understand the mechanism for nanoparticles materials toxicity and their impacts on natural environment.

CONCLUSIONS AND FUTURE PERSPECTIVES

Nanotechnology applications have the potential to change agricultural production by allowing better management and conservation of inputs to plant production. Researchers in nanotechnology can do a lot to benefit society through applications in agriculture and food systems (Sugunan and Dutta, 2004). Introduction of any new technology always has an ethical responsibility associated with it to be apprehensive to the unforeseen risks that may come along with the tremendous positive potential. Public awareness about the advantages and challenges of nanotechnology will lead to better acceptance of this emerging technology. Rapid testing technologies and biosensors related to the control of pests and

cross contamination of agriculture and food products will lead to applications of nanotechnology in the near future.

Nanotechnology application in agriculture and food systems is still at the nascent stage and a lot more applications can be expected in the years to come. Nanoparticles present an extremely gorgeous platform for a diverse range of biological applications. As it provides the single step process for biosynthesis of nanoparticles, it attracts more researchers to go for future developments in the area of electrochemical sensor, biosensors, medicine, healthcare and agriculture. New research also aims to make plants use water, pesticides and fertilizers more efficiently, to reduce pollution and to make agriculture more environmental friendly (Suman et al., 2010).

As in the case of every nonconventional technology, for example, genetic engineering, some fear that nanotechnology can give people too much control. Agriculture technology should take advantage of the powerful tools of nanotechnology for the benefit of mankind. Nanotechnology can endeavour to provide and fundamentally streamline the technologies currently used in environmental detection, sensing and remediation. The potential uses and benefits of nanotechnology are enormous. These include agricultural productivity enhancement involving nanoporous zeolites for slow release and efficient dosage of water and fertilizer, nanocapsules for herbicide delivery and vector and pest management and nanosensors for pest detection (Scrinis and Lyons 2007; Scott 2007).

Some nanotechnology applications are near commercialization: nanosensors and nanoscale coatings to replace thicker, more wasteful polymer coatings that prevent corrosion, nanosensors for detection of aquatic toxins, nanoscale biopolymers for improved decontamination and recycling of heavy metals, nanostructured metals that break down hazardous organics at room temperature, smart particles for environmental monitoring and purification, nanoparticles as a novel photocatalyst for environmental catalysis, among others. Thus nanotechnology will revolutionize agriculture including pest management in the near future. Over the next two decades, the green revolution would be accelerated by means of nanotechnology. Nanoparticles help to produce new pesticides, insecticides and insect repellents (Owolade et al., 2008).

Nanotechnology has great potential in agriculture as it can enhance the quality of life through its applications in fields like sustainable and quality agriculture and the improved and rich food for community. All over the world, this technology has become the future of any country. One has to be very cautious with any novel technology to be introduced about its probable unforeseen and unexpected jeopardy that could land through its optimistic possibilities. Though, it is also significant for the future of a state to create skilled prospect manpower for this novel technology. Therefore, it becomes vital to inform the common man about its benefits at the first step, which will incredibly augment in the awareness and innovation of novel applications in all spheres. The outlook of nanoscience in agriculture is vague owing to a lot of grounds, for example, the unconstructive response from people towards genetically modified (GM) crops, need of a lot of required cleverness in government agricultural research and technology units for nano type of explorations and poorly-equipped new instruments and new-fangled technologies. There is a terrible call to slash down the jagged outline existing among the society, common man and budding scientific notions and if we achieve something in overcoming this line, then an unexpected bright and beneficial future will be at the door step of society.

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Review

Plant regeneration in eggplant (*Solanum melongena* L.): A review

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Eggplant is highly responsive to various tissue culture techniques. Somatic embryogenesis and direct organogenesis are widely studied protocols in this crop, but potential of regeneration varies with genotype, explant and culture media supplemented with different combination and concentration of growth hormones. The genotype is the most important factor affecting somatic embryogenesis and organogenesis. Embryogenic competence occurs even within explant segments. Among growth regulators, auxins and cytokinins are of more significance as their ratio determines callogenesis, rhizogenesis, embryogenesis and regeneration in eggplant. Organogenesis and somatic embryogenesis related gene expression has been studied and transcripts have been analyzed through molecular studies. Efficient plant regeneration protocols would make a platform for exploitation of useful somaclonal variations, mutation breeding, induction of di-haploids, and genetic transformation with economically important genes for the improvement of eggplant.

Key words: Callus, somatic embryogenesis, organogenesis, hypocotyl, cotyledon, leaf.

INTRODUCTION

Eggplant (*Solanum melongena* L., 2n = 2x = 24) is a widely adaptive and highly productive vegetable crop of tropical and subtropical regions world, which suffers from various abiotic and biotic stresses particularly insect-pests (Singh et al., 2000; Kaur et al., 2004). To control the pests, various biological and biochemical control measures have been recommended, but cryptic nature of the pest is a big hindrance in efficient management. Consequently, growers use excessive and un-recommended pesticides, which is a matter of concern for food safety, environmental degradation, pest resistance and economics of the crop. The non-availability of resistance in cultivated, cross-incompatibility with wild relatives (*Solanum mammosum*, *Solanum incanum* and *Solanum grandiflorum*) and inadvertent linkage drag of undesirable genes (Baksh and Iqbal, 1979) are problems in developing intrinsic plant resistance through conventional breeding approach. Thus, use of biotechnological techniques can be an alternative approach to tackle such issues.

In eggplant, somatic embryogenesis was first reported from immature seed embryos of two different cultivars by culturing on MS (Murashige and Skoog, 1962) medium with supplementation of indole-3-acetic acid (IAA) (Yamada et al., 1967). Although, this crop is most amenable to *in vitro* culture, still its genetic make-up, explant and culture media affect its regeneration potential (Kantharajah and Golegaonkar, 2004). Genotype and explant are the most important factor affecting somatic embryogenesis and its

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Abbreviations: **MS,** Murashige and Skoog; **BAP,** 6-benzylamino purine; **NAA,** naphthalene acetic acid; **IAA,** indole,3,acetic acid; **IBA,** indole,3,butyric acid; **ZT,** zeatin; **KN,** kinetin; **NOA,** naphthoxy acetic acid; **TDZ,** thidiazuron. **2,4-D,** 2, 4-dichlorophenoxyacetic acid; **BA,** 6, benzyladenine; **GA3,** gibberellic acid; **TIBA,** 2,3,5-triiodobenzoic acid; **PCR,** polymerase chain reaction; **ADC,** arginine decarboxylase.

further regeneration (Afele et al., 1996; Sharma and Rajam, 1995(a or b?); Dobariya and Kachhadiya, 2004; Franklin et al., 2004; Huda et al., 2007; Mir et al., 2008). The response of growth hormones in the culture media is also variable within genotype and explant for somatic embryogenesis and organogenesis (Slater et al., 2003).

The plant tissue culture methods also provide base for the improvement of crop. To induce somaclonal variations, *in vitro* mutations, herbicide tolerance, di-haploid induction, genetic transformation of economically important genes and development of somatic hybrids, efficient plant regeneration protocol is required. Such advance techniques in combination with conventional breeding give a momentum to the improvement of a crop. Thus, realizing the prospects for future research, relevant literature to "Plant regeneration in eggplant (*Solanum melongena* L.)" has been reviewed.

PLANT REGENERATION

Eggplant is highly amenable to cell, tissue and organ culture (Kantharajah and Golegaonkar, 2004). Plant regeneration from tissues of eggplant can be achieved via embryogenesis (Ammirato, 1983) and organogenesis (Flick et al., 1983). It can be done directly from cultured explants or from calli of cell suspension (Fassuliotis et al., 1981), anther (Khatun et al., 2006), microspore (Miyoshi, 1996; Lian et al., 2004) and protoplasts (Saxena et al., 1981, 1987; Kim and Shin, 2005; Oda et al., 2006; Borgato et al., 2007).

Somatic embryogenesis

Somatic embryogenesis is the process of a single cell or a group of cells initiating the developmental pathway. It was first reported in eggplant from immature seed embryos cultured on MS medium supplemented with IAA (Yamada et al., 1967). In general, it is independent or inversely related to organogenesis (Matsuoka and Hinata, 1979). The different factors such as genotype, explant, combination of growth hormones and some other factors affect somatic embryogenesis in eggplant (Kantharajah and Golegaonkar, 2004).

The genotype is the most important factor affecting somatic embryogenesis and significant quantitative differences in their capacity to form embroids among different species like *S. melongena*, *S. melongena* var. *insanum, Solanum gilo*, *Solanum integrifolium* and their F1 hybrids, cultivars, and inbred lines (Alicchio et al., 1982; Gleddie et al., 1983; Ali et al., 1991; Rao, 1992; Anisuzzaman et al., 1993; Huda et al., 2007; Mir et al., 2008, Zayova et al., 2008; Chakravarthi et al., 2010; Kaur et al., 2011a and 2013). The differential responses for regeneration of adventitious shoots and somatic embryos, number of days to shoot initiation and mean number of shoots per callus (Sharma and Rajam, 1995a; Afele et al., 1996; Dobariya and Kachhadiya, 2004) are also there among cultivars. The molecular investigation using polymerase chain reaction (PCR) of different cultivars for the induction of somatic embryos indicated that embryogenic response is due to differences in mRNA expression and consequently gene expression patterns (Afele et al., 1996).

The type of explant is also an important factor for induc-tion of somatic embryos in eggplant (Kantharajah and Golegaonkar, 2004). The use of immature seed embryo (Yamada et al., 1967; Swamynathan et al., 2010), hypo-cotyl (Alicchio et al., 1982; Sharma and Rajam, 1995a; Zayova et al., 2008; Swamynathan et al., 2010, Ray et al., 2010, Kaur et al., 2011a and 2013), cotyledon (Alicchio et al., 1982; Fari et al., 1995b; Zayova et al., 2008; Tarre et al., 2004; Huda et al., 2007; Swamynathan et al., 2010; Kaur et al., 2011a and 2013), leaf (Alicchio et al., 1982; Macchia et al., 1983; Gleddie et al., 1986; Rao and Singh 1991; Ray et al., 2010; Kaur et al., 2011a and 2013), root (Jahan and Syed, 1998; Franklin et al., 2004; Mir et al., 2008; Swamynathan et al., 2010; Ray et al., 2010), anther (Khatun et al., 2006), microspore (Miyoshi, 1996 and Lian et al., 2004) and protoplasts (Saxena et al., 1981 and 1987, Kim and Shin, 2005, Oda et al., 2006; Borgato et al., 2007) have showed different potential for somatic embryogenesis. The differences in regenerative potential of callus, number of shoots and time required for rege-neration in sub-cultures are observed also (Dobariya and Kachhadiya, 2004). The embryogenic competence varies even within hypocotyl and leaf segments (Sharma and Rajam, 1995b; Magioli et al., 2001), which can be due to gradient phytohormones (Ulvskov et al., 1992), develop-mentally regulated genes (Momiyama et al., 1995), distri-bution of polyamine content, arginine decarboxylase (ADC) activity and metabolism correlated with the position in eggplant (Fobert and Webb, 1988; Sharma and Rajam, 1995a, 1995b; Yadav and Rajam, 1997; Yadav and Rajam, 1998). Size and age of explant did not affect callus-initia-tion response, but showed marked influence on shoot regeneration response (Prakash et al., 2012).

Growth hormones like auxins, cytokinins, gibberellins and abscisic acid play role in plant regeneration. However, auxins and cytokinins are of more significance as their ratio determines callogenesis, rhizogenesis, embryogenesis and regeneration. Among auxins, naphthalene acetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2, 4-D), and IAA generally favour callogenesis and naphthoxy acetic acid (NOA), indole butyric acid (IBA) promotes rhizogenesis (Kamat and Rao, 1978; Fobert and Webb, 1988) in eggplant. However, different concentrations of NAA required for callusing (0.8 mgL^{-1}), rooting (0.016 mgL^{-1}), embryoid formation (8.0 mgL^{-1} NAA) and shooting (no NAA) (Matsuoka and Hinata, 1979; Swamynathan et al., 2010). Growing medium supplemented with IBA resulted in white, friable, and slow growing callus with roots; NAA gave green and fast growing callus; 2, 4-D

induced early callus (Macchia et al., 1983; Anwar et al., 2002). Prolonged and continued callus sub-culture on medium containing 2,4-D progressively lose its ability to regenerate (Reynolds, 1986). Callus induction and somatic embryogenesis on different medium supplemented with different auxins (Alicchio et al., 1982; Gleddie et al., 1986; Saito and Nishimura, 1994; Sharma and Rajam, 1995a; Fari et al., 1995b; Magioli et al., 2001; Picoli et al., 2000; Mir et al., 2008) is listed in Table 1. Among several cytokinins, kinetin (Kin) is effective for shoot bud regeneration (Kamat and Rao, 1978; Alicchio et al., 1982). Other cytokinins 6-bemzylamino purine (BAP) or thidiazuron (TDZ) (Kaparakis and Alderson, 2002), BAP (Picoli et al., 2000), 6-BA (Li et al., 2003) also produced highest percentage of somatic embryos in different explants of eggplant as listed in Table 1. The cytokinins not only inhibit the NAA-induced embryogenic response, but also act synergistically to promote callus growth (Gleddie et al., 1983).

Cytokinin-auxin interactions either promoted or inhibited the development of shoots and roots depending upon their ratio in the medium (Kamat and Rao, 1978). The rege-neration also depends upon the type and concentration of cytokinin. The high concentrations of benzyladenine and all concentrations of kinetin promoted organogenesis, while low concentrations of benzyladenine induced somatic embryogenes as well as organogenesis (Reynolds, 1986). Generally, higher level of auxins and lower of cyto-kinine favours somatic embryogenesis. MS / LS medium supplemented with combination of 10 mgL^{-1} 2, 4-dichloro-phenoxyacetic acid and 1 mgL^{-1} kinetin (Reynolds, 1986), 2ip (γγ-isopentyladenine) and IAA (Fassuliotis, 1975), 8 mgL^{-1} NAA and 0.1 mgL^{-1} Kin (Rao and Singh, 1991, Swamynathan et al., 2010), Zeatin @ 2 mgL^{-1} and NAA @ 0.01 mgL^{-1} (Fari et al., 1995), 1 mgL^{-1} NAA and 2 mgL^{-1} BAP (Salih and Al-Mallah, 2000), NAA or IBA at 0.5 mgL^{-1} (Anwar et al., 2002), 6-BA+ ZT (Zeatin) and 6-BA+IAA or ZT+ IAA (Yu et al., 2003; Li et al., 2003), 2.0 mgL^{-1} NAA + 0.05 mgL^{-1} BAP, 2.0 mgL^{-1} NAA and 0.5 mgL^{-1} BAP, 2 mgL^{-1} 2,4-D + 0.05 mgL^{-1} BAP and 2 mgL^{-1} NAA+2.5 mgL^{-1} BAP (Rahman et al., 2006; Huda et al., 2007; Hossain et al., 2007; Zayova et al., 2008; Chakravarthi et al., 2010) induced the callus in eggplant (Table 1).

Gene expression during initial stage of somatic embryogenesis in eggplant revealed that 2,4-D induces specific alteration in gene expression due to differential display of RNA (Momiyama et al., 1995). In spite of this, an antioncogen homolog and the activation of retro-transposon were described during early stages of somatic embryogenesis (Momiyama et al., 1996). Differential display and restriction fragment length poly-morphism (RFLP) analysis resulted in the identification of one organogenesis and two somatic embryogenesis related transcripts (Bucherna et al., 2001).

The frequency of embryogenesis depended on optimal ratio of NO_3^- : NH_4^+ (2:1) in the medium. The optimal sucrose concentration of the medium was 0.06 M, whereas, elevated or reduced level inhibited the embryo-genesis in eggplant (Gleddie et al., 1983). Sucrose concentrations of 0.2 - 0.5% induced somatic embryo-genesis, 1% led to embryogenesis and shoot regene-ration and 2% provoked maximum shoot regeneration, whereas, increased sucrose levels from 3 to 5% decreased the regenerating ability. The lowered sucrose concen-tration from 2 to 0.2% also caused complete bleaching, which can be used for selection of herbicide-resistant mutants (Farooqui et al., 1997). The pesticides like Endlosulfan, Rogor and Kitazin in relation to their concentrations also affected callus induction and multiple shoot formation in eggplant. The callus growth decreased with increasing level of pesticides in medium. Some concentrations (50 - 500 ppm) of pesticides in the medium also formed abnormal callus growth and shoot induction. Among pesticides, Rogor (25 ppm) induced maximum callus (76.0%) and shoots (11.0), whereas, Kitazin 45% EC showed more inhibitory effect than the Endosulfan and Rogor (Sammaiah et al., 2011a, 2011b).

Plant regeneration from tissue culture of *S. melongena* L. can be achieved via embryogenesis (Ammirato, 1983) and organogenesis (Flick et al., 1983). Light could help the development of adventitious rooted shoots from callus (Macchia et al., 1983; Salih and Al-Mallah, 2000). High concentration of 2ip and low concentration of IAA led to differentiation of leaflets with morphogenetic variation in leaves and cytological studies of plants indicated them genetically aberrant (Fassuliotis, 1975). LS medium without hormones also regenerated plant from callus (Alicchio et al., 1982). Also, MS medium supplemented with different concentrations and combinations of cytokinins and auxins (Table 1) produced more shoot primordial and rooted shoots in calli derived from cotyledon, hypocotyls, leaf and root explants (Macchia et al., 1983; Anwar et al., 2002; Yu et al., 2003; Franklin et al., 2004; Rahman et al., 2006; Chakravarthi et al., 2010). Plants regenerated through somatic embryogenesis had somaclonal varia-tions. Frequencies of somaclonal variations in leaf shape, plant height, fruit shape and pollen fertility was higher with NAA than that of 2, 4-D (Hitomi et al., 1998). There-fore, the future research would determine the importance of new somaclonal lines for genetic variability of eggplant (Zayova et al., 2010, 2012).

Organogenesis

Organogenesis is the morphogenesis of plantlets directly from explants without the intervention of callus in the culture. This omits the callus and embryoid phases, reduces use of auxin from the *in vitro* culture and leads to direct formation of new shoots from the explants. Anatomically and histolosically, longitudinal sections of leaf explants formed numerous meristematic zones within the tissue, that subsequently converted into shoot buds (Mukherjee et al., 1991). The formation of shoot buds was characterized

Table 1. Somatic embryogenesis in eggplant.

Explant	Somatic embryogenesis	Shoot induction	References
Immature embryo cultures	MS + IAA		Yamada et al. (1967)
Hypocotyl	MS + 0.016 mgL^{-1} - 0.8 mgL^{-1} NAA (callus), MS + 8.0 mgL^{-1} NAA(embryogenesis)	Hormone free MS	Matsuoka and Hinata (1979)
Hypocotyl, otyledon, leaf	LS+ 0.4 mgL^{-1} 2,4-D	Hormone free LS	Alicchio et al. (1982)
Leaf	MS+10 mgL^{-1} NAA	Basal MS	Gleddie et al. (1986)
Leaf	Kao/ NT (liquid)+ 10 mgL^{-1} NAA or Kao/ NT(liquid)+ 2 mgL^{-1} 2,4-D, Kao/ NT(liquid)+ 1 mgL^{-1} 2,4-D	Kao/ NT (solid)+ 10 mgL^{-1} NAA or Kao/ NT(solid)+ 2 mgL^{-1} 2,4-D, Kao/ NT(solid)+ 1 mgL^{-1} 2,4-D	Gleddie et al. (1986)
Stem segments	MS+ 10mgL^{-1} 2, 4-D +1 mgL^{-1} kin	MS+ +1 mgL^{-1} kin	Reynolds (1986)
Cotyledon	MS+1.0-5.0 mgL^{-1}NAA	Hormone free MS	Fobert and Web (1988)
Hypocotyl	MS+ 0.5-2.0 mgL^{-1} 2,4-D	Hormone free MS	Ali et al. (1991)
Leaf	MS+ 8 mgL^{-1}NAA + 0.1 mgL^{-1} Kin	Basal MS	Rao and Singh (1991)
Leaf	MS +0.5-2.0 mgL^{-1} NAA	Basal MS	Rao (1992)
Cotyledon	50 αM 2,4-D	half-strength MS solid medium without hormones	Saito and Nishimura(1994)
Hypocotyl, cotyledon and leaf	MS+ 32.2 μM (hypocotyls) and, MS + 10.7 μM (cotyledon and leaf)	-	Sharma and Rajam (1995a)
cotyledon	TMG+ 2 mgL^{-1} Zeatin + 0.01 mgL^{-1} NAA(callus), TMG+4 mgL^{-1} NAA(SE)	TMG+ 2 mgL^{-1} Zeatin + 0.01 mgL^{-1} NAA(callus), TMG+4 mgL^{-1} NAA(SE)	Fari et al. (1995)
Leaf	10.73 *mM* NAA+0.5m M putriscine	-	Yadav and Rajam (1997)
Stem and leaf	MS+ 1 mgL^{-1} NAA + 2 mgL^{-1} BAP	MS+ 1 mgL^{-1} NAA + 2 mgL^{-1} BAP	Salih and Al-Mallah (2000)
Hypocotyl, cotyledon, leaf, epicotyl	MS + 54 αM	½ MS+1% phytagel	Magioli et al. (2001)
Hypocotyl and cotyledon	MS +2.5-10.0 mgL^{-1} NAA	-	Picoli et al.(2000)
Leaf	MS +2 mgL^{-1} 6-BA+0.5 mgL^{-1} IBA, MS +2 mgL^{-1} 6-BA+0.5 mgL^{-1} NAA	MS +2 mgL^{-1} 6-BA+0.5 mgL^{-1} IBA, MS +2 mgL^{-1} 6-BA+0.5 mgL^{-1} NAA	Anwar et al. (2002)

Table 1. Contd.

Cotyledon, hypocotyl	MS +1.0-2.5 mgL^{-1} 6-BA	MS +1.0-2.5 mgL^{-1} 6-BA	Yu et al. (2003)
cotyledon	54 mM NAA	MS basal	Tarre et al. (2004)
Root	MS + 0.45 mM TDZ (Thidiazuron) and 13.3 mM BA (6-benzyladenine)	MS + 0.45 mM TDZ (Thidiazuron) and 13.3 mM BA (6-benzyladenine)	Franklin et al. (2004)
Cotyledon and young leaf explant	MS+1 mgL^{-1} BA MS+2 mgL^{-1} KIN MS+1 mgL^{-1} BA+1mgL^{-1} KIN MS+2 mgL^{-1} BA+1mgL^{-1} KIN MS+2 mgL^{-1} KIN+1 mgL^{-1} BA MS+2 mgL^{-1} BA+2 mgL^{-1} KIN	MS+1 mgL^{-1} BA MS+2 mgL^{-1} KIN MS+1 mgL^{-1} BA+1mgL^{-1} KIN MS+2 mgL^{-1} BA+1mgL^{-1} KIN MS+2 mgL^{-1} KIN+1 mgL^{-1} BA MS+2 mgL^{-1} BA+2 mgL^{-1} KIN	Dobariya and Kachhadiya (2004)
Cotyledon and midrib	MS+ 2.0 mgL^{-1} NAA and 0.05 mgL^{-1} BAP	MS+ 2.0 mgL^{-1} Zeatin and 1.0 mgL^{-1} BAP	Rahman et al. (2006)
Cotyledon	MS+ 2.0 mgL^{-1} NAA and 0.05 mgL^{-1} BAP, MS+ 1.0 mgL^{-1} BAP+ 0.5 mgL^{-1} GA3	MS+ 2.0 mgL^{-1} NAA and 0.05 mgL^{-1} BAP, MS+ 1.0 mgL^{-1} BAP+ 0.5 mgL^{-1} GA3	Huda et al. (2007)
Cotyledon	MS+ 2.0 mgL^{-1} NAA + 0.05 mgL^{-1} BAP, MS+ 2.0 mgL^{-1} 2,4-D+ 0.05 mgL^{-1} BAP,	MS+ 0.75 mgL^{-1} NAA+ 1.5 mgL^{-1} BAP, MS+ 2.0 mgL^{-1} NAA+ 0.5 mgL^{-1} IBA	Hossain et al. (2007)
Hypocotyl, cotyledon and root	MS+ 1.0mgL^{-1} NAA (hypocotyls), 1.5 mgL^{-1} NAA (cotyledon) and 2.0 mgL^{-1} NAA (root)	MS+ 2.5 mgL^{-1} IAA + 0.5 mgL^{-1} BAP	Mir et al. (2008)
Cotyledon hypocotyl	MS + 2.0 mgL^{-1} NAA + 0.5 mgL^{-1} BAP	Hormone free MS	Zayova et al. (2008, 2012)
immature seed embryo, cotyledon, shoot	MS+ 10.5 mgL^{-1} NAA(cotyledon), MS+ 8.0 mgL^{-1} NAA+ 0.1 mgL^{-1} KN (seed embryos)	Hormone free MS medium	Swamynathan et al. (2010)
Hypocotyl, root, leaf	MS + 2.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA	MS + 2.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA	Ray et al., 2010
Cotyledon	MS+ 2 mg/ mgL^{-1} l NAA+2.5 mgL^{-1} BAP	MS+2.5 mgL^{-1} each BAP and KN	Chakravarthi et al. (2010)
Cotyledon	MS + 2 mgL^{-1} NAA	MS+0.5 mgL^{-1} IAA +3.0 mgL^{-1} BAP	Sammaiah et al. (2011a& 2011b)
Hypocotyl, cotyledon and root	MS+2.5 mgL^{-1} /l IAA + 0.5 mgL^{-1} BAP	MS+2.5 mgL^{-1} IAA + 0.5 mgL^{-1} BAP	Mir et al. (2011)
Hypocotyl, cotyledon and leaf	MS + 1.5 mgL^{-1} IBA + 1.0 mgL^{-1} BAP	MS + 2.5 mgL^{-1} BAP + 1.0 mgL^{-1} kin + 0.2% activated charcoal	Kaur et al. (2013)
shoot tip, hypocotyls, leaves, stem	MS+0.6 mgL^{-1} 2, 4-D	MS+0.2 mgL^{-1} BAP, MS+0.6 mgL^{-1} NAA, MS + 0.4 mgL^{-1} IAA	Robinson and Saranya (2013)

Table 2. Organogenesis in eggplant.

Explant	Direct organogenesis	References
Hypocotyl	MS + 2.8-11.4 µM IAA, MS + 4.7 µM KIN, MS + 2.3-4.6 µM ZT	Kamat and Rao **1978**
Leaf	MS + 2.0 mgL^{-1} Kin+ 88mM sucrose, MS + 2.0 mgL^{-1} Kin+ 5.5 and 11mM glucose	Mukherjee et al., (1991)
Leaf	MS + 1.0 mgL^{-1} BAP +0.5 mgL^{-1} ZT	Perrone et al., 1992
Hypocotyl, cotyledon and leaf	MS + 11.1 µM BA and 2.9 µM IAA	Sharma and Rajam, 1995a
Cotyledon	TMG + 2 mgL^{-1} Kin	Fari et al., 1995
Leaf	MS + 0.1 µM TDZ and MS + 10 or 20 µM2ip	Billings et al., 1997
Leaf	MS + 0.001-1 μgml^{-1}TDZ and MS + 5-20 μgml^{-1} 2ip	Jelenkovic and Billings 1998
Leaves and cotyledons	MS + 0.2 w*m* TDZ	Magioli et al., 1998
Cotyledon and hypocotyl	MS + 0.1 mgL^{-1} IAA	Picoli et al., 2000
Leaf and stem	MS + 0.5 mgL^{-1} NAA	Taha and Tizan, 2002
Cotyledon and leaf	MS + 0.1 or 0.2 µ*M* TDZ	Gisbert et al., 2006
Cotyledon, hypocotyl, shoot tip , root	MS + 1.0 mgL^{-1} BAP + 1.0 mgL^{-1} Kin	Sarker et al., 2006
Meristem	MS(liquid)+ 2.0 mgL^{-1} BAP, MS(semisolid)+ 2.0 mgL^{-1} BAP+1 mgL^{-1} NAA, MS(semisolid)+ 1.0 mgL^{-1} BAP	Sharmin et al., 2008
Cotyledonary nodes	MS + 2.0 mgL^{-1} BAP + 1.0 mgL^{-1} 2iP	Kanna and Jayabalan, 2010
Hypocotyls, cotyledon and leaf	MS + 2.5 mgL^{-1} BAP + 1.0 mgL^{-1} KN	Kaur et al., 2011
Cotyledon	MS+ 1.0 mgL^{-1} Zeatin	Prasad et al., 2011
Leaf	MS+ 1.0 mgL^{-1} TDZ+ 4.02 g/l nitrogen, +2.36% sucrose	Naveenchandra et al., 2011
Cotyledon, hypocotyl and leaf	MS + 2.0 mgL^{-1} BAP + 0.5 mgL^{-1} Kn	Shivraj and srinath, 2011
Cotyledon nodal segments and shoot tip	MS + 2.0 mgL^{-1} BAP + 1.0 mgL^{-1} Kn	Bhat et al., 2013
Hypocotyl (inverted)	MS + 0.5 mgL^{-1} TDZ	Mallaya and Ravishankar, 2013

by the appearance of shoot apex with the developing leaf primordial (Sarker et al., 2006). Genotype played important role in organogenesis of the shoots directly from the explants. Different varieties and species such as *Solanum aethiopicum, Solanum macrocarpon* showed different potential in direct plant regeneration, where, 70 - 100% explants with a mean of two to seven shoots per explant were obtained (Gisbert et al., 2006; Sarker et al., 2006; Shivraj and Srinath, 2011).

The direct regeneration potential also varied with the tissue system used on a well defined medium. Different explants had differential response to regeneration (Sharma and Rajam, 1995a; Magioli et al., 1998; Zhang, 1999; Taha and Tizan, 2002; Sarker et al., 2006; Gisbert et al., 2006; Kanna and Jayabalan, 2010; Shivraj and Srinath, 2011; Kaur et al., 2011) on different media combinations containing cytokinins and auxins. Hypocotyl and cotyledon explants had different morphogenetic potential for numbers of adventitious shoots (Sharma and Rajam, 1995a; Zhang, 1999). Explant age also affected regeneration as younger leaves showed better organogenesis than mature ones (Zhang, 1999).

Different growth regulators such as auxins and cytokinins have been used for direct organogenesis (Table 2). Among these, auxins had influenced the regeneration of shoot buds and roots in eggplant (Kamat and Rao, 1978). Presence of any cytokinin in the media led to shoot organogenesis from leaf explants (Gleddie et al., 1983; Polisetty et al., 1994). However, combinations and concentrations of auxins and cytokinin should be optimum for

having maximum number of regenerated shoots in egg-plant (Mukherjee et al., 1991; Fari et al., 1995; Magioli et al., 1998; Zhang, 1999; Picoli et al., 2000; Sarker et al., 2006). Combinations of two cytokinins had shown proficient shoot differentiation (2 to 7 shoots per explant) in eggplant (Iannamico et al., 1993; Billings et al., 1997; Jelenkovic and Billings, 1998; Gisbert et al., 2006; Shivraj and Srinath, 2011; Kanna and Jayabalan, 2010).

Low sugar concentrations enhanced shoot regeneration, where, higher concentration of glucose and lower of sucrose showed better effects (Mukherjee et al., 1991; Polisetty et al., 1994). Shoot regeneration process had also been affected by the gelling agents and agar was found superior to gerlite (Perrone et al., 1992). Peptone had no effect on reducing hyperhydric shoots of *S. melongena* and *S. integrifolium*. Culture vessels with gas-permeability by membrane filter reduce the percentage of hyperhydric shoots and increased survival rate than sealed vessels (Takamura et al., 2006).

Elongation and rooting of plantlets

Small shoots require elongation *in vitro*. Hormone free MS or 1/2MS has been most frequently used for the elongation plantlets in eggplant (Gleddie et al., 1983; Magioli et al., 1998; Franklin and Sita, 2003; Franklin et al., 2004; Gisbert et al., 2006; Sarker et al., 2006; Borgato et al., 2007; Mir et al., 2011). Sometimes, MS fortified with gibberellic acid (GA3) (0.1 to 1.5mgL^{-1}) (Shivraj and Srinath, 2011), 0.5 mg/l 2,3,5-triiodobenzoic acid (TIBA) and 0.1 mg/l GA3 (Naveenchandra et al., 2011) Zeatin and Augmentin (Billings et al., 1997) elongated eggplant shoots also.

Eggplant developed roots upon transfer to medium containing IAA (Fassuliotis, 1975), hormone-free MS medium (Saxena et al., 1981; Gleddie et al., 1983; Taha and Tizan, 2002; Gisbert et al., 2006; Sarker et al., 2006) and MS medium containing 0.1 -1.5 mgL^{-1} 3-indol butyric acid (Jahan and Syed, 1998; Borgato et al., 2007; Sharmin et al., 2008; Chakravarthi et al., 2010; Shivraj and Srinath, 2011; Zayova et al., 2012; Robinson and Saranya, 2013, Bhat et al., 2013). Half strength MS medium containing 0.08 mgL^{-1} NAA also developed roots of 90% shoots (Kanna and Jayabalan, 2010). Half-strength MS medium supplemented with 0.6 w*m* IAA (Magioli et al., 1998) and 5.0 mg sucrose and 2.5 gl^{-1} gellan gum (Kim and Shin, 2005; Oda et al., 2006) induced rooting of plantlets. Quarter strength hormone free MS medium induce roots also (Dobariya and Kachhadiya, 2004), however, MS+ 3.0 mgL^{-1} BAP was used for better root induction with respect to average number (14 - 15) and mean length (12 cm) (Rahman et al., 2006).

Hardening and field establishment

Most of the species grown *in vitro* require acclimatization process in order to ensure that sufficient number of plants survive and grow vigorously on transferring to the soil. It took 3-4 months from initiation to establishment in pots *ex vitro* for 99% survival rate (Polisetty et al., 1994), however, rooted plants can be acclimatized in 14 days with 80% success (Salih and Al-Mallah, 2000; Taha and Tizan, 2002; Chakravarthi et al., 2010; Kanna and Jayabalan, 2010; Shivraj and Srinath, 2011; Kaur 2011a, b). Rooted shoots were transferred for establishment in polythene bags filled with a potting mixture of sand, soil and FYM in 1:2:1 ratio (Dobariya and Kachhadiya, 2004). The plantlets were successfully established in polycarbonated polyhouse with 100% survival rate (Bhat et al., 2013). When root system was developed well, plants were hardened in the glass house and transferred to the field for flowering, fruiting and seeding (Kamat and Rao, 1978; Gleddie et al., 1983; Jahan and Syed, 1998; Magioli et al., 1998; Franklin et al., 2004; Sarker et al., 2006).

CONCLUSIONS

Research work has mainly been focused on the develop-ment of regeneration protocol, somaclonal variations and their physiological as well as morphological aspects in eggplant. An efficient plant regeneration protocol is a pre-requisite for the exploitation of various biotechnological techniques. However, practical utility of the basic protocol is still far away. It can serve as a platform for the transfer of economically important traits through genetic engi-neering, inducing somaclonal variations, *in vitro* muta-tions, double-haploids induction, development and utilize-tion of somatic hybrids, determining herbicide or pesticide tolerance limits in eggplant. Therefore, a remark-able pro-gress can be made in eggplant improvement through the combination of conventional and biotechnological approaches.

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Full Length Research Paper

Comparative effects of some medicinal plants on blood glucose concentration and lipid levels in alloxan-induced diabetic rats

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The comparative effects of the chloroform extracts of the leaves of *Psidium guajava* (Myrtaceae), *Anacardium occidentale* (Anacardiaceae) and *Eucalyptus globulus* (Myrtaceae) and fruits of *Xylopia aethiopica* (Annonaceae) on blood glucose concentration and lipid levels of diabetic rats were investigated using standard methods. The results show 74, 82 and 83% reductions in the blood glucose concentrations upon the administration of *A. occidentale* (100 mg/kg body weight), *E. globulus* (100 mg/kg body weight) and *X. aethiopica* (250 mg/kg body weight) extracts respectively as from the 10th hour of treatments in relation to the 74 and 69% reductions in glibenclamide and diabetic untreated groups respectively while the synergic treatment group [*A. occidentale* + *E. globulus* (100 mg/kg body weight)] showed 83% decrease in the blood glucose concentration as from the 10th hour upon the administration of the combined extracts when compared with the values obtained for the glibenclamide and diabetic untreated groups. *P. guajava* extract had the greatest significant (p<0.05) reduction in the total cholesterol concentration of the treated rats. *P. guajava* + *X. aethiopica* treatment group in a similar manner showed the most significant (p<0.05) decrease in the triglyceride concentration of the treated rats. Hence, the individual performances of these extracts on blood glucose concentration and blood lipids confirm their ability to reduce blood glucose and diabetic complications.

Key words: Chloroform extract, *Psidium guajava* (Myrtaceae), *Anacardium occidentale* (Anacardiaceae), *Eucalyptus globulus* (Myrtaceae), *Xylopia aethiopica* (Annonaceae).

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder of carbohydrate, proteins and fats occurring in the endocrine system (Jarald et al., 2008) as a result of absolute or relative deficiency of insulin secretion as in the case of type 1 diabetes mellitus or with/without varying degree of insulin resistance (Devlin, 2006) as in the case of type 2 diabetes mellitus. This disorder is characterized by hyperglycemia, producing typical symptoms such as polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger). When diabetes is not properly treated or controlled, it causes persistent hyperglycemia that culminates in chronic complications for example, microvascular complications (atherosclerosis, myocardial infection and stroke). Other complications are: diabetic ketoacidosis, non ketotic hyperosmolar coma amongst others (Merlin et al., 2005).

Atherosclerosis is a disease of the arteries, characterized by a gradual accumulation of cholesterol, cholesterol esters, collagen, elastic fibers and proteoglycans in the arterial wall. This accumulation of cholesterol and its

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esters is caused by the increased production of this metabolite, an associated condition of hyperlipidemia. Hyperlipidemia occurs upon a diabetic state as a result of resistance to the action of lipolytic hormone on fat deposits leading to an elevated mobilization of fatty acids from the peripheral deposits where insulin inhibits the hormone-sensitive lipase (Murray et al., 2000).

It is reported that diabetes mellitus (DM) is rapidly becoming a pandemic with population growth, aging, urbanization, increasing prevalence of obesity and physical inactivity as factors influencing the rise in the population diabetics (Wild et al., 2004). On the other hand, the drugs currently used in the management of diabetes mellitus especially type 2 are plagued with several limitations that include resistances, adverse effects, lack of responsiveness in large population of diabetics, liver toxicity, worsening of heart diseases, hypoglycemia and weight gain to mention but a few (Michael et al., 2005). In addition, most of these therapeutic agents have not effectively controlled hyperlipidemia, an associated condition of diabetes mellitus (Derek, 2011). These drawbacks coupled with the high prevalence of diabetes amongst rural population globally has reveille for the development of safe indigenous inexpensive botanical sources for antidiabetic (crude or purified) drugs (Venkatesh et al., 2003). Botanical sources are materials for plant- based drugs/agents that have been employed in treating various diseases for several years (Mushtaq et al., 2009). The present study was undertaken to investigate the comparative effects of the chloroform extracts of the leaves of *Psidium guajava, Anacardium occidentale and Eucalyptus globulus* and fruits of *Xylopia aethiopica* on blood glucose concentration and lipid levels of diabetic rats.

MATERIALS AND METHODS

The plant samples

The leaves of *A. occidentale*, *E. globulus* and *P. guajava* were collected from the premises of University of Nigeria, Nsukka while the fruits of *X. aethiopica* were purchased from a local market in Delta State. The plant samples were identified by Prof. (Mrs.) May Nwosu of the Department of Botany, University of Nigeria, Nsukka, Enugu State, Nigeria; where the voucher specimens were deposited in the herbarium.

Preparation of the crude extract

The leaves of *A. occidentale, E. globulus, P. guajava* and fruits of *X. aethiopica* were air dried to constant weight at room temperature and then reduced to powder. Six hundred grams of each plant material was macerated in 2.7 l of analytical grade chloroform. After 48 h, the resulting extracts were filtered and concentrated with rotary evaporator at reduced pressure and the yield of extracts calculated. A standard weight 8 g of each extracts was dissolved in 16 ml of 10% dimethyl sulphuroxide (DMSO). The doses of each extracts administered was estimated by the methods of Tedong et al. (2007), where volumes given were calculated as follows:

$$V\ (ml) = \frac{D \times P}{C}$$

Where, D, dose used (g/kg body weight of test animals); P, body weight (kg); C, concentration (g/ml); V, volume (ml).

Animals

Seventy five (75) male Wistar albino rats of weight (180 -230 g) and 128 male mice of weight (20-30 g) were used for this study. They were housed and maintained at a 12 h light and dark cycle and fed with rat diet *ad libitum*. The mice were used for acute oral toxicity study while the rats were made diabetic by a single dose of 180 mg/kg body weight of alloxan monohydrate intraperitonially.

Acute oral toxicity test (LD_{50})

A lethal dose toxicity study of each plant material was carried out by the method described by Lorke (1983).

Measurement of plasma glucose concentrations

Monitoring of blood glucose concentrations was carried out by life scan ultra one touch ultra-mini 2 glucose meter using blood samples from pricked tails of rats.

Determination of blood lipids

Determination of cholesterol concentration was done according to the methods of Abell et al. (1952) and Richmond (1973) while that of triglyceride level was by the methods of Tietz (1990) and Jacobs and VanDemark (1960).

Statistical analysis

Data generated from this study were represented as mean ± SEM. Variables were analyzed by one-way analysis of variance (ANOVA) and comparison done by multiple comparisons using Duncan test.

RESULTS

Effects of the various plant extracts on blood glucose concentration at different time intervals

As shown in Figure 1, reductions in blood glucose concentration were recorded after 10 h of establishment of diabetes and treatment with the various plant extracts. Fourteen percent (14%) decrease in blood glucose was observed up to the fortieth hour in the group treated with 100 mg/kg body weight of *A. occidentale* when compared with diabetic untreated group while the group administered 250 mg/kg of the same extract had 70% reduction in blood glucose concentration at the 10th to 40th hour. The group administered 100 mg/kg of *E. globulus* showed 82% decline in blood glucose concentration at the 10th to 40th hour when compared with the 74 and 6.9% recorded for glibenclamide and diabetic untreated groups, respectively. 60% decrease in blood glucose was observed in the group administered 250 mg/kg of the same extract. *P. guajava* extract (100 mg/kg) showed 50% reduction in blood glucose from the tenth to fortieth hour when compared with the values obtained for glibenclamide and

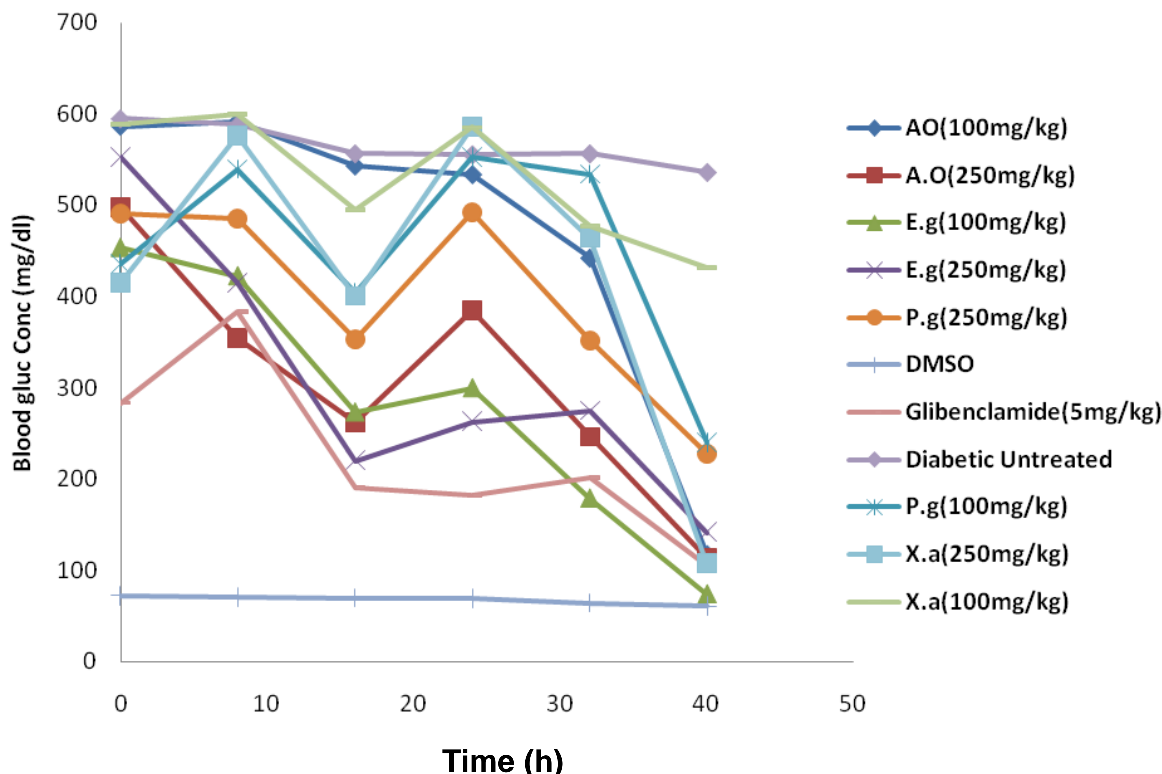

Figure 1. Effects of the various plant extracts on blood glucose concentration at different time intervals.

diabetic untreated groups at the said time interval. However, the group administered 250 mg/kg of the same extract had 50% fall in blood glucose concentration relative to those of glibenclamide and diabetic untreated groups. Blood glucose concentration was reduced by 23% in the group given 100 mg/kg of *X. aethiopica* from the 10th to 40th hour while an effective decrease in blood glucose concentration up to 82% occurred in the group administered 250 mg/kg of the same extract as from the 10th to 40th hour when compared with the values obtained for glibenclamide and diabetic untreated groups. The glibenclamide-treated group exhibited only 74% decline in blood glucose concentration at the tenth to fortieth hour of treatment in relation to the 74% for *A. occidentale* (100 and 250 mg/kg), 82 and 62% for *E. globulus* (100 and 250 mg/kg respectively) and 23 and 82% for *X. aethiopica* (100 and 250 mg/kg, respectively).

Effects of the combined plant extracts on blood glucose concentration at different time intervals

Figure 2 shows that 83% decline in blood glucose was observed in the group administered 100 mg/kg of *A. occidentale* + *E. globules* while 79% reduction occurred in the group administered 250 mg/kg of the same combined extracts when compared with the 74 and 6.9% obtained for glibenclamide and diabetic untreated groups, respectively, from the tenth to the fortieth hour. 66% reduction was observed in the group administered 100 mg/kg of *P. guajava* + *X. aethiopica* when compared with the values recorded for glibenclamide and diabetic untreated groups while 58% decrease was observed in the group given 250 mg/kg of the same combined extracts when compared with the values obtained for glibenclamide and diabetic untreated groups from the tenth to the fortieth hour.

Effects of varying doses of the different plant extracts on total cholesterol concentration

As shown in Figure 3, there were significant ($p<0.05$) differences between the total cholesterol concentration of group 1 and those of groups 4, 5, 6, 7, 9, 10, 11, 12, 13 and 14 whereas there were no significant ($p>0.05$) differences between that for group 1 and those of groups 2, 3, 8 and 13. The administration of 250 mg/kg of *A. occidentale* extract caused significant ($p<0.05$) decrease in total cholesterol concentration relative to that of the diabetic untreated group. The total cholesterol concentrations of other test groups were however, not significantly ($p>0.05$) different from that of the 250 mg/kg of *A. occidentale* group. Group 3 was found to be significantly ($p<0.05$) different from groups 4, 5, 6, 7, 9, 10, 11, 12, 13 and 14. No significant ($p>0.05$) difference occurred between group 3 and groups 1, 2, 8 and 15.

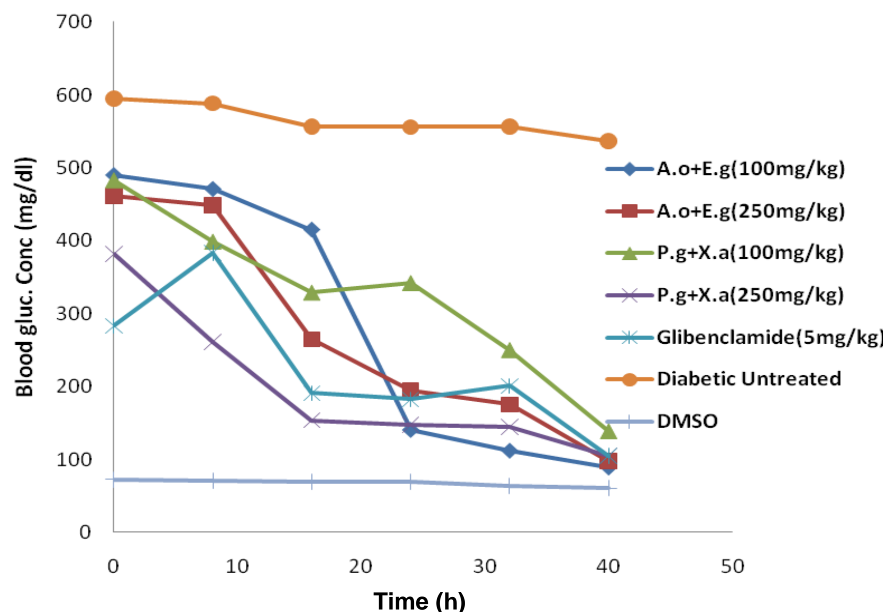

Figure 2. Effects of the combined plant extracts on blood glucose concentration at different time intervals.

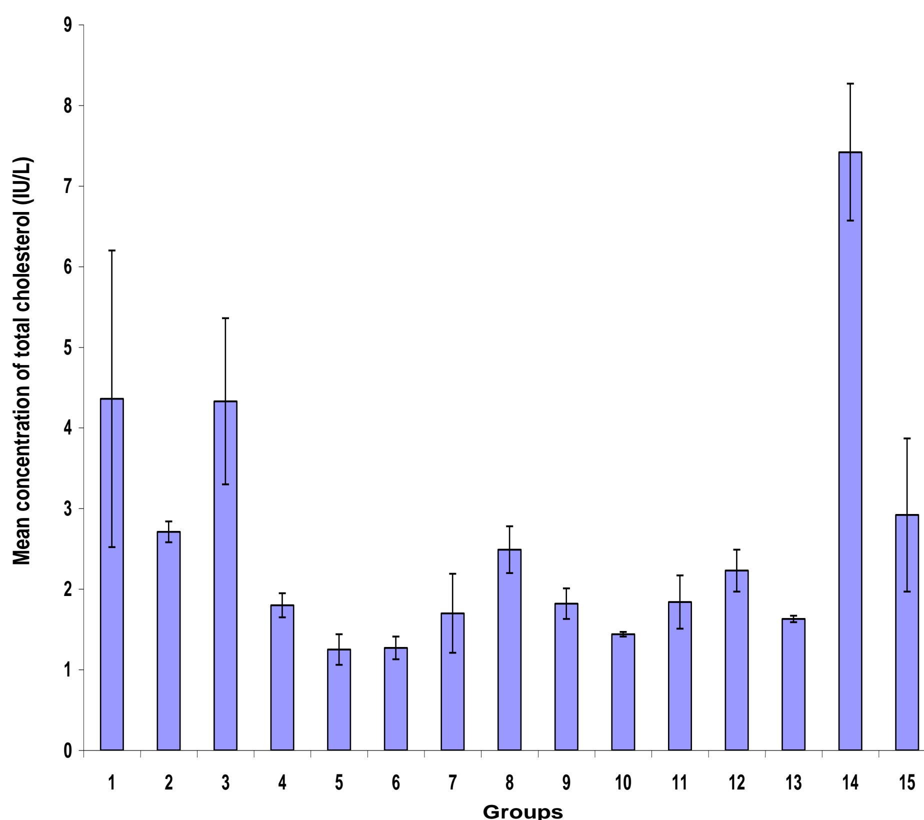

Figure 3. Group 1=*Anacardium occidentale* (100 mg/kg); Group 2=*Anacardium occidentale* (250 mg/kg); Group 3=*Eucalyptus globulus* (100 mg/kg); Group 4=*Eucalyptus globulus* (250 mg/kg); Group 5=*Psidium guajava* (100mg/kg); Group 6=*Psidium guajava* (250 mg/kg); Group 7=*Xylopia aethiopica* (100 mg/kg); Group 8=*Xylopia aethiopica* (250 mg/kg); Group 9=*A. occidentale* + *E. globulus* (100 mg/kg); Group 10= *A. occidentale* + *E. globulus* (250 mg/kg) ; Group 11= *P. guajava* + *X. aethiopica* (100 mg/kg); Group 12 = *P. guajava* + *X. aethiopica* (250 mg/kg); Group 13 = Glibenclamide (5 mg/kg); Group 14 = Diabetic Untreated; Group 15 = DMSO Control.

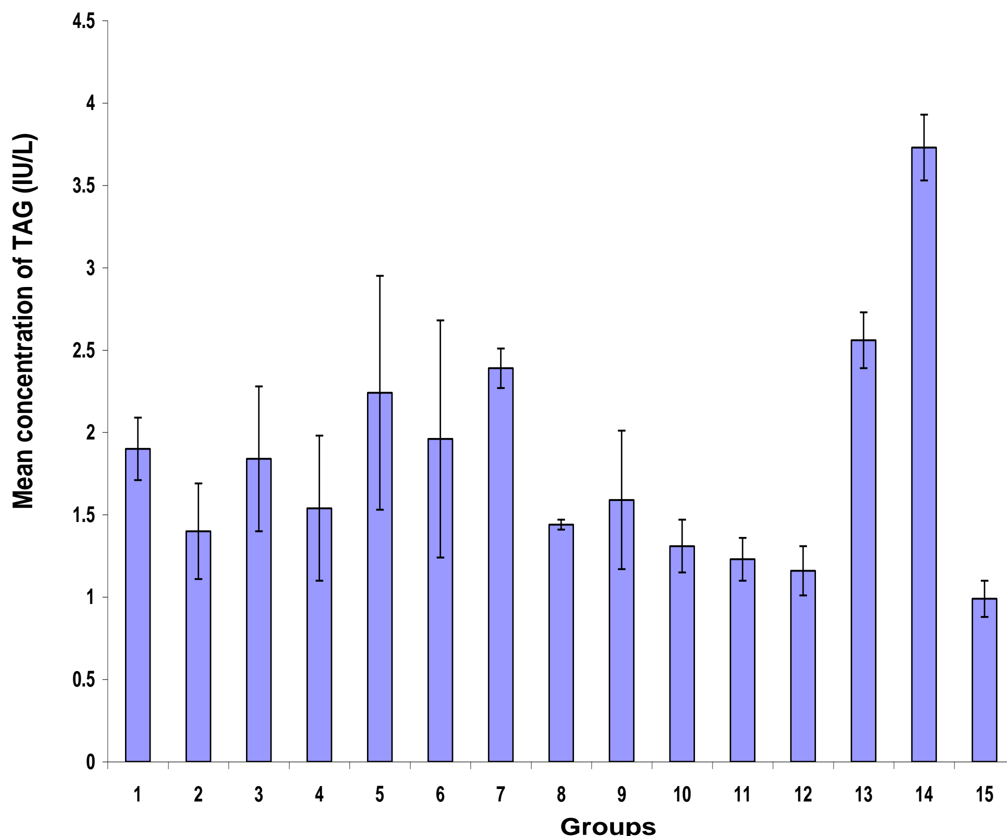

Figure 4. Group 1=*Anacardium occidentale* (100mg/kg); Group 2= *Anacardium occidentale* (250mg/kg); Group 3=*Eucalyptus globulus* (100mg/kg); Group 4=*Eucalyptus globulus* (250mg/kg); Group 5=*Psidium guajava* (100mg/kg); Group 6=*Psidium guajava* (250mg/kg); Group 7=*Xylopia aethiopica* (100mg/kg); Group 8=*Xylopia aethiopica* (250mg/kg); Group 9= *A. occidentale* + *E. globulus* (100mg/kg); Group 10= *A. occidentale* + *E. globulus* (250mg/kg) ; Group 11= *P. guajava* + *X. aethiopica* (100mg/kg); Group 12 = *P. guajava* + *X. aethiopica* (250mg/kg); Group 13 = Glibenclamide (5mg/kg); Group 14 = Diabetic Untreated; Group 15 = DMSO Control.

Effects of varying doses of the different plant extracts on triglyceride concentration

Figure 4 shows that the diabetic untreated group had the highest triglyceride value. Other groups apart from the DMSO, upon the administration of the extracts (especially 250 mg/kg) had decreases in the triglyceride concentrations. There was significant ($p<0.05$) difference between the triglyceride concentration of the 100 mg/kg of *A. occidentale* group and that of the diabetic untreated group. However, there were no significant ($p>0.05$) differences between the triglyceride concentrations of *A. occidentale* (250 mg/kg) group, *E. globulus* (100 and 250 mg/kg) groups, *P. guajava* (100 and 250 mg/kg) groups, *X. aethiopica* (100 and 250 mg/kg) groups, *A. occidentale* + *X. aethiopica* (100 and 250 mg/kg) groups and glibenclamide group and that of the diabetic untreated group. Triglyceride concentration was significantly ($p<0.05$) reduced in group 4 when compared with those of groups 7, 13 and 14 whereas there were no significant ($p>0.05$) differences between the triglyceride concentrations of groups 1, 2, 5, 6, 8, 10, 11, 12 and 15 and that of group 4.

DISCUSSION

Results on the performances of the various extract on blood glucose concentration indicate that *A. occidentale* and *E. globulus* extracts effectively decreased blood glucose concentration upon their administration. This finding is in concert with the investigations of Kamtchouing et al. (1998) and Sokeng et al. (2001) who reported the hypoglycemic effect of *A. occidentale* aqueous leaf extract in streptozotocin-induced diabetic rats. Also, Tedong et al. (2007) showed that the hexane extract of *A. occidentale* significantly ($p<0.05$) decreased blood glucose concentration in diabetic rats. In addition, oral administration of *E. globulus* leaf extract to diabetic rats led to significant ($p<0.05$) reduction in the blood glucose concentration and restored liver glycogen to a high concentration (Soussi et al., 2009). The antihyperglycemic properties demonstrated by these plants in the present study may be attributed to the enhancement of glucose absorption by peripheral tissues.

Figures 3 and 4 indicate that the concentrations of total cholesterol and triglycerides of the diabetic rats were sig-

nificantly ($p<0.05$) increased when compared with those of the DMSO control group. However, administration/ treatment of the various plant extracts reversed these biochemical parameters. This study shows that there were greater reductions in the total cholesterol concentrations of the groups treated with *P. guajava* (100 and 250 mg/kg) when compared with that of the glibenclamide group. The overall performance of *P. guajava* in the reductions of blood lipids correlates with the findings of Deguchi and Miyazaki (2010) who reported that a single dose ingestion of *P. guajava* leaf tea for eight weeks resulted in decreases of serum concentrations of total cholesterol and triglycerides in hypercholesterolemia and hypertriglyceridemia subjects.

In conclusion, results of this study show that the chloroform extracts of the leaves of *P. guajava, A. occidentale and E. globulus* and fruits of *X. aethiopica* exhibited remarkable effects in restoring blood glucose concentration to normal and ameliorating diabetic complications.

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Full Length Research Paper

Qualitative trait loci analysis for seed yield and component traits in sunflower

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The present investigation was carried out to identify the molecular markers associated with various characters in sunflower using recombinant inbred lines. Linkage analysis was carried out and five linkage groups were obtained with 19 simple sequence repeats (SSR) markers. Linkage map construction, single marker analysis (SMA) and composite interval mapping analysis were carried out with SSR primers and quantitative traits. In SMA, out of 50 SSR markers, a total of 29 SSR markers were found to be significantly linked to various traits. The adjusted R2 for the regression equation varies from 3.2 to 29.8%. Two traits namely, days to flowering and seed color recorded above 20% R2 value. Hull weight recorded above 10% R2 value. In inclusive composite interval mapping (ICIM), the quantitative trait loci (QTL) analysis resulted into two QTLs namely, seed and volume weight. QTL analyses were performed through inclusive composite interval mapping (ICIM). The QTL analysis revealed each one QTL for traits namely, stripes on seed margin, stripes between seed margin, 100-seed weight and seed yield. The LOD ranged from 1.5 to 1.9. The adjusted R^2 value ranged 10.6 (seed yield) to 65.0 (stripes between seed margin) percent. Among these QTL, QTL on stripes on seed margin and stripes between seed margin may be considered as potential as they recorded very high phenotypic variation accounted. As the distance between the flanking marker is more than 5 cm, fine mapping of this QTL region with more markers may be attempted to utilize these QTL in the marker assisted back cross programme.

Key words: Sunflower, Simple sequence repeats (SSR), quantitative trait loci (QTL), hundred seed weight, stripes on seed margin, stripes between seed margin, seed yield.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is grown mostly as a source of vegetable oil and proteins in the world. Sunflower oil yield is determined by the product of seed yield per unit area and the oil percentage in the grain. Therefore, consideration of seed yield and oil content are important when breeding for high oil yield. Yield in sunflower, as in all other crops, depends on many characters, especially yield components which are controlled by several genes. Molecular markers in applied breeding programs facilitate the appropriate choice of parents for crosses to map or tag the gene blocks associated with economically important traits often termed as Quantitative Trait Loci (QTL). In the present research programme, attempts were made to identify QTL for various yield and yield component traits in sunflower. In the course of plant improvement, plant breeders deal with several qualitative traits.

However, the most difficult problem is the manipulation of metric traits with complex inheritance. Many strategies are available which rely upon the statistical analysis of field data to evaluate what has occurred on the genotypic level, but these inferences are often not precise as to the

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Table 1. Characteristics of parental lines.

Character	TNHSF239-68-1-1-1 (female)	17B (male)
Seed stripes	No stripes on the surface of seeds	Stripes present on surface, stripes strongly expressed in the margins
Pollen colour	Yellow	White
Seed colour	Black	Brown color
100 seed weight (g)	3.7	5.2
Hull weight/100 seeds (g)	1.5	2.5
Hull (%)	25	35
Oil content (%)	Very high (40 to 42%)	Very low < 33%

number of genes involved and their mode of action. Tracking polygenes with genetic markers can be traced back to the early 1920's when Sax (1923) reported the association of quantitatively inherited seed size with monogenes controlling seed coat pigmentation and pattern in bean (Dudley, 1993; Paterson and Tanksley, 1997). Effort to construct high-density linkage maps of molecular genetic polymorphism (marker loci) is currently underway for sunflower (Gentzbittel et al., 1995; Jan et al., 1998; Tang et al., 2002). Simple sequence repeats (SSR), also called microsatellites, are widely used as molecular markers. Their polymorphism has shown high efficiency and they are used for genetic mapping, population and evolutionary studies, as well as for finger-printing and pedigree analyses.

Statistical associations between alleles at molecular marker loci and alleles at quantitative trait loci (QTL) can be used to select indirectly, but with potentially very high accuracy, for DNA segments containing favourable QTL allele. This process effectively increasing the heritability of economically important agronomic characters such as yield, plant status and its components, quality traits, resistance and environmental stresses (Dudley, 1993; Paterson and Tanksley, 1997). QTL can be followed in a segregating population with the help of molecular markers. The selection for QTL using genetic markers can be effective if a significant association is found between the quantitative trait and the genetic markers and using these associations to develop improved lines or populations. QTL regions obtained from one population can later be introgressed into other varieties, which may be more suited for specific environments (Dudley, 1993). These studies helped to bring forth the potential of exploiting non-adapted and wild germplasm using backcross QTL analysis for the enhancement of elite crop varieties (MAB: marker-assisted backcrossing). The quick discovery and transfer of these QTL from non-adapted to adapted germplasm ultimately opens the door for the expansion of the genetic base of sunflower (Vischi et al., 2001).

Within the broad field of genomics, the QTL approach can be further validated or supported by other areas such as transcriptional profiling, physical mapping, and other functional genomics technologies (Alibert et al., 2001).

MATERIALS AND METHODS

Plant materials

Two sunflower inbred lines namely, TNHSF239-68-1-1-1 and 17B with significant differences (Table 1) for various traits namely, stripe on seeds surface (both on margin and between margins), seed color, hull weight and oil content were selected as female and male parent respectively to develop the $F_{2:5}$ population. These parents were crossed during June to October, 2009. The F_1 plants were raised during January to April, 2010 and confirmed with polymorphic SSRs. The $F_{2:5}$ population was raised during June to October, 2011 and leaf samples were collected to extract DNA.

Pheotypic data

Recombinant inbred lines of 94 in $F_{2:5}$ generation was used in the present study. The experiment was laid out with two replications in randomized block design (RBD) with a plot size of 2.4 m^2 with spacing of 60 × 30 cm during June to October, 2011 at Oilseeds Farm, Department of Oilseeds, Tamil Nadu Agricultural University, Coimbatore. Data were recorded on five out of seven plants were selected randomly in each replications for recording morphological traits namely, days to 50% flowering, plant height (cm), head diameter (cm), pollen color (score), stripes on seed margin (score), stripes between seed margin (score), seed color (score), volume weight (g/100 ml), 100- seed weight (g), hull weight (g/100 seed), kernel weight (g/100 seed), hull (%), oil content (%), seed yield (g/plant) and oil yield (g/plant). The oil content of the seeds was estimated by using Pelicon Soxoplus apparatus and expressed in percentage. The mean data from each replication were subjected to statistical analysis as per the standard method. The mean data over replication were used as phenotypic data for QTL analysis.

DNA isolation, SSRs and PCR condition

Genomic DNA of individual progenies and parents were extracted by CTAB method (Doyle and Doyle, 1987) and the quality was checked by using 0.8 % (w/v) agarose gel electrophoresis. A total of 156 SSRs were to study the parental polymorphism. The polymerase chain reaction (PCR) mixtures (5 µl) contained 10 ng template DNA, 1 X *Taq* Polymerase buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM of forward and reverse SSR primers and 0.03 IU of Taq polymerase. Amplification was performed in 0.2 ml well PCR plates (96 wells/plate) in a thermal cycler (Applied Biosystems). The samples were initially incubated at 94.0°C for 3 min and then subjected to 20 times of the following cycle: 94.0°C for 30 s, 63.0°C for 30 s (-0.5°C reduction per cycle) and 72.0°C for 1 min. This was followed by another 20 cycle of 94.0°C for 15 s, 55.0°C for 30 s and 72.0°C for 1 min. Final extension was 72.0°C for 10 min. Amplified

products were analyzed using 6% non denaturing polyacrylamide gel at constant current of 350 V for about 4 h and silver stained (Benbouza et al., 2006).

Statistical analyses

Mean and variability analysis

Plant breeders are commonly facing with problems of handling segregating populations and selection procedures. Mean and variability are the important factors for selection. Mean serves as a basis for eliminating undesirable crosses. Variability helps to choose a potential cross since variability indicates the extent of recombination for initiating effective selection procedures. Selection for the improvement of quantitative traits can be effective only when segregating generations posses the potential variability. The probability of obtaining superior lines can be worked out in early generations through the estimates of first and second order degree of statistics (Jinks and Pooni, 1976). The various genetic parameters like variability, GCV, PCV, heritability and genetic advance as percent mean were calculated by adopting the formulae given by Johnson et al. (1955). The software used for this study is TNAU Stat.

Single marker analysis

Single-marker analysis (also 'single-point analysis') is the simplest method for detecting QTLs associated with single markers. The statistical methods used for single-marker analysis include t-tests, analysis of variance (ANOVA) and linear regression. Linear regression is most commonly used because the coefficient of determination (R^2) from the marker explains the phenotypic variation arising from the QTL linked to the marker. This method does not require a complete linkage map and can be performed with basic statistical software programs. Markers were subjected to single marker analysis, to identify the marker trait association using simple regression analysis. The phenotypic mean and marker data were considered as dependent and independent respectively. The significant threshold for association of marker to the trait was set at $P \leq 0.05$ for single marker analysis. The adjusted R^2 value was used as percent of variance explained by the marker on the particular trait of interest.

Linkage and QTL analysis

Genotyping and phenotyping data obtained from the mapping population was subjected to Linkage analysis using QTL IciMapping software version 3.2 (Wang et al., 2012). Linkage groups were established using a minimum LOD score of 3.0, ordering by RECORD, rippled by SARF criterion with a window size of 5. The resultant linkage map was used to estimate the QTL using ICIM through the QTL ICI mapping software version 3.2. The QTL were estimated using ICIM-ADD mapping method, with mapping parameters of 1 cM step and 0.001 probabilities in stepwise regression. The LOD threshold used was 1.5 as manual input.

RESULTS AND DISCUSSION

Analysis of variance for various characters

Analysis of variance showed significant differences for all the characters except plant height and head diameter (Table 2). It indicates the presence of significant variability in the experimental materials. Burli et al. (2001) reported significant differences among the parents and crosses for days to 50% flowering. A significant difference for seed yield was reported by Mohan and Seetharam (2005) and Loganathan et al. (2006). Similarly, significant differences for 100-seed weight and oil content were reported by Ashok et al. (2000).

Mean and variability analyses

In the present study, mean and variability parameters were estimated for various traits and presented in Table 3. Among the traits, pollen colour, stripes on seed margin, stripes between seed margin, seed colour, hull weight, kernel weight, seed yield and oil yield recorded high coefficient of variation. Traits namely, days to 50% flowering, plant height and volume weight recorded low coefficient of variation. Parameters namely, skewness and kurtosis helps the breeder to understand the nature of distribution of individuals in the population. Among the traits, days to 50% flowering, head diameter, seed colour and hull weight showed positively skewed distribution which indicates the more proportion of individuals in the lower values. In case of kurtosis, head diameter, hull weight, seed yield and oil yield had lepto kurtic nature. Traits namely, pollen colour, stripes between margin and seed colour had platy kurtic nature and hence selection could be effective for these traits. All other traits recorded normal distribution.

The mean, GCV, PCV, heritability (broad sense) and GA as percentage of mean worked out for 15 characters and presented in Table 4. These result indicated that sufficient level of variability were observed for most of the traits in this population. Heritability value alone may mislead during selection. Therefore, heritability and genetic advance together should be taken into consideration for selection (Johnson et al., 1955). The range of heritability (in broad sense) was from 1.65% (plant height) to 80.14% (pollen colour). High heritability and high genetic advance as percentage of mean were recorded for the traits pollen color, stripes on margin, stripes between margins, seed color, hull weight, kernel weight and 100-seed weight. High heritability and high genetic advance as percentage of mean indicates the presence of additive gene action. Directional selection for these traits would be more effective for desired genetic improvement.

Polymorphism survey on parents

In the present study, TNHSF239-68-1-1-1 and 17B have been chosen as parents. These parents have differential phenotypes for surface stripe (both on margin and between margins), oil content and hull weight. This

Table 2. Analysis of variance of F5 progenies for various characters.

Source of variation	Degrees of freedom	Days to flowering	Plant height (cm)	Head diameter (cm)	Pollen color	Stripes on margin	Stripes between margin	Seed color	Volume weight (g/100 ml)
Treatment	142	9.12 **	241.99	3.97	0.44 **	1.05 **	1.02 **	5.04 **	30.45 **
Error	142	3.93	234.13	3.52	0.04	0.24	0.27	1.68	6.87
Total	285								

Source of variation	Degrees of freedom	100-seed weight (g)	Hull weight (g/100 seed)	Kernel weight (g/100 seed)	Hulling percentage	Oil content (%)	Seed yield (g/plant)	Oil yield (g/plant)
Treatment	142	1.69 **	0.21 **	1.08 **	51.88 **	54.97 **	171.19 **	23.28 **
Error	142	0.41	0.07	0.32	27.58	15.78	88.86	12.53
Total	285							

**Significant at 1% level.

population was made in an attempt to identify marker linked to these traits. The parents were surveyed with 156 SSR primers to assess the parental polymorphism. Among the 156 SSR primers studied, 50 (36%) were polymorphic between parents. These polymorphic primers were utilized for profiling the $F_{2:5}$ progenies.

Construction of linkage map

Genotyping was carried out on 94 F_5 progenies of the cross TNHSF239-68-1-1-1 × 17B with 50 polymorphic SSR markers. Linkage analysis was performed using QTL IciMapping software version 3.2 (Wang et al., 2012). Linkage groups were established using a minimum LOD score of 3.0, ordering by RECORD, rippled by SARF criterion with a window size of 5. A total of 5 linkage groups were obtained with a total of 19 markers (Figure 1). The first linkage group has six markers namely, ORS307, ORS677, ORS847, ORS727, ORS1245 and ORS1040. The second linkage group has four markers namely, ORS552, ORS959, ORS605 and ORS371. The third linkage group has four markers namely, ORS595, ORS1144, ORS537 and ORS1237. The fourth linkage group has three markers namely, ORS996, ORS707 and ORS1012 and fifth linkage group has two markers namely, ORS1017 and ORS799. The total length covered by these 19 markers is 145.48 cm with an average length of 7.65 cm.

Single marker analysis

Among the 141 F_5 individuals, only 94 individuals were subjected to determine the association of marker to the respective phenotype. The markers were subjected to single factor regression analysis using the marker (as independent) and the respective phenotype (as dependent) as suggested by (Sax, 1923). The significant regression value b indicating that the particular marker is linked to trait. The R^2 value is considered as the percent of variability of the traits explained by the marker. Among the 50 SSR markers, a total of 29 SSR markers were found to be linked to various traits (Table 5). The number of associated marker varies from six SSRS (head diameter and seed color) to one SSRS (stripe on margin and stripes between margins). The adjusted R^2 for the regression equation varies from 3.2 to 29.8%. Two traits namely days to flowering (ORS509) and seed colour (ORS533) recorded above 20% R^2 value. Hull weight (ORS785) recorded 11.5% R^2 value. Similar results were reported by Anandhan et al. (2010). The markers associated with these traits are of potential use in marker assisted backcross programme.

QTL analysis

Inclusive composite interval mapping (ICIM)

Genotyping and phenotyping data obtained were analyzed for mapping QTL by using the method inclusive composite interval mapping (ICIM) through QTL ICI mapping software version 3.2.

Table 3. Mean and variability parameters for seed yield and component traits in sunflower RILs.

Character	Mean	CV (%)	Skewness	Kurtosis	Minimum	Maximum
Days to 50% flowering	55.38	3.84	0.86 **	0.99	51.00	62.50
Plant height (cm)	137.99	7.42	-0.32	-0.07	110.95	160.18
Head diameter (cm)	12.61	12.50	2.59 **	23.08 **	6.06	23.23
Pollen colour	1.40	33.13	0.39	-1.72 **	1.00	2.00
Stripes on seed margin	2.01	37.01	0.04	-0.95	1.00	4.00
Stripes between seed margin	1.88	38.04	0.17	-1.09 *	1.00	3.50
Seed colour	5.45	29.50	-0.56 *	-1.19 *	2.00	7.00
Volume weight (g/100 ml)	37.37	9.63	-0.14	0.00	28.00	46.00
!00-seed weight (g)	4.81	19.25	0.12	-0.63	2.89	7.01
Hull weight (g/100 seed)	1.55	20.50	0.85 **	1.69 **	1.01	2.86
Kernel weight (g/100 seed)	3.26	22.70	0.08	-0.84	1.80	4.94
Hull (%)	32.60	15.36	0.35	-0.07	19.68	46.90
Oil content (%)	34.94	15.35	-0.27	0.09	19.01	48.97
Seed yield (g/plant)	23.29	35.83	0.84	1.44 **	6.75	55.70
Oil yield (g/plant)	8.13	39.95	1.05	1.90 **	2.45	21.08

Table 4. Estimates of variability and genetic parameters of F_5 progenies for various characters.

Character	Mean	PCV (%)	GCV (%)	Heritability (%)	GA (%)
Days to 50% flowering (days)	55.45	4.61	2.91	39.79	0.93
Plant height (cm)	137.21	11.25	1.44	1.65	0.38
Head diameter (cm)	12.55	15.42	3.79	6.05	0.24
Pollen color (score)	1.44	34.58	30.96	80.14	102.59
Stripes on margin (Score)	1.93	41.76	32.96	62.29	52.38
Stripes between margins (score)	1.83	44.08	33.36	57.28	48.84
Seed color (score)	5.55	33.06	23.33	49.83	13.05
Volume weight (g/ 100 ml)	37.34	11.57	9.2	63.18	2.77
100-seed weight (g)	4.72	21.78	16.96	60.63	20.59
Hull weight (g/ 100 seed)	1.49	25.39	17.71	48.63	46.95
Kernel weight (g/100 seed)	3.24	25.80	19.05	54.53	25.63
Hull percentage	31.87	19.78	10.94	30.58	1.09
Oil content (%)	34.93	17.03	12.67	55.39	2.43
Seed yield (g/plant)	23.93	47.64	26.81	31.66	1.53
Oil yield (g/plant)	8.34	50.76	27.81	30.01	4.06

The QTL analysis revealed each one QTL for traits namely, stripes on seed margin, stripes between seed margin, 100-seed weight and seed yield. The LOD ranged from 1.5 to 1.9. The adjusted R^2 value ranged 10.6 (seed yield) to 65.0 (stripes between seed margin) percent (Table 6). Tang et al. (2006) identified 40 QTL for 100-seed weight, kerneland pericarp weight and kernel to pericarp weight ratio in 14 DNA marker intervals on 10 linkage groups using composite interval mapping. With the foregoing discussion, it can be concluded that QTL on stripes on seed margin and stripes between seed margin may be considered as potential as they recorded very high phenotypic variation accounted. As the distance between the flanking marker is more than 5 cm, fine

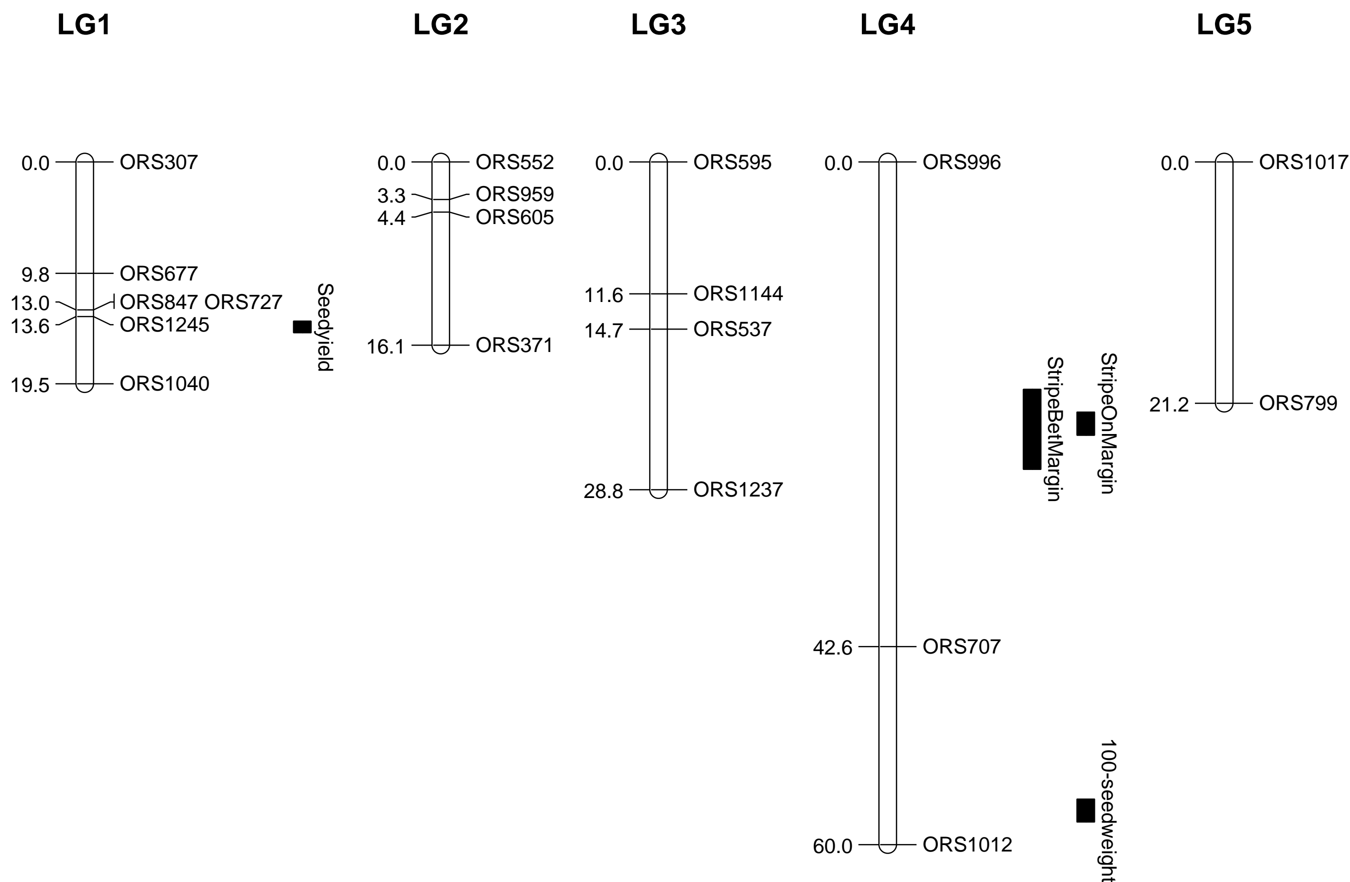

Figure 1. Genetic linkage map of sunflower and QTL position in the cross TNHSF239-68-1-1-1 × 17B.

Table 5. Single marker analysis for SSR primers and oil yield and yield components in the cross of TNHSF239-68-1-1-1 × 17B-1.

Character	Marker	Adjusted R^2 value (%)
Days to flowering	ORS502	7.8
	ORS509	21.8
	ORS533	4.8
	ORS799	8.5
Plant height	ORS613	4.1
	ORS878	3.4
	ORS1040	4.3
	ORS1144	4.3
Head diameter	ORS677	3.2
	ORS799	4.5
	ORS959	3.8
	ORS1040	3.7
	ORS1144	4.1
	ORS1245	4.2
Pollen color	ORS552	3.8
	ORS852	8.1
	ORS1237	3.8
Stripes on margin	ORS366	4.5
Stripes between margins	ORS1245	4.9

Table 5. Contd.

Seed color	ORS310	4.0
	ORS366	3.5
	ORS533	29.8
	ORS727	4.2
	ORS885	3.6
	ORS1037	10.0
Volume weight	ORS523	3.7
	ORS606	3.8
	ORS707	4.1
	ORS733	4.2
Hundred seed weight	ORS552	7.8
	ORS833	3.4
Hull weight	ORS502	4.0
	ORS552	5.5
	ORS785	11.5
	ORS885	4.9
	ORS1144	5.4
Kernel weight	ORS366	4.7
	ORS733	6.9
	ORS1017	3.6
	ORS1037	5.2
	ORS1065	4.8
Hull percentage	ORS833	4.6
	ORS1245	3.2
Oil content	ORS378	3.6
	ORS833	4.7
	ORS878	3.9
	ORS959	4.2
Seed Yield	ORS334	5.8
	ORS552	3.6
	ORS785	4.0
Oil yield	ORS334	3.8
	ORS503	3.2

Table 6. QTL analysis for various traits in sunflower.

Trait name	Chromosome	Position	Left marker	Right marker	LOD	PVE (%)	Add
Stripes on seed margin	4	23	ORS996	ORS707	1.5	54.6	-0.5
Stripes between seed margin	4	24	ORS996	ORS707	1.9	65.0	-0.6
100-seed weight (g)	4	57	ORS707	ORS1012	1.5	18.0	-0.4
Seed yield (g/plant)	1	14	ORS1245	ORS1040	1.52	10.6	2.77

mapping of this QTL region with more markers may be attempted to utilize these QTL in the marker assisted back cross programme.

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Review

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) and its usefulness in soil microbial ecological studies - A review

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The reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) is a highly specific polymerase chain reaction (PCR) method that allows one to detect very low transcription levels of functional gene(s) in soil. RT-qPCR helps us to know the active members of the microbial community, and their activities can be linked with other ecological processes in soil. If after the extraction of RNA from soil, the mRNA is converted to cDNA which is then sequenced, one would analyze directly the active members of the microbial community.

Key words: Complementary DNA (cDNA), messenger RNA (mRNA), reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), soil microbial study, microbial community.

INTRODUCTION

If one can see or know where microbes in soil live, what roles in soil processes they play and how their abundance and activity are influenced by soil physical and chemical properties, the soil will no longer be a black box. Molecular methods are evolving newer methods that help to answer questions like “who is the active member in the soil microbial community and where are their activities located”? Through DNA/DNA hybridization experiment, studies showed that one gram of soil contained more than 4,000 different genomes of bacteria (Torsvik et al., 1990). Most of the diversity was found in the fraction that could not be isolated and cultured by standard and sophisticated plating techniques. Thus, there is lack of knowledge on the unculturable portion of the microbial population in terms of who they are and the taxanomy, their contribution to nutrient and energy flow, soil respiration, gene transfer, degradation of pollutants, disease and quorum sensing, all of whose mechanisms have yet to be better understood. Researchers have developed new methods that allow the use of RNA extractions and manipulations to study microbial gene expression in the environment. One of such method is the reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). RT-qPCR is a combination of reverse transcription and quantitative PCR and is highly specific that allows one to detect very low transcription levels in soil (Wang and Brown, 1999). It allows for enumeration of the number of mRNA copies of a gene under defined environmental conditions. RT-qPCR involves total RNA extraction from soil or an environmental sample, followed by RNA purification and conversion to cDNA. The isolated RNA sample containing mRNA of the gene of interest is then subjected to optimized RT-qPCR conditions that allow for amplification of the gene of interest.

The presence of a gene is an indicator of potential gene

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Abbreviations: FRET, Fluorescence resonanace energy transfer; **TG,** target gene; **CG,** control gene; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **RT-qPCR,** reverse transcriptase-quantitative polymerase chain reaction.

expression, not an actual measurement of it. DNA analyses target not only active microorganisms, but also inactive microorganisms (Lindahl, 1993). Baelum et al. (2008) also demonstrated that analyzing soil microbial communities for a specific metabolic activity based on DNA sequences may be biased by detection of nonactive populations encoding homologous genes, but not actively expressing them. Measurement of mRNA (where tractable) is a far better indicator of *in situ* metabolic activity than measurement of the number of copies of the gene that are present in an environmental sample. There is an increasing need to investigate gene expression directly in soil since it can provide a more detailed understanding of the dynamics of the functional population, the activities of specific groups and the conditions required for induction of the activities in soil and devising optimal strategies for sustainable low-input farming and forestry.

Environmental studies based on functional gene transcripts are potentially complicated by many critical factors. Many processes proceed slowly in the environment due to a variety of environmental parameters and limiting factors and therefore require only low amounts of mRNA. Hence, it is important that the genes of interest are actually expressed to a detectable level, and studies of slow soil processes with correspondingly low transcription levels may always be limited. The ability to detect and quantify functional gene transcripts in a complex environmental matrix will become an important tool for microbial ecologists to link phylogeny to ecological function and should help to improve understanding of microbial processes in general. The objective of this review is to assess the importance of RT-qPCR in soil related studies and the extent RT qPCR can be used as a tool to "open" the black box of soil.

STUDYING OF GENE EXPRESSION IN SOIL

Total soil RNA must first be extracted from soil or from the environmental sample. Thus, a robust protocol for extraction of total RNA, (more specifically, nondegraded mRNA) is essential. However, only a small number of studies have reported successful analyses of mRNA isolated from soil or sediment (Fleming et al., 1998; Hurt et al., 2001; Mendum et al., 1998; Miskin et al., 1998; Ogram et al., 1995; Tsai et al., 1991). To obtain pure RNA from the initial total nucleic acid extracts, DNA must be selectively removed by combined use of DNase digestion and acid phenol extraction (Bornemann and Triplett, 1997). This method proved to be DNA free upon visual inspection after gel electrophoreses, however, positive amplification was observed in the control RT- qPCR not containing the reverse transcriptase, indicating that there was residual contamination with DNA (Burgmann et al., 2003). Replacing the acid phenol extraction step with heat inactivation of the added DNase resulted in extracts with the RT-qPCR procedure being frequently unsuccessful. Similarly, the use of commercially available spin columns for DNA removal from the initial extracts also resulted in unsatisfactory RNA clean up.

Later, Burgmann et al. (2003) extracted total RNA from soil based on optimized nucleic acid extraction with a highly efficient and fast bead beating method (Burgmann et al., 2001) with only small amounts of soil used (0.5 g). The extraction buffer allowed both soil DNA and total soil RNA isolation due to the nuclease (protein)-denaturing capacities of cethyltrimethyl ammoniac bromide (CTAB) and dithiothreitol (DTT) and rapid application of the phenol-chloroform extraction procedure (Cheung et al., 1994; Farrel, 1998). The extracted RNA was protected in phenol-chloroform-treated raw extracts kept at -80°C for four months. Detection of active members of natural soil microbial communities was feasible when the method of Burgmann et al. (2003) was used to extract soil RNA. Novinscak and Filion (2011) recommended that the Burgmann et al. (2003) protocol appeared to be the most robust and recommendable for extracting RNA and allowing microbial gene transcript quantification in soils varying in clay content. Later, Botero et al. (2005) increased the accessibility of prokaryotic RNA from environmental samples by modifying the purified RNA with the addition of a poly A tail. Subsequent amplification and cloning to create cDNA library followed by screening of the library revealed clones representing sequences from bacterial ribosomal RNA and mRNA.

Difficulties in extraction of RNA from soil

Some of the difficulties involved in the extraction of total RNA from soil have been the extraction of RNA at a sufficient yield and purity to allow for subsequent molecular analysis. This is challenging because soil contains substances that interfere with RNA extraction and subsequent downstream applications (England et al., 2001; England and Trevors, 2003; Sayler et al., 2001; Trevors, 1996). Clay particles and organic matter bind to nucleic acids, interfere with the extraction processes, thus reducing nucleic acid yields. Humic acids inhibit the enzymes used in RT-qPCR which are reverse transcripttase and DNA polymerase (Mendum et al., 1998). It has been suggested that humic acids consisting of polyphenolic substances are inhibitory because phenols bind to proteins by forming hydrogen bonds which could change the conformation of the enzyme (Kreader, 1996). Overcoming inhibition, challenges/limitations often requires trying different specialized soil extraction kits, modifying existing extraction procedures to optimize RNA yields and minimizing inhibitors, using small amounts of soil, trying different polymerases, using additives such as bovine serum albumin (BSA), using less template, diluting the inhibitors below levels that are not inhibitory or even devising a new or modified extraction purification method for the particular sample being researched and also using positive and negative controls to avoid the false positives.

Once extracted, the stability of RNA is affected by several factors including short half-life. mRNA has a short half life, prokaryotic mRNA half-life is an average of 1.3 min at 37°C (Arriano, 1993). Once mRNA is outside the cell, it is quickly degraded and is susceptible to RNases. Storage methods alter mRNA stability. Lypholization and storage at -20°C and storage in glycerol stocks at -80°C for soil conservation were equally effective methods of soil preservation for subsequent RNA isolation (Sessitsch et al., 2002).

Other difficulties involving the use of RNA from soil samples includes the designing of degenerate primer sets that captures a particular gene of interest from an entire community in a soil sample. This becomes more of a challenge when diverse bacterial groups carry out the same metabolic process of interest. The presence of genomic DNA contamination in the RNA sample is troublesome because the primers used in the PCR reaction can also bind to genomic DNA and result in inaccurate quantification result. Most of the studies have been conducted on nitrogen processes including N fixation, nitrification, denitrification among others. Almost no studies have been done on the other biogeochemical cycles in soil such as carbon and sulphur cylces.

Conversion of mRNA to cDNA

Small amounts of RNA converted into cDNA by reverse-transcription reaction could be amplified with the advent of PCR. Poly -T primers bind to the polyA-tail on the mRNA. Reverse transcriptase is the enzyme that synthesizes a strand of complementary DNA. Nucleotides that can be labeled or unlabelled are incorporated into the new cDNA molecule. After the reverse transcriptase has assembled the nucleotide into a cDNA molecule, the mRNA is degraded by RNase out. Different kits have been used for the reverse transcriptase reaction such as superscript II reverse transcriptase (Invitrogen, Parsley, United Kingdom) and Omniscript reverse transcription have been used by Freitag and Prosser (2009), Treusch et al. (2005), Baelium et al. (2008) and so forth. The produced cDNA copies are used as templates in a PCR reaction along with probes designed to amplify genes of interest. The cDNA pool from sample could be used to analyze tanscripts from other genes as well.

Real time polymerase chain reaction (PCR)

The cDNA produced can be used for real-time PCR analysis. Real- time PCR has an increased capability for quantifying the number of transcript copies present in the environmental sample. Real-time PCR integrates the amplification and analysis steps by monitoring the DNA produced during each PCR cycle. The focus on real time PCR is the logarithmic phase of product accumulation rather than the end point abundance of PCR product which is a more accurate estimate of the amount of transcripts obtained, since it is less affected by the amplification efficiency of the reaction or depletion of reagent (Gruntzig et al., 2001).

A typical PCR amplification plot shows the reaction going through exponential and linear phases in which the PCR is initially not limited by enzymatic activity or substrates. Eventually, the enzyme activity and /or the substrates (for example, dNTPs) become limiting or exhausted. At this point, the reaction reaches a plateau and theoretically, all of the samples will reach the same total amount of amplified DNA. This both obscures any difference in initial cDNA abundance and is also quite variable. While end point qPCR requires that PCR products be detected and quantified by gel electrophoresis after completion of the reaction, real-time qPCR technology allows quantification of PCR products in “real time” during each PCR cycle yielding a quantitative measurement of PCR products accumulated during the course of reaction. Real-time reactions are carried out in a thermocycler that permits measurement of a fluorescent detector molecule decreasing the PCR post-processing steps and minimizes experimental error. This is most commonly achieved through the use of fluorescence-based technologies including 1) Probe sequences that fluoresce upon hydrolysis (TaqMan, Applied Biosystems, Foster City, CA,USA) or hybridization (Light Cycler, Roche, Indianapolis, IN, USA); 2) fluorescent hairpins or 3) intercalating dyes (SYBR Green). These approaches require less RNA than the end point assays.

TaqMan chemistry uses the 5’-3’exonuclease activity of Taq DNA polymerase, which degrades a nonextendable fluorescent DNA probe following hybridization and extension in PCR reaction (Heid et al., 1996). TaqMan probes are labeled with both fluorescent reporter (FAM) and a fluorescent quencher (rhodamine (TAMARA) that are bound to the 5’ and 3’ ends of the probe sequence and fluorescence resonanace energy transfer (FRET) from the reporter to the quencher. Following annealing of the forward and the reverse primers to the target sequence, the TaqMan probe is designed to anneal between these primer sites.

If a product is present, the probe binds to the product and is degraded, hence the reporter remains unquenched. Probe hydrolysis results in desuppression of the reporter and a subsequent cumu-lative increase in fluorescence proportional to the amount of transcriptome present. This oligonucleotide primer/probe approach increases accuracy and specificity of PCR product detection due to the requirement for precise, gene-specific matching of three independent nucleotide sequences (Wang and Brown, 1999).

SYBR Green is an example of an intercalating dye that fluoresces upon binding to double-stranded DNA. During PCR, multiple molecules of SYBR Green bind to the product and emit a strong fluorescent signal that is easily detected. Inercalating dyes are inexpensive and simple to

use compared to sequence probes and because they are not sequence specific, they can be used for any reaction. Limitations of real-time PCR are similar to microarrays. A specific probe used in the amplification reactions may fail to capture the sequence diversity that is present in environmental populations. Humic acid, clay material and enzymatic inhibitors that co-extract with the RNA may interfere with the PCR reaction. Finally, the RNA extract from environmental samples is often not of sufficient yield to be representative of the soil microbial population.

Data analysis of real-time PCR reactions

Data can be analyzed by the use of the absolute and the relative methods. For absolute quantization (or the absolute method), an RNA standard curve of the gene of interest is required in order to calculate the number of copies. In this case, a serial dilution of a known amount (number of copies) of pure RNA is diluted and subjected to amplification. The unknown signal is compared with the curve so as to extrapolate the starting concentration. The major limitation of the absolute method is its inability to account for any procedure that may introduce inter - or intra sample variability (Bustin et al., 2005).

The relative quantification method ($2^{-\Delta\Delta CT}$ method) is commonly used to analyze data obtained from real time PCR reactions (Livak and Schmittgen, 2001). This method relies on two assumptions (a) the PCR reaction is occurring with 100% efficiency, in other words, with each cycle of PCR, the amount of product doubles. This assumption is also one of the reasons for using a low cycle number when the reaction is still in the exponential phase and substrates are not limiting and there is no degradation of products. This requires setting the crossing threshold (C_T) at the earliest cycle possible. The C_T is the number of cycles that it takes each reaction to reach an arbitrary amount of fluorescence. The second assumption of the $2^{-\Delta\Delta CT}$ method is that there is a gene /genes that are expressed at a constant level between the samples. This endogenous control will be used to correct for any difference in sample loading. The choice of the endogenous control is important. When C_T is known for a reaction, it can be used to generate the relative expression level.

Assuming that there are two samples which are the control and treated samples, hence we have the gene expression level of target sample (target gene- TG) and ii) endogenous gene expression level of control gene (control gene -CG). C_T value can be used to generate the relative gene expression level; C_T for the gene of interest (TG) is measured and C_T for control gene (CG) is also measured, and the difference between the C_Ts for the target gene and control gene is found,

$$C_T(TG) - C_T(CG) = \Delta C_T$$

$$-\Delta\Delta CT = \text{Control condition } \Delta C_T - \text{treated condition } \Delta C_T$$

Or

$$2^{-\Delta\Delta CT} = \text{Control condition } \Delta C_T - \text{treated condition } \Delta C_T$$

The negative value of this subtraction, the -ΔΔCT is used as the exponent of 2 in the equation and represents the difference in ‘corrected’ number of cycles to threshold.

Choice of endogenous controls

Intersample variation between biological and technical replicates can interfere with data analysis and therefore must be normalized to one or more endogenous control genes. Properly selected, the control genes will normalize differences in the amount and quality of starting material as well as in reaction efficiency. Normalization uses endogenous housekeeping or reference genes with the assumption that their expression is i) similar between all samples in a given study ii) is resistant to experimental controls iii) undergoes all steps of the PCR with the same kinetics as the target gene (Bustin, 2000; Thellin et al., 1999). Housekeeping genes such as β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin, or tubulin are commonly used since they are ubiquitously expressed in cells and tissues. Quantification of mRNA from functional genes might be normalized to transcripts of housekeeping gene that is constitutively and evenly transcribed during all growth phases and states of metabolic activity (Eleaume and Jabbouri, 2004; Johnson et al., 2005). However, no such gene has been suggested across all of prokaryotic diversity and such normalization would require extensive *a* prior knowledge of the suites of genes present in natural soil microorganisms which is currently not available (Baelium et al., 2008).

DeCoste et al. (2011) first used the human GAPDH gene transcripts as exogenous spike-in RNA in their experimental system. The choice and logic of using the GAPDH mRNA centered on the fact that it was highly characterized (Schmittegen and Zakrajsek, 2000), available in copious amounts from commercial sources and being of human origin, assumed to exhibit negligible cross-reactivity to coextracted soil components. However, the qRT-PCR results with the GAPDH mRNA exogenous spike-in were inconsistent and very difficult to interpret, possibly due to higher cross-reactivity than that expected of GAPDH primers/probes to coextracted nontarget sequences and other unexplainable factors. De Coste et al. (2011) later extracted total soil RNA and spiked in a defined quantity of *in vitro*-synthesized myIC RNA (synthetic RNA internal amplification control) for relative quantification and generated specific and reproducible results in their controlled exprememtal set up.

Baelium et al. (2008) normalized their data to quantify gene expression *in situ* by utilizing the ‘DNA equivalents’ which is described as mRNA:DNA. They believed that this general approach represented the most reliable norma-

lization protocol available to quantify gene expression *in situ* of environmental samples.

THE USE OF THE RT-qPCR AS A TOOL IN SOIL RELATED STUDIES

Devers et al. (2004) investigated the expression of atrazine degrading genes (*atz*) using real-time RT-PCR in *Pseudomonas* sp. strain ADP and *Chelatobacter heintzii*. Their results showed that all the atrazine degrading genes were expressed in *Pseudomonas* sp. while only *atzA* was basally expressed in *C. heintzii*. When atrazine was added to the test medium, *atz gene* expression increased in *Pseudomonas* sp., while in *C. heintaii*, only *atzA* and *atzB* were up regulated in response. The atrazine degradation rate was also two-fold lower for *C. heintzii*. This study indicates that the host microorganism may be a factor in determining the degree of gene expression under basal and stimulated conditions and this is an important consideration in bioremediation.

Based on functional gene DNA sequences, studies showed that in a soil dominated by bacteria carrying class I *tfdA* genes, only bacteria harboring class III *tfdA* genes were able to proliferate during the degradation of MCPA. However, in this DNA based study, it was not possible to tell whether the class I *tfdA* gene was actually expressed in the standing population of bacteria harboring this gene in the presence of MCPA or if these bacteria were inactive for MCPA degradation. Hence, methodologies to detect and quantify actual gene expression related to specific microbial function in soil are needed (Baelum et al., 2006).

RT-qPCR helps to link gene expression with a specific measurable microbial activity. Studies have been conducted to link gene expression with activity, but most of these were conducted on bacterial strains isolated from soil which necessitates that the strain be culturable (Tao et al., 1999; Devers et al., 2004). To better understand ecosystem processes, there is the need to identify and characterize environmental factors affecting gene expression and ultimately the activity of soil microorganisms. Also, linking gene expression with methods that detect the presence and activity of corresponding protein either by antibody-based techniques or biochemical tests would further enhance our understanding on ecosystem processes in soil.

CONCLUSION

Major strides to date have been made with the use of RT-qPCR in soil related studies. With the use of RT-qPCR, one will know the active members of the microbial community as far as functional genes are concerned. More insight will be obtained if after the extraction of total RNA from soil, the mRNA is converted to cDNA and then sequenced. The active microbial members, diversity index, evenness, abundance and species richness in the microbial community will be known. Thus, apart from knowing the abundance of the relative genes expressed, the biodiversity changes and the microbial community structure of the active population would be known. The use of mRNA provides information about the metabolic state at the moment of testing and if combined with analysis of rDNA, very detailed information may be obtained about the involvement of certain populations in a particular metabolic activity (Gottschal et al., 1997).

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Full Length Research Paper

Diversity analysis of sweet potato (*Ipomoea batatas* [L.] Lam) germplasm from Burkina Faso using morphological and simple sequence repeats markers

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Collecting and characterizing plant material has been basic for crop improvement, and diversity has long been seen as vital for rational management and use of crops. Thirty (30) morphological characters and thirty (30) simple sequence repeat (SSR) markers were used to assess the diversity among 112 sweet potato (*Ipomoea batatas* [L.] Lam) cultivars in Burkina Faso and to develop a core collection. Eight morphological characters were able to differentiate the 112 accessions and to identify 11 duplicates while 28 SSR markers were more informative in discriminating the accessions and to identify five duplicates. The diversity assessment using the two approaches revealed high diversity with a coefficient of 0.73 using the phenotypic data, while moderate diversity with a coefficient of 0.49 was obtained using the SSR markers. These results show no correlation between the two approaches (with dissimilarity index of 0.95). A core collection was constituted using the SSR based data while the eight discriminative phenotypic descriptors will be used in the identification of cultivars.

Key words: Accessions, genetic diversity, germplasm, molecular markers, morphological characters, simple sequence repeat, sweet potato.

INTRODUCTION

Sweet potato (*Ipomoea batatas* [L.] Lam), a hexaploid crop (2n = 6X = 90) is one of the most economically important crops in the world. In Burkina Faso, the major production areas are near the borders with Mali, Ghana,

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Abbreviations: SSR, Simple sequence repeat; **PCR,** polymerase chain reaction; **PIC,** polymorphic information content; **PT,** plant type; **GC,** ground cover; **VID,** vine internode diameter; **VIL,** vine internode length; **PVC,** predominant vine colour; **SVC,** secondary vine colour; **VTP,** vine tip pubescence; **GOL,** general outline of leaf; **LLN,** leaf lobes number; **LLT,** leaf lobes type; **MLS,** mature leaf size; **ALVP,** abaxial leaf vein pigmentation; **PL,** petiole length; **PP,** petiole pigmentation; **SCLL,** shape of central leaf lobe; **MLC,** mature leaf colour ; **ILC,** immature leaf colour; **FH,** flowering habit; **PSC,** predominant skin colour; **IPSC,** intensity of predominant skin colour; **SSC,** secondary skin colour; **PFC,** predominant flesh colour; **SFC,** secondary flesh colour; **DSFC,** distribution of secondary flesh colour; **SRF,** storage root formation; **SRS,** storage root shape; **LPSR,** latex production in storage roots; **OSR,** oxidation in storage roots; **SRSD,** storage root surface defects; **SRCT,** storage root cortex thickness; **UPGMA,** unweighted pair group method using arithmetic average.

Togo and Benin suggesting that important exchanges of planting material has occurred between these neighbouring countries. Cultivar names differ from one location to another, therefore placing limitations on accurate identification on locally available sweet potato germplasm that is vital to the rational management and use of the crop. Collection, characterization and maintenance of local germplasm are the bases of varietal improvement (Mok and Schmiediche, 1998).

Morphological characterization has been used extensively on various crop plants diversity assessments in many places of the world (Bos et al., 2000; Kaplan, 2001; Lacroix et al., 2005; Li et al., 2009; K'Opondo, 2011). Despite the environmental influences on plant morphology, this direct inexpensive and easy to use method of estimations was perceived as the strongest determinant of the agronomic value and taxonomic classification of plants (Li et al., 2009) and the first step in the assessment of plant diversity. On sweet potato, this tool has been used successfully to analyse genetic diversity necessary for the germplasm conservation, to reduce accession number by identification and elimination of duplicates and to enhance crop breeding (Huaman, 1992; Mok and Schmiediche, 1998; Tairo et al., 2008; Li et al., 2009; Karuri et al., 2009; Yada et al., 2010a).

According to La Bonte (2002), when trait expression is environmentally unstable or difficult to evaluate, molecular markers become more useful than traditional phenotypic evaluations. During the last decade a lot of molecular information has been accumulated and used for genetic diversity assessment on sweet potato germplasm (Jarret et al., 1992; Kowyama et al., 1992; Jarret and Austin, 1994; Bruckner, 2004; Tseng et al., 2002; Hu et al., 2003; He et al., 2006; He et al., 2007; Soegianto et al., 2011). The most widely used molecular marker procedures for population genetic analysis of both animals and plants during the past few years are the simple sequence repeat (SSR) markers or microsatellites (Shih et al., 2002; Veasey et al., 2008; Zhang et al, 2001; Karuri et al., 2010; Yada et al, 2010b; Li et al., 2009) (Weising et al., 1995). These markers are highly polymorphic, co-dominant, and can easily be detected on high-resolution gels.

Limited success has been achieved with morphological diversity analysis alone (Yada et al., 2010a). Therefore, to optimize the characterization efficiency, morphological characterization has now been combined with molecular techniques. SSR markers have been used in combination with morphological descriptors to analyse genetic diversity in sweet potato germplasm and useful core collections have been developed using this combination (Li et al., 2009; Karuri et al., 2010).

The objective of this research was to quantify the diversity in sweet potato germplasm collected in Burkina Faso using morphological descriptors and SSR molecular markers.

MATERIALS AND METHODS

Collection of plant materials

One hundred and forty-four (140) sweet potato accessions (Table 1) were collected from December 2008 to January 2009 and January 2010 from the main production areas located in the Cascades, Western, Central-West, Southern, Central-South, Central-East and Eastern regions of Burkina Faso using the method described by Huaman (1991). One hundred and seven (107) accessions survived and were maintained at the INERA research station of Kamboinse located in the centre of the country in the Soudanian zone characterized by an annual rainfall ranged from 600 to 1100 mm. Three varieties introduced from the International Potato Center (CIP) East Africa CIP-440001 (known as Resisto), CIP-199062-1 and TIB-440060, one from China (TN-Leo) and Tiebele-2 an orange fleshed sweet potato of unknown origin were added and used as control.

Morphological characterization

The experiment

The 112 accessions were grown at the INERA station of Kamboinsé during the rainy season, from July to October 2009. Based on the records of the first year, the experiment was replicated from July to October 2010 and the materials were planted in groups of relatedness to allow further morphological comparisons between those accessions which were morphologically alike. Planting was done on ridges of 3 m long with distance between ridges of 1 m. On each ridge, 11 cuttings were planted at a spacing of 30 cm. The fields were maintained by frequent weeding. NPK (14-23-14) fertilizer was applied 21 days after planting when the cuttings were well established. Additional watering was done by irrigation to complement rainfall.

Data collection

Morphological data were collected 60 days after planting based on the average of three measurements from the middle portion of the main stem as recommended by Huaman (1992). Qualitative characters were scored using a scale of 0 to 9. The following variables were scored: Plant growth characteristics: plant type (PT), ground cover (GC); mature vine characteristics: vine internode diameter (VID), vine internode length (VIL), predominant vine colour (PVC), secondary vine colour (SVC), vine tip pubescence (VTP); mature leaf characteristics: general outline of leaf (GOL), leaf lobes number (LLN), leaf lobes type (LLT), mature leaf size (MLS), abaxial leaf vein pigmentation (ALVP), petiole length (PL), petiole pigmentation (PP), shape of central leaf lobe (SCLL), mature leaf colour (MLC), immature leaf colour (ILC); flowering habit (FH); Storage root characteristics: predominant skin colour (PSC), intensity of predominant skin colour (IPSC), secondary skin colour (SSC), predominant flesh colour (PFC), secondary flesh colour (SFC), distribution of secondary flesh colour (DSFC), storage root formation (SRF), storage root shape (SRS), latex production in storage roots (LPSR), oxidation in storage roots (OSR), storage root surface defects (SRSD), storage root cortex thickness (SRCT). Measurements were done on three plants chosen randomly from the 11 plants per plot and averaged for the variable.

Data analysis

The computer program Genstat 14th edition was used to analyse the morphological data. Stepwise discriminant analysis was

Table 1. List of accessions collected in Burkina Fasoand the varieties introduced used for the characterisation.

Code	Name	Site	Number	Code	Name	Ssite	Number	Code	Name	Site
BF1	Unknown	Koubri	38	BF49	Dagouam	Mantiagogo	75	BF93	Massakoun-Gbeman	Beregadougou
BF2	Unknown	Koubri	39	BF51	Bagre	Tiebele/Tigalo	76	BF94	Unknown	Banfora
BF3	Unknown	Koubri	40	BF52	Unknown	Garango	77	BF95	Wosso-Gbe 2	Sourou
BF4	NangnouNoondo	Koubri	41	BF53	Unknown	Garango	78	BF97	Diabo Local	Diabo
BF7	Unknown	Koubri	42	BF54	Unknown	Garango	79	BF98	Garango	Diabo
BF8	Unknown	Koubri	43	BF55	Unknown	Garango	80	BF99	Sawiyague	Lo-Longo
BF9	Gelwango	Tingandgo	44	BF56	Unknown	Garango	81	BF100	NalougourouNono	Tiebele
BF10	Tiébélé	Tingandgo	45	BF57	Unknown	Maoda	82	BF108	Bobo rouge	Reo
BF11	Patate	Tingandgo	46	BF58	Unknown	Maoda	83	BF112	ShiraJaa	Reo
BF12	Saafaré	Tingandgo	47	BF59	Nakalbo	Koupela	84	BF114	Dayejopouri	Goundi
BF13	Tiébélé	Tingandgo	48	BF60	Unknown	Koupela	85	BF115	Dayepoan	Goundi
BF14	Jaune 2	Kombissiri	49	BF61	Unknown	Koupela	86	BF116	Kokonetioulou	Poun
BF15	Patate	Kombissiri	50	BF62	Unknown	Maoda	87	BF117	Dayebioun	Poun
BF16	Bananbato	Kombissiri	51	BF63	Fandaga	Badara	88	BF119	Dayepouan	Poun
BF17	Saafaréblanc	Kombissiri	52	BF64	Wosso	Badara	89	BF120	Dayebioun	Poun
BF18	Saafaré rose	Kombissiri	53	BF65	Unknown	Badara	90	BF126	Zimien-botouhin	Mboa
BF19	Jaune 1	Kombissiri	54	BF66	Unknown	Badara	91	BF127	Zipo-kouka	Mboa
BF20	Nayiré	Yale	55	BF67	Unknown	Badara	92	BF128	Zipo-botouhin	Mboa
BF21	Nayiré	Yale	56	BF68	Unknown	Oradara	93	BF129	Zimien-kouka	Mboa
BF23	Nayi-mina	Sagalo	57	BF71	Denbaya	Oradara	94	BF130	Ziro-dodobo	Mboa
BF24	Nayir-vapapao	Sagalo	58	BF72	Fardan-wouleman	Oradara	95	BF131	Nagnou-pla	Komsaya
BF25	Nayir-sian	Sagalo	59	BF74	Wosso-Gbe	Sourou	96	BF132	Nagnou-ziè	Komsaya
BF27	Nayir-po	Leo	60	BF75	Djakani	Sourou	97	BF133	Unknown	CREAF
BF32	Kabakourou	Leo	61	BF77	Gambagre	Sikorla	98	BF135	Nankansongo	Lolongo
BF33	Nayir-papao	Sissili	62	BF78	Badara	Sikorla	99	BF136	Nankanpongo	Lolongo
BF34	Kabakourou	Sissili	63	BF80	Massako-fing	Sikorla	100	BF137	Iloropongo	Lolongo
BF35	Nayir-manan	Sissili	64	BF81	Massoko 2	Sikorla	101	BF138	Nayoumondo-1	Kombissiri
BF36	Nayir-mian	Sissili	65	BF82	Bagayogo	Sikorla	102	BF139	Nayournondo-2	Kombissiri
BF38	Unknown	Kombissiri	66	BF83	Massakoun-Gnin	Sitiena	103	BF140	Djacané	Sarkandiara
BF40	Unknown	Kombissiri	67	BF85	Massakoun 2	Sitiena	104	BF141	Sèguè-Bana	Sarkandiara
BF41	Unknown	Kombissiri	68	BF86	Massakoun-Plaa	Kiribina	105	BF142	Ouagnougui	Gonsin
BF42	Nankan-poupiou	Lo	69	BF87	Wosso-Gbe	Banfora	106	BF144	Unknown	Sikorla

Table 1. Contd.

BF43	Nankan-pongo	Lo	70	BF88	Fandaga-Woule	Banfora	107	BF145	Unknown	Ouagadougou
BF44	Nankan-soungo	Lo	71	BF89	Fandaga-Gbeman	Banfora	108	TN.LEO	TN.LEO	Introduced
BF45	BinagaNapouni	Mantiagogo	72	BF90	Wosso-Woule	Banfora	109	CIP-199062-1	CIP 199062-1	Introduced
BF46	Nanlougourou	Mantiagogo	73	BF91	Wosso-Woule	Banfora	110	TIB-440060	TIB	Introduced
BF47	Manga	Mantiagogo	74	BF92	Massakoun-Woule 2	Beregadougou	111	TIEBELE.2	TIEBELE.2	Tiebele/Tigalo
							112	CIP-440001	Resisto	Introduced

performed to select a subset of variables that best discriminate among the classes. The Wilks' Lamda criterion was used to measure the variable contribution to the discriminatory power of the model as described by Daulfrey (1976); least contribution leads to removal of the variable.

The significant level of retaining or adding a discriminative variable was 0.15. Subsequently, principal component analysis was applied to examine the structure of the correlations between variables. The null hypothesis that any r_{ij} was equal to zero was tested by computing the ratio of the explained variance to the unexplained variance. The eigenvalues and eigenvectors of the correlation matrix were derived, and the eigenvectors scaled by the square root of the corresponding eigenvalues to produce the matrix of component loadings. The eigenvalues and their associated eigenvectors, the correlation matrix are used to reduce the number of variables in the statistical analyses (Daulfrey, 1976).

A graphical display of the genetic relationships was also computed by principal coordinate analysis using the Rogers Tanimoto dissimilarity index of DARwin5.0.158 software. Cluster analyses were performed to group observations together using the method of Euclidian distance. Data points with the smaller distances between them were grouped together. A dendrogram was plotted from these computed clusters as a graphical relationship among accessions. From the dendrogram duplicates, samples were identified as a result of complete similarity between accessions.

Molecular characterization

Leaf sampling procedure

Leaf sampling was done as recommend by the DNALandmarks, a Canadian biotechnology laboratory, where the molecular work was done. Using 96-wells blocks, two leaf discs of 5 mm diameter were harvested from young leaves of each accession using a whole paper punch and put into a specific well position.The block was then placed inside a plastic bag with 50 g of silica gel and kept for 24 h to dry.

DNA extraction and SSR amplification

DNA extraction and amplification were done using an internal protocol at DNALandmarks laboratory in Canada. After extraction, the quality of the DNA was tested on 1% agarose gel. The DNA samples were then diluted to 25 ng/ul. The diluted DNA samples were then used for polymerase chain reaction (PCR) amplification with 30 SSR markers which sequences were provided by the International Potato Center (Table 2). PCR reactions were performed following an internal protocol of DNALandmarks with minor modifications (Ghislain et al., 2009). Forward primers were tailed with a M13 primer and the M13 primer (CACGACGTTGTAAAACGAC) labelled with one of the four fluorescence dyes (6FAM, PET, NED or VIC) for multiplexed PCR products detection using the ABI3730xl apparatus. The PCR conditions consisted of an initial denaturation at 95°C for 15 min, annealing at 60°C for 1 min and 72°C followed by 35 cycles of 94°C for 1 min, annealing at 60°C for 1 min, and 72°C for 1 min. This was followed by a final extension step of 20 min at 72°C and a halt at 4°C. The allele sizes were scored using GeneMapper software. Multiple peaks were detected due to the polyploidy nature of sweet potato. Any peak with the peak height greater than one sixth of the highest peak was scored. Allele size was calculated by subtracting 19 (M13 primer length) from the peak size. The raw data were provided for the further analysis. Failed samples were repeated one to two times.

Data analysis

The polymorphic information content (PIC) that is the importance of each SSR marker in distinguishing between accessions was determined (Weir, 1996) as:

$$PIC = 1 - \sum P_i^2$$

Where, P_i is the frequency of the ith allele.

Each SSR fragment was treated as binary matrix in which band presence was coded as present or absent by 1 and 0, respectively. Based on the binary matrix, Jaccard'sdissimilarity index was computed as follows. A graphical display of the genetic relationships was also computed by principal coordinate analysis. Subsequently,

Table 2. The 30 SSR primers used for the genotyping of the 112 sweet potatoaccessions.

Marker	Primer sequences from client	Forward_primer with M13 tailed *
IbL16_F	GTCTTGCTGGATACGTAGAACA	cacgacgttgtaaaacgacGTCTTGCTGGATACGTAGAACA
IbL16_R	GGGAGAAGTAAGAGAACCGATA	-
IbL32_F	GGGATGAAGGAGAGAATGAGTA	cacgacgttgtaaaacgacGGGATGAAGGAGAGAATGAGTA
IbL32_R	TTGAAAACCTAGAGAGAAAGGG	-
IbL46_F	CTGAAATTAGGGATTGAAGAGG	cacgacgttgtaaaacgacCTGAAATTAGGGATTGAAGAGG
IbL46_R	TCCAATCACTCCTTGTTTTCTC	-
IbO2_F	TGTGGATCTGTTCTTTGAACC	cacgacgttgtaaaacgacTGTGGATCTGTTCTTTGAACC
IbO2_R	TTCCATGTGGAGTGTGAAGTAT	-
IBS100_F	TGCTATAGTTACGTGGACGAAG	cacgacgttgtaaaacgacTGCTATAGTTACGTGGACGAAG
IBS100_R	TTTAATGCTGATGTGGATGC	-
IBS12_F	CAGTTATCAATTCCCACCTACC	cacgacgttgtaaaacgacCAGTTATCAATTCCCACCTACC
IBS12_R	TTGCTGTGTTATAGGCTTTGTC	-
IBS134_F	CTTCAATCACCTGAAACTCTGA	cacgacgttgtaaaacgacCTTCAATCACCTGAAACTCTGA
IBS134_R	AATATCGCTATGTTCTTGGGaC	-
IBS137_F	TcAACAGACGTCTTCACTTACC	cacgacgttgtaaaacgacTcAACAGACGTCTTCACTTACC
IBS137_R	TCGATAGTATGATGTGAATCGC	-
IBS139_F	CTATGACACTtCTGAGAGGCAA	cacgacgttgtaaaacgacCTATGACACTtCTGAGAGGCAA
IBS139_R	AGCCTTCTTGTTAGTTTCAAGC	-
IBS144_F	TCGAACGCTTTCTACACTCTT	cacgacgttgtaaaacgacTCGAACGCTTTCTACACTCTT
IBS144_R	CTGTGTTTATAGTCTCTGGCGA	-
IBS147_F	TGTGTACATGAGTTTGGTTGTG	cacgacgttgtaaaacgacTGTGTACATGAGTTTGGTTGTG
IBS147_R	GAAGTGCAACTAGGAAACATGA	-
IBS156_F	TTGATTCCACTATGACTTGAGC	cacgacgttgtaaaacgacTTGATTCCACTATGACTTGAGC
IBS156_R	ACACCAACCCTTATATGCTTTC	-
IBS166_F	TCCGTCTTTCTTCTTCTTCTTC	cacgacgttgtaaaacgacTCCGTCTTTCTTCTTCTTCTTC
IBS166_R	ATACACTAACTGCATCCAAACG	-
IBS18_F	GCCAAGGATGAAGGATATAGAa	cacgacgttgtaaaacgacGCCAAGGATGAAGGATATAGAa
IBS18_R	ACAAcCAAACTAGCTAAAAGCC	-
IBS19_F	TCCTATGAGTGCCCTAAGAATC	cacgacgttgtaaaacgacTCCTATGAGTGCCCTAAGAATC
IBS19_R	CTCCTTCGTCTTCTTCTTcTTC	-
IBS199_F	TAACTAGGTTGCAGTGGTTTGT	cacgacgttgtaaaacgacTAACTAGGTTGCAGTGGTTTGT
IBS199_R	ATAGGTCCATATACAATGCCAG	-
IBS24_F	AGTGCAACCATTGTAATAGCAG	cacgacgttgtaaaacgacAGTGCAACCATTGTAATAGCAG
IBS24_R	TCCTTTCtTcATCATGCACtAc	-
IBS33_F	ATCTCTtCATACcAATCGgAaC	cacgacgttgtaaaacgacATCTCTtCATACcAATCGgAaC
IBS33_R	CaATgaTAGCGGAGATTGAAG	-
IBS72_F	CTACTCTCTGCTGGTTTATCCC	cacgacgttgtaaaacgacCTACTCTCTGCTGGTTTATCCC
IBS72_R	CTAGTGGTCTCTCTTCCTCCAC	-
IBS82_F	GACATAATTTGTGGGTTTAGGG	cacgacgttgtaaaacgacGACATAATTTGTGGGTTTAGGG
IBS82_R	GAAATGGCAGAATGAGTAAGG	-
IBS84_F	CAAAGATGAAGCAAGTAAGCAG	cacgacgttgtaaaacgacCAAAGATGAAGCAAGTAAGCAG
IBS84_R	ACTAATGTTGATCTACGGACCC	-
IBS85_F	AACTACTCATGGGGAGAACAAC	cacgacgttgtaaaacgacAACTACTCATGGGGAGAACAAC
IBS85_R	CTAACGAAAGTTTGGACATCTG	-
IBS86_F	AGAAACTGAAAACTAAGCTCGC	cacgacgttgtaaaacgacAGAAACTGAAAACTAAGCTCGC
IBS86_R	GCTATGCGTTTACAGAAACAAG	-
IBS97_F	GTTACCAGGAATTACGAACGAT	cacgacgttgtaaaacgacGTTACCAGGAATTACGAACGAT
IBS97_R	CTCTCTACAAAAACTCACAGCG	-
IbU13_F	GCAACCAATCTACAGCAAACTA	cacgacgttgtaaaacgacGCAACCAATCTACAGCAAACTA
IbU13_R	CAGATAAAGTCCCCATTTCTTC	-
IbU20_F	GGAGAGCAAGTGGAGAAAGTAT	cacgacgttgtaaaacgacGGAGAGCAAGTGGAGAAAGTAT

Table 2. Contd.

IbU20_R	ACTCCTAGACCCACAATTGAAC	-
IbU31_F	CCGCAGAAAAAGTTCAGATT	cacgacgttgtaaaacgacCCGCAGAAAAAGTTCAGATT
IbU31_R	GCAACTTTTCTTCTTCCGTAAC	-
IbU33_F	TTTGAAGAAGATGAGAGCGAC	cacgacgttgtaaaacgacTTTGAAGAAGATGAGAGCGAC
IbU33_R	TCAGAAAGACGATACACTAGAGAGA	-
IbU4_F	GGCTGGATTCTTCATATTTAGC	cacgacgttgtaaaacgacGGCTGGATTCTTCATATTTAGC
IbU4_R	GCTTAATGGATCAGTAACACGA	-
IbU6_F	GGGGTAGAGAGAAGAGAGTGAC	cacgacgttgtaaaacgacGGGGTAGAGAGAAGAGAGTGAC
IbU6_R	CCAGGTGAGAGTGTCTTTCAA	-

Table 3. Selected morphological characters by The STEPDISC procedure.

Step	Entered	Partial R-square	F value	Pr> F	Wilks' Lambda	Pr< Lambda	Average squared canonical correlation	Pr> ASCC
1	Predominant Flesh Color (PFC)	0.8498	299.81	<.0001	0.15022095	<.0001	0.42488952	<.0001
2	Leaf Lobe Number (LLN)	0.4128	36.90	<.0001	0.08821579	<.0001	0.62429365	<.0001
3	Leaf Lobe Type (LLT)	0.1204	7.12	0.0013	0.07759628	<.0001	0.65429960	<.0001
4	Mature Leaf Size (MLS)	0.1035	5.94	0.0036	0.06956707	<.0001	0.66236509	<.0001
5	Vine Tip Pubescence (VTP)	0.0711	3.91	0.0232	0.06461809	<.0001	0.67647456	<.0001
6	Storage Root Surface Defects (SRSD)	0.0525	2.80	0.0655	0.06122257	<.0001	0.68154041	<.0001
7	Petiole Pigmentation (PP)	0.0514	2.71	0.0716	0.05807721	<.0001	0.69485966	<.0001
8	Storage Root Formation (SRF)	0.0508	2.65	0.0759	0.05512967	<.0001	0.69785323	<.0001

Number of observation =109, Variables in the analysis=30, Class level=3, Significance level to enter=0.15, Significance level to stay=0.15.

a dendrogram was generated with the unweighted pair group method using arithmetic average (UPGMA) algorithm of DARwin5.0.158 software (Perrier et al., 2003 and Perrier and Jacquemoud-Collet, 2006).

RESULTS AND DISCUSSION

Morphological characterization

Discriminant analysis

Eight morphological traits with sufficient discriminative power to differentiate the accessions were identified based on their significant p-value forWilk's Lambda (P < 0.0001) and p-values for the average squared canonical correlations (P <0.0001) (Table 3). These were: PFC, LLN and LLT (Figure 3), MLS, VTP, SRSD, PP and SRF. The correlation matrices from Table 4 shows that these eight descriptors were not correlated with one another; this therefore indicates that using them will not create redundancy in the measurements. The F values revealed that the PFC and the LLN, respectively, with 299.81 and 36.90 had the greatest discriminating power associated with highly significant F values. Among the 22 variables discarded were the PSC commonly used by farmers to identify cultivars; the FH very important in breeding and other visible traits such as PT, MLC, ILC, GOL, and PVC.

Principal component analysis

Four principal components (PC) were identified which accounted for 67.22% of the total variation among the accessions (Table 5).The first PC accounted for 23.08% whereas the second, the third and the forth PC axes accounted respectively for 18.08, 13.32 and 12.73%. The first PC with reference to its high loadings (Table 6) was positively associated with traits such as leaf lobe number and predominant flesh colour. The second PC was associated with storage root characteristics (predominant flesh colour, storage root surface defects); the third with leaf characteristics (mature leaf size and petiole pigmentation) as well as with storage root formation, while the forth was associated with traits related to stems (leaf lobe type, petiole pigmentation and vine tip pubescence).

Cluster analysis

From the hierarchical cluster analysis, leaf lobe number, leaf lobe type, petiole pigmentation, vine tip pubescence, predominant flesh colour, storage root formation, storage surface defectand storage root surface defect showed a high polymorphism of 0.75 within the 112 sweet potato accessions (Figure 1).

Table 4. Correlation matrix for the 8 morphological traits used to distinguish the 112 sweet potato accessions.

Parameter	VTP	LLN	LLT	MLS	PP	PFC	SRF
Vine tip pubescence (VTP)							
Leaf lobe number (LLN)	0.1666						
Leaf lobe type (LLT)	0.0035	0.1039					
Mature leaf size (MLS)	0.2159	0.1699	-0.1856				
Petiole pigmentation (PP)	-0.0089	-0.2091	0.0254	0.0133			
Predominant flesh color (PFC)	0.1763	0.2690	0.0976	0.1906	-0.1685		
Storage root formation (SRF)	-0.2387	-0.2462	0.1031	-0.0850	0.0980	-0.0117	
Storage root surface defects (SRSD)	0.0157	0.2267	0.1249	-0.0096	-0.1464	0.2728	0.1879

Table 5. Eigenvalues of the correlation matrix**.**

Eigen values	Difference	Proportion	Cumulative
1.84627182	0.39948563	0.2308	0.2308
1.44678619	0.38093347	0.1808	0.4116
1.06585272	0.04728026	0.1332	0.5449
1.01857246	0.26427255	0.1273	0.6722
0.75429991	0.04126424	0.0943	0.7665
0.71303567	0.06889746	0.0891	0.8556
0.64413821	0.13309520	0.0805	0.9361
0.51104301		0.0639	1.0000

Table 6. Eigenvectors from the eight principal component axes used to classified the 112 sweet potato accessions.

Parameter	Prin1	Prin2	Prin3	Prin4	Prin5	Prin6	Prin7	Prin8
Vine tip pubescence (VTP)	0.366	-.311	0.090	0.433	-.641	0.248	0.240	0.208
Leaf lobe number (LLN)	0.528	0.038	-.231	0.030	0.564	0.177	0.125	0.548
Leaf lobe type (LLT)	0.058	0.448	-.271	0.650	0.085	-.386	0.275	-.262
Mature leaf size (MLS)	0.313	-.343	0.575	-.074	0.298	-.309	0.395	-.328
Petiole pigmentation (PP)	-.306	-.137	0.413	0.601	0.362	0.314	-.351	0.050
Predominant flesh color (PFC)	0.484	0.209	0.248	0.022	-.162	-.397	-.686	0.075
Storage root formation (SRF)	-.255	0.498	0.512	-.084	-.129	-.127	0.316	0.534
Storage root surface defects (SRSD)	0.306	0.522	0.201	-.121	-.017	0.625	0.038	-.432
Eigen value	1.846	1.447	1.066	1.019	0.755	0.713	0.644	0.511
% Variation	23.08	18.08	13.32	12.73	9.43	8.91	8.05	6.39
Cumulative %	23.08	41.16	54.49	67.22	76.65	85.56	93.61	100

The accessions were grouped into eleven (11) clusters based on their average linkage and the Euclidean test. Clusters IV, VIII, IX and XI can be considered as outliers as they contained only one accession each, BF90, BF120, BF81 and BF137, respectively. Cluster I consisted of 37 accessions, cluster II had 11 accessions, cluster III had 10 accessions, cluster V of 6 accessions, cluster VI and VII had 21 accessions each, whereas cluster X had two accessions. Cluster II and cluster III were entirely constituted by orange fleshed accessions mostly with three leaf lobes, while the other clusters did not show any distinguishable relationship or pattern. The three East African OFSPs:Resisto (CIP 440001) belonged to cluster II while CIP-199062-1 and TIB-440060 belonged to cluster III. Cluster I was associated mostly with accessions with yellow flesh and a leaf lobe number of nine except for BF16 and BF42 which had 13 and 11 leaf lobes, respectively. Cluster V was constituted by accessions with white flesh and seven leaf lobes, cluster VI had individuals characterized by white flesh and one leaf lobe while cluster VII had white flesh with a very divergent number of leaf lobes ranging from one to

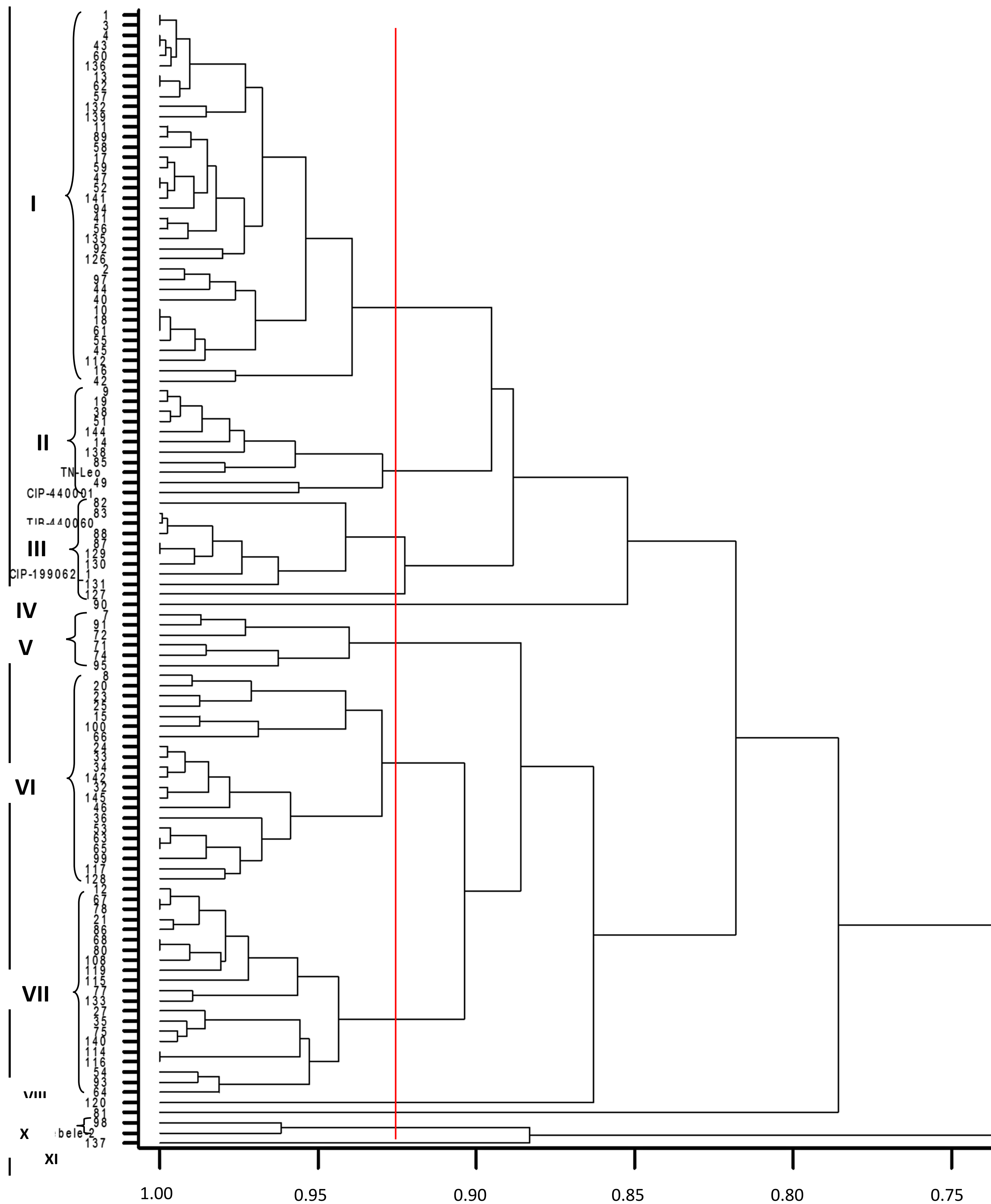

Figure 1. Dendrogram of the 112 sweet potato accessions revealed by average linkage cluster analysis based on the eight discriminant phenotypic characters.

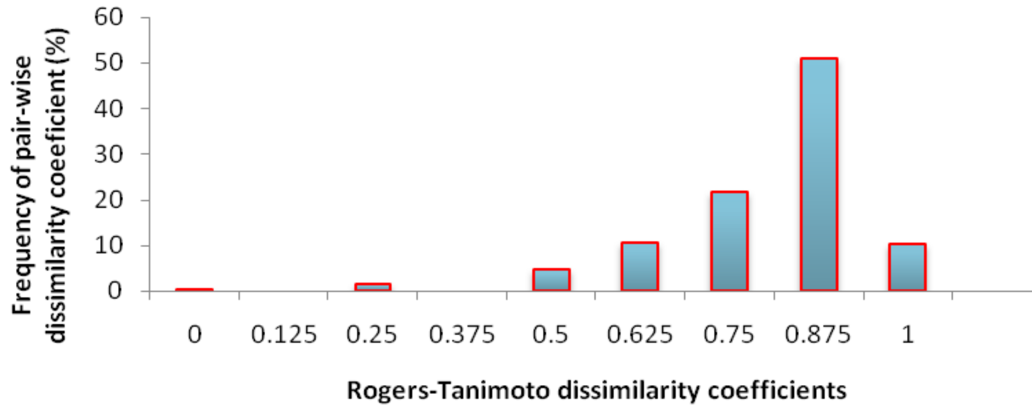

Figure 2. Rogers-Tanimoto Dissimilarity index using: Mean = 0.73, Min value = 0, Max value = 1 (DARwin5.0.158).

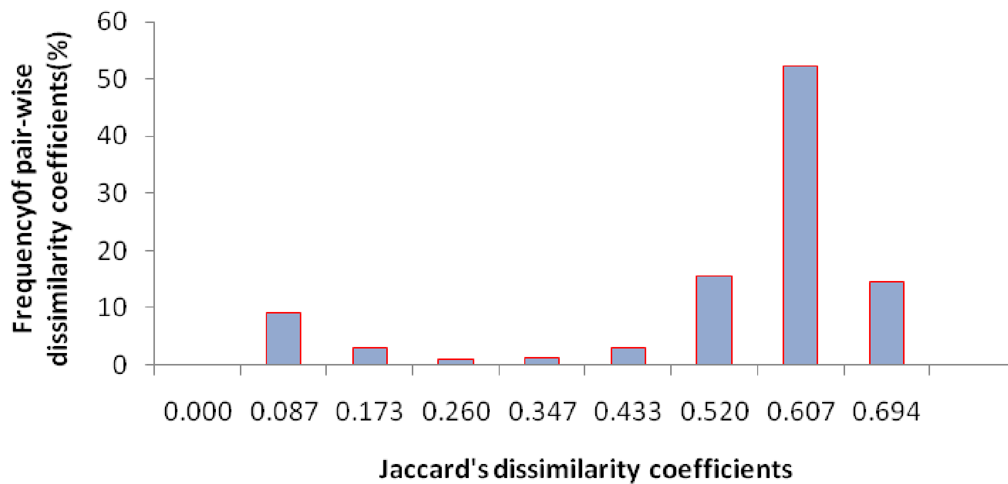

Figure 3. Jaccard's dissimilarity index using: Mean = 0.49, Min value = 0, Max value = 0.69 (DARwin5.0.158).

five with most of the accessions having five leaf lobes. The Rogers-Tanimoto pairwise dissimilarity coefficients computed as single and modality data using DARwin 5.0.158 revealed a dissimilarity index ranging from 0 to 1 with an average value of 0.73 (Figure 2) suggesting a very high diversity among these 112 accessions. Most of accessions had dissimilarity indices ranging from 0.75 to 0.875 explaining 72.51% of the total frequency of dissimilarity with a maximum pair-wise dissimilarity of 1.

Identification of duplicates

From the hierarchical cluster analysis (Figure 4), duplicates were identified. Accessions BF1 and BF3 from two close villages in the central region were identical. Accession BF13 from the central south was identical to accession BF62 from the Eastern region; two accessions BF78 and BF67 from the "Hauts-Bassins" region were identical as well as accessionsBF129 from the "Hauts-Bassins" and BF87 from the "Cascades". BF80 and BF68 from the Hauts-Bassins were also identical as were BF65 and BF63 from the same region. BF10 and BF18 from the central south and BF61 from the Eastern region were also identical. BF116 and BF114 from the Central west were morphologically identical as were BF52 and BF47 from the Central South.

Molecular characterization

Number of alleles detected

Among the 30 SSR markers, 27 were detected between one to six alleles while the remaining three markers detected between seven to eight alleles.

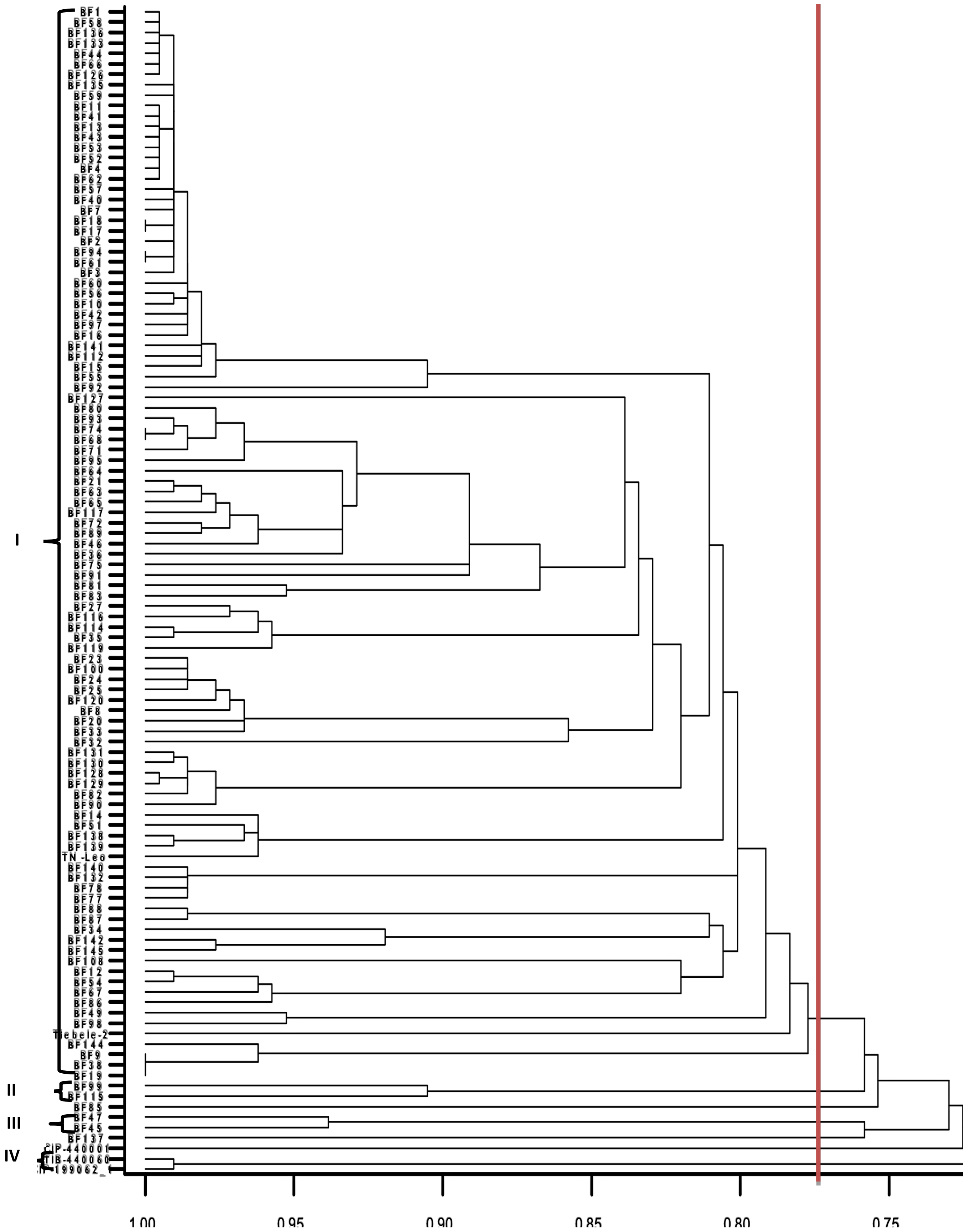

Figure 4. SSR UPGMA based dendrogram of 112 sweetpotato accessions from Burkina Faso.

Table 7. Markers, number of alleles per locus, total number of alleles and PIC for 30 SSR.

Marker	Number of alleles per locus	Total alleles	PIC
IBL16	8	232	0.715
IbL32	5	374	0.762
IbL46	7	252	0.713
IbO2	10	644	0.881
IBS12	8	267	0.782
IBS18	7	324	0.774
IBS19	7	322	0.796
IBS24	7	329	0.795
IBS33	5	313	0.734
IBS72	4	253	0.746
IBS82	7	382	0.764
IBS84	6	433	0.789
IBS85	8	240	0.776
IBS86	7	301	0.726
IBS97	7	346	0.744
IBS100	6	347	0.771
IBS134	4	287	0.708
IBS137	7	337	0.781
IBS139	12	431	0.873
IBS144	8	374	0.812
IBS147	8	336	0.788
IBS156	6	133	0.283
IBS166	10	232	0.739
IBS199	12	441	0.841
IbU4	9	370	0.813
IbU6	8	395	0.819
IbU13	6	333	0.786
IbU20	1	111	0.000
IbU31	4	135	0.547
IbU33	7	329	0.763
Mean			0.727

The SSR marker IbO2 detected one to six alleles from 61 samples, seven alleles from 48 samples and eight alleles from three samples. The markers IBS139 and IBS199 detected one to six alleles each from 111 samples and seven alleles from one sample. The samples BF32 and BF99 showed between seven to eight alleles by the three SSR markers.

Polymorphic information content (PIC)

The thirty SSR markers revealed the usefulness of a marker in distinguishing between accessions with PIC values ranging from 0.00 for IbU20 to 0.881 for IbO2with an average of 0.727 (Table 7). Except for two SSR markers that had PIC values lower than 0.50 (IbU20 with 0 and IBS156 with 0.283), twenty eight (28) markers had high power of polymorphism (PIC>0.50). The high PIC values observed in this study indicated that the twenty eight SSR markers used were informative.

Genetic dissimilarity analyses and identification of duplicates

The frequency of pair-wise dissimilarity coefficients of the 112 sweet potato accessions based on the Jaccard's coefficient is shown in Figure 3. These SSR-basedpair-wise dissimilarity coefficients ranged from 0 to 0.69 with a mean of 0.49 suggesting a relatively moderate diversity among the 112 sweet potato accessions. Most of the dissimilarity coefficients were between 0.52 and 0.69 explaining 82.35% of the total frequency.

Nine accessions were identified with a pair-wise

dissimilarity of 0 and therefore were considered as duplicates. This observation is confirmed by the dendrogram (Figure 4) generated using the unweighted pair group method (UPGMA). Thus, BF61 and BF94 with yellow flesh which were collected from“Cascades” and the Central-East region, respectively, were genetically identical; BF17 and BF18, two yellow fleshed accessions collected in two different communities in the Bazega province (Central-South region), were identical. BF38, BF19 and BF9, three orange fleshed accessions from the Bazega province, were also identical and different from the OFSP introduced from CIP-Eastern Africa.BF74 and BF68, with white flesh from the Kenedougou province, constituted a unique accession. After removing the duplicates, the initial number of 112 accessions was reduced to 107. These 107 sweet potato accessions will constitute a national core collection of sweet potato germplasm.

Comparison between morphological and SSR data

Using the morphological characters, the 112 accessions were grouped into 11 clusters with dissimilarity indices ranging from 0 to 1 with a mean of 0.73 suggesting a very high genetic diversity among the accessions. The use of the morphological data reduced the number of accessions from 112 to 101. Conversely, using the SSR-based analysis, 7 clusters were obtained.The dissimilarity indices ranged from 0 to 0.69 with a mean of 0.49, therefore, showing a relatively moderate diversity among the 112 accessions. The accession numbers were reduced from 112 to 107 using SSR markers. The accessions BF87 and BF88; BF63 and BF65; BF114 and BF116 identified as group of duplicates by morphological descriptors were closely related (nested on the dendrogram) using the SSR markers. Except for the groups of duplicates BF47 and BF52; BF67 and BF78 that were seen far away by the SSR markers, the other morphologically duplicates accessions belonged to the same molecular cluster.

In the other side, the duplicates identified using the SSR marker procedure BF17 and BF18 belonged to the same morphological cluster, as did BF94 and BF61. The duplicates BF74 and BF68 were seen morphologically far away, while BF9, BF19 and BF38 identified as the same accessions by the molecular procedure were found nested closely on the morphological dendrogram. The consensus between the morphological and the molecular based treeswas performed by using the strict rule consensus method consisting of simple counts of the frequency of occurrence of clusters in the set of trees (Perrier Perrier and Jacquemoud-Collet, 2006). It was observed that between the two trees, 4.7% of the clusters were in agreement. This weak consensus between the two trees suggested that there was no correlation between the morphological and the molecular data.

The Quartet tree distance estimate used as a measure of dissimilarity between the two trees was 0.95 demonstrating the absence of correlation between the two approaches used in the genetic diversity estimation.

DISCUSSION

The high diversity (mean of 0.73) detected within the 112 accessions regarding dissimilarity coefficient values suggests that the sweet potato accessions used in the current work would be a good source of selection for sweet potato breeding materials. Diversity studies have been done on sweet potato using morphological descriptors in various parts of the world and similarities or differences have been ascribed to sample size, number and type of descriptors used, the origin of accessions and the method of analysis. Using forty morphological descriptors in Uganda on 1256 accessions, 20 discriminatory descriptors were identified (Yada et al., 2010a). These 20 descriptors contained seven of the eight descriptors identified in this present study. Predo-minant skin color, commonly used in identification of cultivars in farmers’ fields in Burkina Faso was not useful in differentiation among the accessions. Contrary to the results of this present work, Yada et al. (2010a) found this descriptor as discriminatory. In Kenya, Karuri et al. (2010) identified two descriptors (general outline of leaf, and, the shape of central leaf lobe) that differentiated among 89 accessions and separated them into two clusters. Karuri et al. (2010) found in agreement with the results of the current work, that flower habit was not significantly discriminative. High diversity index was also observed in a population of sweet potato in Kenya (Karuri et al., 2010), Uganda and India (Vimala and Hariprakash, 2011) using morphological traits. However, Tairo et al. (2008) observed lowdiversity of 0.52 among 280 sweet potato accessions in Tanzania.

Considering that SSR-based data are more accurate than the morphological data, the moderate diversity obtained in this study suggests that high priority should be given to further collect and/or introduce divergent materials, since variation in the collections is needed for a successful breeding program. Results from similar studies using SSR markers in sweet potato diversity analysis have been reported and most of the differences in results have been ascribed to sample size, the number of SSR markers used and the source of materials. Moderate genetic diversity values have been reported in Uganda (Yada et al., 2010b) among 192 accessions using 10 SSR markers; Gichuru et al. (2006) also reported low diversity in East African sweet potato cultivars while Soegianto et al., (2011) in Java reported similarity ranging from 15 to 78% between Indonesian accessions. Considering Eastern Africa as the second zone of diversity of sweet potato after the Central America (Villordon et al., 2007), one would expect a high

diversity. The reason for the low diversity has been attributed to narrow geographic zone of collection of the cultivars. High SSR-based diversity has been noticed by Veasyet al. (2008) in Brazil, in Taiwan by Shih et al. (2002) and in China by Li et al. (2009) where the Jaccard's coefficient of similarity ranging from 0.400 to 0.938 was observed.

The weak agreement between the morphological based tree and the SSR based tree was also confirmed by different duplicates identified by each of these approaches. The findings of the present study are in agreement with those of Karuri et al. (2010) in Kenya who compared morphological and SSR-based evaluation of diversity.

A low correlation of -0.05 was observed between the two data sets. Further studies have reported low correlation between morphological and molecular markers in many crops (Koehler-Santos et al., 2003; Ferriol et al., 2004; Bushehri et al., 2005). The suggested reasons were that it could be a result of the independent nature of morphological and molecular variations. According to Vieira et al. (2007), this low correlation could also be due to the fact that a large portion of variation detected by molecular markers is non-adaptive as compared with phenotypic characters, which are influenced by the environment. The core collection obtained using the SSR markers' approach will be used for breeding purposes but the identified eight phenotypic characters will be used for the physical identification of the cultivars within the core collection.

Conclusion

Findings of the present study reveal that sweet potato germplasm in Burkina Faso presented moderate to high diversity based on molecular and phenotypic assessment approaches. The results obtained will serve as a guide for the basis germplasm management and improvement in the Burkina Faso and in the Sahelian zone of West Africa. However, further diversity is needed that can be achieved through introduction or more collection. The power of eight morphological descriptors and 28 SSR markers in the differentiation of cultivars was identified and could be useful in subsequent studies. Despite the poor correlation between morphological and molecular markers, both techniques can be use defectively in sweet potato characterization. The constitution of core collection will be done based on the SSR based data, but the eight phenotypic characters will be useful in distinguishing the cultivars in the field.

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Full Length Research Paper

Ashwagandha (*Withania somnifera* L. Dunal) crop as affected by the application of farm yard manure (FYM) and inorganic phosphorus in typic Torripsamment of Hisar

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The vegetative parameters of ashwagandha (viz. plant height, number of primary branches, plant spread and dry weight of shoot) were enhanced significantly with the application of 12.5 mg P_2O_5 kg^{-1} soil, whereas; dry weight of roots was enhanced up to the application level of 25 mg P_2O_5 kg^{-1} soil. FYM at the rate of 12.5 t ha^{-1} in combination with 12.5 mg P_2O_5 kg^{-1} soil significantly improved all the vegetative parameters, whereas, FYM at the rate of 12.5 t ha^{-1} in combination with 25 mg P_2O_5 kg^{-1} soil significantly enhanced the dry weight of the roots. The alkaloids yield (mg pot^{-1}) in ashwagandha roots increased significantly with the application of 25 mg P_2O_5 kg^{-1} soil. The application of FYM at the rate of 12.5 t ha^{-1} improved the alkaloids yield (mg pot^{-1}) but the significantly highest yield of total alkaloids (mg pot^{-1}) was found in the treatment combination of 12.5 t FYM ha^{-1} + inorganic-P at the rate of 25 mg P_2O_5 kg^{-1} soil as compared with other eleven treatments. Nutrients (NPK) uptake by ashwagandha shoot increased significantly with the application level of 12.5 mg P_2O_5 kg^{-1} soil over control whereas in case of ashwagandha roots, the increase in nutrients uptake at the level of 25 mg P_2O_5 kg^{-1} soil over control. Application of FYM at the rate of 12.5 t ha^{-1} + 12.5 mg P_2O_5 kg^{-1} soil was the best treatment combination for ashwagandha roots in terms of nutrients uptake. Application of fertilizer-P significantly improved the status of organic carbon, Available-P in post harvest soil but decreased the Available-N, Available-K and DTPA extractable micronutrients (Fe, Cu, Zn and Mn) up to the level of 25 mg P_2O_5 kg^{-1} soil. FYM at the rate of 12.5 t ha^{-1} helped in maintaining the soil fertility status after harvest of the crop alone or in combination with fertilizer-P.

Key words: Ashwagandha, phosphorus, farmyard manure, alkaloids, nutrients uptake, soil fertility.

INTRODUCTION

Ashwagandha (*Withania somnifera* L. Dunal), commonly called 'Winter cherry', is a dryland medicinal crop having tremendous marketing potential owing to demand of its roots to the tune of 7000 tonnes and estimated production of 1500 tonnes (Umadevi et al., 2012). Ashwagandha root drug find an important place in Ayurveda (Khanna et al., 2006; Kulkarni and Dhir, 2008) for the treatment of rheumatic pain, inflammation of joints, nervous disorders, impotence and immature ageing and is considered as 'Indian ginseng' (Khanna et al., 2006; Kulkarni and Dhir, 2008). It belongs to the family 'Solanaceae' and is native to Indian subcontinent. The plant grows erect to a height of 35-75 cm having small green-coloured flowers and orange-red ripe fruit. The past studies have concentrated

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Table 1. Effect of FYM and inorganic phosphorus on growth parameters and yield of alkaloids.

FYM levels (t/ha)	Plant height (cm)				Overall Mean	No. of primary branches				Overall Mean	Plant spread (cm^2)				Overall Mean	Dry weight (g) of roots (dry weight of shoots in g given in bracket)				Overall Mean	Yield of alkaloids (mg pot^{-1})				Overall Mean
	P levels (kg ha^{-1})					P levels (kg ha^{-1})					P levels (kg ha^{-1})					P levels (kg ha^{-1})					P levels (kg ha^{-1})				
	P0	P12.5	P25	P37.5		P0	P12.5	P25	P37.5		P0	P12.5	P25	P37.5		P0	P12.5	P25	P37.5		P0	P12.5	P25	P37.5	
F0	27.6	31.4	33.2	34.6	31.7	1.4	1.9	2.3	2.5	2.0	124.7	191.1	232.3	248.8	199.2	2.97 (4.28)	3.58 (4.97)	3.74 (5.43)	3.86 (5.47)	3.43 (5.04)	9.8	13.6	15.3	16.6	13.8
F12.5	29.4	31.6	35.5	36.1	33.1	1.6	2.1	2.4	2.6	2.2	145.3	221.7	243.3	260.1	217.6	3.50 (5.04)	3.92 (5.36)	3.96 (5.58)	4.03 (5.66)	3.79 (5.38)	12.9	16.1	17.8	18.9	16.4
F37.5	29.7	33.8	36.2	36.8	34.1	1.7	2.2	2.5	2.6	2.3	162.9	243.3	248.8	265.9	230.2	3.69 (5.23)	4.02 (5.52)	4.05 (5.77)	4.08 (5.79)	3.92 (5.55)	14.0	16.9	19.0	20.4	17.6
Overall mean	28.9	32.3	35.0	35.8		1.6	2.1	2.4	2.6		144.3	218.7	241.5	258.3		3.39 (4.85)	3.84 (5.28)	3.92 (5.59)	3.99 (5.64)		12.2	16.9	17.4	18.6	

CD, p=0.05; Plant height (cm): Phosphorus = 6.9, FYM = 3.4, phosphorus X FYM = 6.1; No. of primary branches: phosphorus = 3.6, FYM = 1.3, phosphorus X FYM = N.S; plant spread (cm^2): phosphorus = 12.3, FYM = 9.6, phosphorus X FYM = 17.2; dry weight (g) of roots (dry weight of shoots in g given in bracket): phosphorus = 0.08(0.43), FYM = 0.14(0.31), phosphorus X FYM = 0.16(N.S.); yield of alkaloids (mg pot^{-1}): phosphorus = 1.8, FYM = 1.4, phosphorus X FYM = 3.5.

on root growth and biochemical composition in different accessions. There is limited literature available on nutritional studies of ashwagandha with respect to its vegetative and quality parameters. The yield and quality of any crop heavily relies on the knowledge of nutritional requirement and its supply accordingly. Keeping this in view, the present study was an attempt to study the effect of inorganic source of nutrients alone and in combination with farm yard manure (FYM) on plant growth parameters, alkaloids yield, nutrients uptake and post-harvest soil nutrient status under ashwagandha.

MATERIALS AND METHODS

A pot experiment was conducted to study the response of graded levels of P in combination with FYM on plant growth parameters and medicinal quality of ashwagandha (*Withania somnifera* L. Dunal, cv. JA 20) at screen-house, Department of Soil Science, CCS Haryana Agricultural University, Hisar (29°05′ N, 75°38′ E, 222m elevation). The soil (Typic Torripsamment) used was loamy sand in texture, pH 8.0, EC (1:2) 0.34 dS m^{-1}, Organic Carbon 0.26 %, Available N, P, K were 112.6, 12.0 and 192.0 kg ha^{-1} respectively. Available (DTPA extractable) Zn, Cu, Fe and Mn were 0.34, 0.26, 4.05 and 6.87 mg kg^{-1} respectively. The O.C (%), total NPK in FYM were 38.4, 0.87, 0.29 and 0.73%, respectively. The treatment combination comprised of four levels of phosphorus (0, 12.5, 25 and 37.5 mg P_2O_5 kg^{-1} soil) applied through KH_2PO_4 and three levels of FYM (0, 12.5 and 25 t ha^{-1}). Experimental data was statistically analyzed by two factor completely randomized design using ANOVA with three replications by using OPSTAT. Basal doses of nitrogen, potassium and zinc were applied through urea, KCl and $ZnSO_4$ solutions. Nitrogen was applied in two splits, that is, half at the time of sowing and another half at 21 days after sowing. 5 kg soil was placed in each earthen pot after giving respective treatments on a polythene sheet. The crop was harvested after 120 days of sowing and observations were recorded separately in each pot. All the samples were analyzed by following standard laboratory procedures. Total alkaloids (%) in ashwagandha were determined by extraction with chloroform as described by Rajpal (2002).

RESULTS AND DISCUSSION

Effect on growth parameters

It can be seen from Table 1 that application of fertilizer-P at the rate of 12.5, 25 and 37.5 mg kg^{-1} soil resulted in increase in plant height by 11.8, 21.1 and 23.9% over control. Though P at all levels increased the plant height but the increase was significant up to application level of 12.5 mg P_2O_5 kg^{-1} soil (32.3cm) over control (28.9 cm). Application of FYM also helped in increasing the height of ashwagandha at all levels but the significant increase of 4.4% was observed at level of 12.5 t ha^{-1} over control. Wafaa et al. (2006) also reported almost similar results. Phosphorus fertilizer along with FYM resulted in increased plant height at all levels of FYM up to 12.5 mg P_2O_5 kg^{-1} soil. Aishwath (2004) also reported increase in plant height by application of phosphorus fertilizer.

Data also indicated that number of primary branches increased significantly with 12.5 mg P_2O_5 kg^{-1} soil (2.1) to the extent of 31.3% over control (1.6). However the increase between that of 25 mg P_2O_5 kg^{-1} soil (2.4) and 37.5 mg P_2O_5 kg^{-1} soil (2.6) was found to be non-significant. Kothari et al. (2005) also reported increase in number of branches by application of P-fertilizer. FYM at the rate of 12.5 t ha^{-1} (2.2) also increased the average number of primary branches over control (2.0) but this increase (10.0% over control) was on a par with that of FYM at the rate of 25 t ha^{-1} (2.3, that is, 15.0% over control). Somnath et al. (2005)

ported significant increase in yield attributing characters by application of FYM.

A perusal of the data (Table 1) indicated that increasing level of P increased the plant spread by 51.6, 67.4 and 79.0% over control by application of 12.5, 25 and 37.5 mg P_2O_5 kg^{-1} soil. Almost similar results were reported by Kothari et al. (2005) and Somnath et al. (2005). FYM helped in increase of plant spread at all levels, that is, 12.5 and 25 t ha^{-1} (217.6 and 230.2 cm^2 respectively) over control (199.2 cm^2). Almost similar results were reported by Somnath et al. (2005). Inorganic phosphorus applied along with FYM also indicated the positive effect on plant spread and the interaction effect was significant up to 12.5 mg P_2O_5 kg^{-1} soil at all levels of FYM application. Our results are in agreement with that of Joy et al. (2005) and Wafaa et al. (2006).

Data also indicated that application of fertilizer-P at the rate of 12.5, 25 and 37.5 mg P_2O_5 kg^{-1} soil which resulted in mean dry weight of ashwagandha roots to the extent of 13.3, 15.6 and 17.7% increase over control. It could be observed that there was progressive increase in the dry weight of ashwagandha roots by increasing the rate of phosphorus application; however, the increase was significant up to application level of 25 mg P_2O_5 kg^{-1} soil. Kothari et al. (2005) and Somnath et al. (2005) also reported increased dry weight by application of phosphorus fertilizer. FYM at the rate of 25 t ha^{-1} (3.92 g pot^{-1}) significantly increased the average fresh weight of roots over control (3.43 g pot^{-1}) and the extent of increase was 14.3%. Along with phosphorus, 12.5 t FYM ha^{-1} was found to be the appropriate dose for increase in dry weight of roots significantly over control. Chauhan et al. (2005) and Joy et al. (2005) also reported increase in dry matter by application of FYM.

A critical study of the data (Table 1) revealed that phosphorus application up to 12.5 P_2O_5 kg^{-1} soil enhanced the dry weight of ashwagandha shoot to 5.28 g and the corresponding increase was 8.9% over control. This increase was on a par with increase by application levels of 25 and 37.5 kg^{-1} P_2O_5 soil. Pratibha and Korwar (2005) also reported increase in biomass by application of phosphorus fertilizer. FYM at all levels increased the dry weight of shoot but the significant increase was observed up to application of FYM at the rate of 12.5 t ha^{-1} over control (6.7%). Positive effect on plant growth parameters had been demonstrated by Qian and Schoenau (2010), Tantawy et al. (2010) and Wenyi et al. (2011) after application of inorganic phosphorus and by Raafat et al. (2011), Raja et al. (2011) and Singh et al. (2011) after application of FYM. However, the extent of effect varies depending upon soil, fertilizer level and crop.

Effect on yield of alkaloids

Data (Table 1) also indicated that application of phosphorus at the rate of 12.5 mg P_2O_5 kg^{-1} soil significantly increased the alkaloids yield (mg pot^{-1}) of ashwagandha roots up to 38.5% over control. Balakumbahan et al. (2005) and Kolodziej et al. (2009) also reported almost similar results of increase in alkaloids by application of phosphorus fertilizer. FYM at the rate of 12.5 t ha^{-1} increased the alkaloids yield up to 18.8% over control. Kumar (2011) and Lakshmi et al. (2011) showed improvement in quality of crops by application of FYM.

Effect on nutrients uptake by shoot and roots of ashwagandha

N uptake by shoot increased significantly by application of fertilizer-P (Table 2) and the maximum average increase was 23.8% by application of 37.5 mg P_2O_5 kg^{-1} soil (103.0 mg pot^{-1}) over control. Application of FYM at the rate of 12.5 and 25 t ha^{-1} increased the N uptake by 8.8 and 14.1% respectively, over control and the increase was on a par between 12.5 (96.5 mg pot^{-1}) and 25 t ha^{-1} (101.2 mg pot^{-1}). Patil et al. (2005) also reported that supplementing nitrogen promoted the uptake of nitrogen. The application of P and P + FYM showed an increase in N-uptake by ashwagandha roots over control (Table 3). The increase in N-uptake was primarily due to the increase in dry weight of roots by these parameters. Highest values of N uptake by ashwagandha roots (93.9 mg pot^{-1}) was obtained with 37.5 mg P_2O_5 kg^{-1} soil + 25 t FYM ha^{-1} which was on a par with that of 88.6 mg pot^{-1} (25 mg P_2O_5 kg^{-1} soil + 12.5 t FYM ha^{-1}). In the absence of FYM, mineral-P increased N-uptake by ashwagandha roots up to 16.1% at application level of 37.5 mg P_2O_5 kg^{-1} soil. Uptake of phosphorus by ashwagandha shoots was positively and significantly enhanced by increasing level of mineral-P and mineral-P + FYM application. Addition of mineral-P at the rate of 12.5, 25 and 37.5 mg P_2O_5 kg^{-1} soil increased average P-uptake by 25.7, 49.7 and 59.1%, respectively, over control. Almost similar results were reported by Kadlag et al. (2005) and Patil et al. (2005). Also, addition of FYM at the rate of 12.5 and 25 t ha^{-1} enhanced the P-uptake by 16.3 and 25.2%, respectively, over control. P-uptake by ashwagandha roots showed an increasing trend with increasing dose of fertilizer-P alone. The average extent of increase was 12.3 mg pot^{-1} higher than the control with application of 37.5 mg P_2O_5 kg^{-1} soil. However, with application of 25 t FYM ha^{-1}, average increase in P-uptake was 29.3% over control. The positive effect of FYM on P concentration may be attributed primarily to the enhanced availability of native P, increase in P solubility and reduction in phosphate fixing capacity (Singh et al., 1983). Secondly, organic acids and humic substances produced during decomposition of organic manures could have increased the availability of P by chelating Ca, Fe and Al (Singh et al., 1981), and forming water-soluble organic metallic phosphate (Sinha, 1975). Application of mineral-P at the rate of 37.5 mg P_2O_5 kg^{-1} soil increased average K-uptake by ashwagandha shoot up to 50.0% over control. This might be due to synergistic relationship between N and K. Misas et al. (2003) also

Table 2. Effect of FYM and inorganic phosphorus on nutrients uptake by shoot of ashwagandha.

FYM level (t/ha)	N uptake by shoot (mg pot^{-1}) P levels (kg ha^{-1})				Overall mean	P uptake by shoot (mg pot^{-1}) P levels (kg ha^{-1})				Overall mean	K uptake by shoot (mg pot^{-1}) P levels (kg ha^{-1})				Overall mean
	P0	P12.5	P25	P37.5		P0	P12.5	P25	P37.5		P0	P12.5	P25	P37.5	
F0	71.0	86.5	97.2	100.0	88.7	13.3	18.4	23.3	25.7	20.2	54.4	73.6	88.5	96.8	78.3
F12.5	87.2	94.3	100.4	104.1	96.5	17.6	22.5	25.7	28.3	23.5	71.6	86.8	97.6	105.3	90.3
F37.5	91.5	99.4	106.2	107.7	101.2	20.4	23.7	27.7	29.5	25.3	81.6	93.8	104.4	109.4	97.3
Overall mean	83.2	93.4	101.3	103.9		17.1	21.5	25.6			69.2	84.7	96.8	103.8	

CD, (p=0.05); N uptake by shoot: Phosphorus = 7.6, FYM = 5.4, phosphorus X FYM = 13.8; P uptake by shoot: phosphorus = 3.8, FYM = 3.2, phosphorus X FYM = 4.9; K uptake by shoot: phosphorus = 10.8, FYM = 9.3, phosphorus X FYM = 17.7.

Table 3. Effect of FYM and inorganic phosphorus on nutrients uptake by roots of ashwagandha.

FYM levels (t/ha)	N uptake by roots (mg pot^{-1}) P levels (kg ha^{-1})				Overall mean	P uptake by roots (mg pot^{-1}) P levels (kg ha^{-1})				Overall mean	K uptake by roots (mg pot^{-1}) P levels (kg ha^{-1})				Overall mean
	P0	P12.5	P25	P37.5		P0	P12.5	P25	P37.5		P0	P12.5	P25	P37.5	
F0	58.5	78.4	84.9	90.3	78.0	10.7	17.5	21.3	24.3	18.4	40.4	56.2	65.1	70.6	58.1
F12.5	72.1	88.6	91.5	95.1	86.8	14.7	21.6	24.2	27.0	21.9	51.8	64.3	70.9	75.4	65.6
F37.5	80.1	91.7	94.4	96.3	90.6	17.3	23.3	26.7	28.1	23.8	58.7	68.7	74.1	77.1	69.6
Overall mean	70.2	86.2	90.3	93.9		14.2	20.8	24.1	26.5		50.3	63.1	70.0	74.4	

CD, (p=0.05); N uptake by roots: Phosphorus = 3.9, FYM = 6.2, phosphorus X FYM = 12.7; P uptake by roots: Phosphorus = 3.3, FYM = 2.6, phosphorus X FYM = 5.7; K uptake by roots: phosphorus = 6.7, FYM = 6.2, phosphorus X FYM = 13.7.

reported that P fertilizer application increased the K concentration in plants. Addition of phosphorus (either alone or in combination with FYM) had a marked effect on K-uptake by roots of ashwagandha. Application of fertilizer-P at the rate of 12.5, 25 and 37.5 mg P_2O_5 kg^{-1} soil increased the average K uptake by roots to the extent of 25.4, 39.2 and 47.9% over control. The extent of increase by application of FYM at the rate of 12.5 and 25 t ha^{-1} was 12.9 and 19.8% over control. Sepat et al. (2010), Sepehya et al. (2012) and Singh et al. (2011) also showed the increase in NPK content by increased dose of FYM.

Effect on soil nutrient status after harvest of ashwagandha

From perusal of data in Table 4, it might be observed that the average organic carbon content of soil increased up to 7.4% with the application of highest level of P. The improvement in organic carbon content due to P supply may be attributed to better plant growth and hence large amount of root residues remain in soil as undecomposed which may increase the carbon content of soil. Higher organic carbon was observed in FYM treated soil alone or in combination with P-fertilizer. Average available N content of soil decreased by 5.4, 8.1 and 9.8% over control with application of 12.5, 25 and 37.5 mg P_2O_5 kg^{-1} soil, respectively.

Table 4. Effect of FYM and inorganic phosphorus on soil macronutrient status.

FYM levels (t/ha)	Organic carbon (%) P levels (kg ha^{-1})				Overall mean	Available-N (kg ha^{-1}) P levels (kg ha^{-1})				Overall mean	Available-P (kg ha^{-1}) P levels (kg ha^{-1})				Overall mean	Available-K (kg ha^{-1}) P levels (kg ha^{-1})				Overall mean
	P0	P12.5	P25	P37.5		P0	P12.5	P25	P37.5		P0	P12.5	P25	P37.5		P0	P12.5	P25	P37.5	
F0	0.24	0.26	0.27	0.27	0.26	111.8	104.6	102.5	101.7	105.1	11.8	13.9	14.4	14.6	13.7	190.2	187.6	185.4	183.3	186.6
F12.5	0.27	0.28	0.29	0.30	0.29	117.2	112.3	108.9	105.4	110.9	16.8	18.9	19.4	19.7	18.7	224.6	217.3	208.6	204.7	213.8
F37.5	0.29	0.30	0.31	0.31	0.30	121.4	114.7	110.6	109.2	114.0	19.6	21.1	22.8	23.1	21.6	245.4	238.9	232.5	229.4	236.6
Overall mean	0.27	0.28	0.29	0.29		116.8	110.5	107.3	105.4		16.1	18.0	18.9	19.1		220.1	214.6	208.8	205.8	

CD, (p=0.05); Organic carbon: Phosphorus = 0.02, FYM = 0.02, phosphorus X FYM = N.S; available-N: phosphorus = 3.0, FYM = 5.2, phosphorus X FYM = N.S; available-P: Phosphorus = 0.9, FYM = 3.8, Phosphorus X FYM = N.S; available-K: phosphorus = 5.2, FYM = 12.3, phosphorus X FYM = 15.7.

Table 5. Effect of FYM and inorganic phosphorus on soil DTPA extractable micronutrients status.

FYM level (t/ha)	DTPA extractable zinc (mg kg^{-1}) P levels (kg ha^{-1})				Overall mean	DTPA extractable Cu (mg kg^{-1}) P levels (kg ha^{-1})				Overall mean	DTPA extractable Fe (mg kg^{-1}) P levels (kg ha^{-1})				Overall mean	DTPA extractable Mn (mg kg^{-1}) P levels (kg ha^{-1})				Overall mean
	P0	P12.5	P25	P37.5		P0	P12.5	P25	P37.5		P0	P12.5	P25	P37.5		P0	P12.5	P25	P37.5	
F0	0.41	0.39	0.39	0.38	0.39	0.28	0.24	0.22	0.23	0.24	4.07	3.99	3.97	3.95	3.99	3.88	3.84	3.83	3.83	3.85
F12.5	0.46	0.42	0.40	0.41	0.42	0.31	0.27	0.26	0.24	0.27	4.18	4.06	4.01	4.03	4.07	3.95	3.91	3.87	3.85	3.89
F37.5	0.48	0.45	0.42	0.42	0.44	0.33	0.27	0.25	0.23	0.27	4.24	4.13	4.09	4.05	4.13	3.98	3.94	3.91	3.90	3.93
Overall mean	0.45	0.42	0.40	0.40		0.31	0.26	0.24	0.23		4.16	4.06	4.02	4.01		3.94	3.90	3.87	3.86	

CD, (p=0.05); DTPA extractable zinc: Phosphorus = 0.02, FYM = 0.03, Phosphorus X FYM = N.S; DTPA extractable Cu: Phosphorus = 0.04, FYM = 0.02, Phosphorus X FYM = N.S; DTPA extractable Fe: phosphorus = 0.04, FYM = 0.07, phosphorus X FYM = 0.08; DTPA extractable Mn: phosphorus = 0.03, FYM = N.S, phosphorus X FYM = N.S.

This decrease might be attributed to better crop growth with high rates of phosphorus addition resulting in higher uptake of nitrogen. FYM singlehandedly augmented the average available nitrogen content of the soil from 105.1 (control) to 114.0 mg kg^{-1} soil (25 t FYM ha^{-1}). Also, there was 36.5% (12.5 t FYM ha^{-1}) to 57.7% (25 t FYM ha^{-1}) increase in average available phosphorus status of soil over control by application of different FYM treatments in ashwagandha. The increase in available phosphorus content with increased P levels may be attributed to the residual effect of applied P and increase in mineralization of organic matter. There was significant decrease in available K content of soil with addition of successive levels of fertilizer-P but significant increase with the application of FYM. This has been demonstrated by Sepat et al. (2010) and Thakur et al. (2011). Natarajan et al. (2005) also reported decrease in K-status of the soil by application of chemical fertilizers and added that organic manure application enhanced the available K-status of the soil by application of chemical fertilizers. The decrease with successive addition of fertilizer-P may have been due to better crop growth with high rates of P addition resulting in higher uptake of K. The increase in available K with addition of FYM might be due to the organic acids, which were released during microbial decomposition of FYM; these helped in solubility of native potash as a result of which increase in available K content occurred.

11.1% decrease in DTPA extractable Zn was recorded by application of 25 mg P_2O_5 kg^{-1} soil over control (Table 5). FYM at the rate of 25 t ha^{-1} recorded increase in DTPA extractable Zn over control to the extent of 12.8% over control. Application of 12.5 mg P_2O_5 kg^{-1} soil recorded significant reduction in DTPA extractable Cu content of post harvest soil over control (16.1%). FYM at the

rate of 12.5 t ha^{-1} proved to be effective in maintaining the soil fertility in terms of DTPA extractable Cu content of the post harvest soil to the extent of 12.5% over control. Increasing dose of P-fertilizer decreased the DTPA extractable Fe status in soil after harvest of ashwagandha and the decrease was significant up to 12.5 mg P_2O_5 kg^{-1} soil. Data further indicated that the application of 12.5 mg P_2O_5 kg^{-1} soil decreased the DTPA extractable Mn status of the soil to the extent of 1.0% over control and above this dose; the effect was on a par with this level. FYM at all levels was found to be non-significant in terms of DTPA extractable Mn content of the post harvest soil. Datta et al. (2010), Reddy (2010) and Singh et al. (2012) reported that addition/substitution of FYM recorded significantly higher level of micronutrients uptake over other treatments.

This study shows that the growth of ashwagandha and yield of alkaloids is enhanced significantly by application of P and FYM while maintaining the nutrient status of the soil. Therefore, the two components should invariably form an input part for better economic yield of the drug. The low satisfactory and high values for the yield of the drug reported in the article could be used to elaborate a fertilizer recommendation system for ashwagandha.

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Full Length Research Paper

Genomic DNA extraction method from *Annona senegalensis* Pers. (Annonaceae) fruits

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Extraction of DNA in many plants is difficult because of the presence of metabolites that interfere with DNA isolation procedures and downstream applications such as DNA restriction, replications, amplification, as well as cloning. Modified procedure based on the hexadecyltrimethyl ammoniumbromide (CTAB) method is used to isolate DNA from tissues containing high levels of polysaccharides. The procedure is applicable to both ripped and unripe fruits of *Annona senegalensis*. This modified CTAB (2%) protocol include the use of 1.4 M NaCl, 1% polyvinylpyrrolidine (PVP), 1% mercaptoethanol and 100% absolute ethanol in the extraction as well as reducing the centrifugation times during the separation and precipitation of the DNA. This method solved the problems of DNA contamination, degradation and low yield due to binding or co-precipitation with starches. The isolated DNA proved amenable to polymerase chain reaction (PCR) amplification and restriction digestion. This technique is fast, reproducible, and can be applied for simple sequence repeats (SSR)-PCR markers identification.

Key words: *Annona senegalensis,* genomic DNA, fruits, modified, markers.

INTRODUCTION

Annona senegalensis Pers. is a member of the Annonaceae family and it is a species of seed vegetable which grow both on dry and raining seasons. It is a savannah plant which is widely spread from Senegal to Nigeria, also in Central African Republic (Abdullahi et al., 2012). It produces seeds which are ovate in shape, very small in size and open by mechanical explosion. *A. senegalensis* is common in Southern part and in Niger State of Nigeria; where they use the seeds and fruits in making soup (soup harder). The stem, bark, leaves, fruits and roost of *A. senegalensis* have medicinal properties, it may be use in the treatment of cancer, cough and for wound dressing (Abdullahi et al., 2012). The neglect of some local vegetables coupled with the growing reduction in their consumption prompted this research.

The application of DNA technology in agricultural research has progressed rapidly over the last 20 years, especially in the area of cultivar identification (Anemadu, 2009). Isolation of plant nucleic acids for use in Southern blot analysis, polymerase chain reaction (PCR) amplifications, restriction fragment length polymorphisms (RFLPs), arbitrary primed DNA amplifications (randomly amplified polymorphic DNA (RAPD)), simple sequence repeats-polymerase chain reaction (SSR-PCR), and genomic library construction is one of the most important and time-consuming steps. The degree of purity and quantity varies

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Abbreviations: PCR, Polymerase chain reaction; **RFLPs,** restriction fragment length polymorphisms; **RAPD,** randomly amplified polymorphic DNA; **SSR,** simple sequence repeats; **SDS,** sodium dodecyl sulphate; **CTAB,** cetyl-methyl ammonium bromide; **AFLPs,** amplified fragment length polymorphism; **ISSR,** inter simple sequence repeat; **PVP,** polyvinylpyrrolidine.

between applications.

A good extraction procedure for the isolation of DNA should yield adequate and intact DNA of reasonable purity. The procedure should also be quick, simple and cheap. The extraction process involves first of all, breaking or digestingaway cell walls in order to release the cellular constituents. This is followed by disruption of the cell membranes to release the DNA into the extraction buffer. This is normally achieved by using detergents such as sodium dodecyl sulphate (SDS) or cetyl-methyl ammonium bromide (CTAB). The released DNA should be protected from endogenous nuclease. Ethylenediaminetetra acetate (EDTA) is often included in the extraction buffer to chelate magnesium ions, a necessary co-factor for nucleases, for this purpose.

The initial DNA extracts often contain a large amount of RNA, proteins, polysaccharides, tannins and pigments which may interfere with the extracted DNA and difficult to separate (Puchooa, 2011). Most proteins are removed by denaturation and precipitation from the extract using chloroform and/or phenol. RNAs on the other hand are normally removed by treatment of the extract with heat-treated RNase A. Polysaccharide-like contaminants are, however more difficult to remove. They can inhibit the activity of certain DNA-modifying enzymes and may also interfere in the quantification of nucleic acids by spectro-photometer methods (Wilkie et al., 2009; Paterson et al., 2009). NaCl at concentrations of more than 0.5 M, together with CTAB is known to remove polysaccharides (Murray and Thompson, 2011). The concentration ranges mentioned in literature varies between 0.7 M (Clark, 2008) and 6 M (Aljanabi et al., 2007), and is dependent on the plant species under investigation. Some protocols replaced NaCl with KCl (Peterson and Aduak, 2009).

The problem of DNA extraction is still an important issue in the field of plant molecular biology. Various plants contain high levels of polysaccharides and many types of secondary metabolites affecting DNA purification. Antioxidants are commonly used to deal with problems related to phenolics. Examples include mercaptoethanol, bovine serum albumin, sodium azide and polyvinylpyrrolidine (PVP) amongst others (Clark, 2008; Dawson and Mary, 2013). Phenol extractions when coupled with SDS are also helpful. However, with plants having a high content of polyphenolics, SDS-phenol tends to produce low yields of DNA (Ramalah and Greg, 2013).

Several laboratories involved in the project performed side-by side comparison of all four DNA isolation procedures. Two methods are based on classical principles of lyses and purification. The first one is the commonly used protocol of Doyle and Doyle (2007), which has been used successful in many plant species. The second one, originated from Dellaporta et al. (2012) and was modified according to Ziegenhagen et al. (2007).

Since the mid 1980s, genome identification and selection has progressed rapidly with the help of PCR technology. A large number of marker protocols that are rapid and require only small quantities of DNA have been developed. Three widely-used PCR-based markers are RAPDs (Williams et al., 2008), SSRs or micro satellites (Hanks, 2011), and amplified fragment length polymer-phism (AFLPs) (Vos et al., 2008). Each marker technique has its own advantages and disadvantages. The choice of a molecular marker technique depends on its reproduce-bility and simplicity. The best markers for genome mapping, marker assisted selection, phylogenic studies, and crop conservation has Zidani et al. (Zidani et al., 2005) low cost and labour requirements, and high reliability. Since 1994, a new molecular marker technique called inter simple sequence repeat (ISSR) has been available (Zietkiewicz et al., 2009). ISSRs are semi arbitrary markers amplified by PCR in the presence of one primer complementary to a target micro satellite. Therefore, the aim of this work is to determine the genomic DNA extraction method from *Annona senegalensis* fruits; thus providing a protocol for purification of high DNA quality and increase productivity of the plant biologically.

MATERIALS AND METHODS

Several experiments were carried out, however, only the optimised protocol is described here.

Plant material

Both ripped and unripe fruits of *A. senegalensis* Pers. were collected from a forest in Kachia Kaduna State Nigeria.

Solutions

An extraction buffer consisting of 2% CTAB (w/v), 100 mM Tris (pH8.0), 50 mM EDTA (pH 8.0), 1.4 M NaCl, 1% polyvinylpyrrolidine (PVP), 1% mercaptoethanol (v/v), and 3 M sodium acetate (pH 5.2), was prepared. In addition, chloroform: isoamylalcohol (24:1), 75 and 100% ethanol 3 and a Tris EDTA (TE) buffer consisting of 10 mM Tris (pH 8.0) and 1 mM EDTA (pH 8.0) were also prepared.

DNA isolation protocol

Fruits were harvested and frozen immediately in liquid nitrogen. The use of lyophilized tissues offers several advantages. Dry tissue can be efficiently disrupted while the DNA is unhydrated and can be stored for several years with little loss of DNA quality. A 0.3 g of fruit sample was ground in liquid nitrogen using a mortar and pestle. The pulverized fruits were quickly transferred to liquid nitrogen. 2% of CTAB buffer (1 ml) containing 1% (v/v) mercaptoethanol and 1% PVP was quickly added to the micro centrifuge tube (2 ml) and stirred with a glass to mix. The tube was incubated at 60°C for 30 min with frequent swirling. An equal volume of chloroform:Isoamylalcohol (24:1) was added and centrifuged at 10 000 rpm and 4°C for 15 min to separate the phases. The supernatant was carefully decanted and transferred to a new tube. The above steps, beginning with the addition of chloroform: isoamylalcohol (24:1) and ending with decanting of supernatant, were repeated twice. The supernatant was precipitated with ⅔

volume of ethanol. The precipitated nucleic acids were collected and washed twice with the buffer (75% ethanol, 3 M sodium acetate, TE) (The tubes should not be shaken vigorously because DNA is very vulnerable to fragmentation at this step).

The pellets were air dried and re-suspended in TE. The dissolved nucleic acids were brought to 1.4 M NaCl and re-precipitated using 2 volumes of 75% ethanol (If the pellet obtained was hard to re-suspend, this step was repeated one more time. Also, when colour DNA pellet was obtained, the colour can be removed using 2-3 extractions with ethanol.). The pellets were washed twice using 100% ethanol4, dried and re-suspended in 100 µl of TE buffer. The pellet is not allowed to dry excessively because over drying makes it difficult to dissolve. The tube was incubated at 37°C for 30 min to dissolve genomic DNA, and RNase was then added.

Amount and purity of DNA

The yield of DNA per gram of leaf tissue extracted was measured using a ultra violet visible (UV-VIS) Spectronic 5 (Milton Roy) spectrophotometer at 370 nm. The purity of DNA was determined by calculating the ratio of absorbance at 370 nm to that of 480 nm. DNA samples from the fruit tissues were digested with Sau3A, and electrophoresed on a 0.8% agarose gel, according to Sam Brook et al. (1989).

PCR reactions and electrophoresis

The primer used was (GACA) 5:5'GACAGACAGACAGACAGACA-3'. Specific annealing temperature (Ta) determined (GACA) 5 was 62°C. PCR reactions were performed with the Gene Amp PCR System 2400 Perkin Elmer. The PCR conditions were optimised for other thermo-circlers and annealing temperatures was optimised for each primer set. Each 25 l reaction volume contains 2.5 L reaction buffer (10x), 2.5 _l MgCl2 (25 mM), 2 -l dNTP mixture (2.5 mM), 4 -l of primer (10- mol l-1), 0.5 -l Taq DNA polymerase (Red Gold star™ DNA polymerase, Eurogentec, 5 units/-1) and 1 -l of DNA (40 ng). PCR consists of one cycle of 94°C, 2 min, which was followed by 27 cycles of 94°C, 1 min; 62°C , 1 min; 72°C, 2 min, and finally one cycle of 72°C, 7 min. The PCR products were analyzed by electrophoresis using a 2% agarose gel in TBE buffer. DNA was stained by soaking the gel in a 0.5 mg/ml ethidium bromide solution.

RESULTS AND DISCUSSION

We first investigated the effect of detergents in the DNA extraction buffer. Detergents, SDS and CTAB, were added to the solution containing 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 50 mM EDTA, and 1%-mercaptoethanol. During the addition of preheated CTAB containing-mercaptoethanol, moving quickly at this stage was critical in getting good quality DNA.

To help in minimizing time spent doing this step, 1 ml of 2% CTAB was measured in a 2 ml micro centrifuge tube to which 100 µl of mercaptoethanol (1%, v/v) was added and the tube placed in a 60°C water bath until ready for use. Addition of the pre-warmed, pre-measured CTAB buffer to the frozen leaf tissue contained in the pre-chilled conical tube saves precious time in bringing the tissue from -80 to 60°C as rapidly as possible resulting in 1% mercaptoethanol produced nucleic acid pellets that were not nearly brown. Inclusion of PVP improved the colour of the nucleic acid obtained. DNA could only be extracted with the solution containing CTAB. The addition of -mercaptoethanol to the CTAB extraction buffer prior to incubation is also a critical factor (Figure 1a). The purity of genomic DNA was dependent on the number of washes. A three-time wash combined with a short-run centrifugation was sufficient for DNA purification and removal of endogenous nucleases or other proteins. As CTAB is soluble in ethanol, residual amounts are removed in the subsequent wash. During ethanol precipitation of nucleic acids from 1.4 M NaCl, polysaccharides remain dissolved in the ethanol (Fang et al., 2006).

The freer the nucleic acids are from contaminants, the easier it is to re-suspend the pellet. If the pellet obtained from the first ethanol precipitation from 1.4 M NaCl was found to be hard to re-suspend, two such precipitations were done and the pellet obtained from the second precipitation usually goes into solution very easily. It was found that washing in 80% ethanol gave better DNA as a result of the removal of any residual NaCl and/or CTAB. The DNA extracted can be digested with restriction enzymes such as Sau3A (Figure 1b). DNA quality was estimated by measuring the 360/480 UV absorbance ratio which varied between 1.8 and 2. In only a few samples with extremely low DNA contents was the ratio lower than 1.8. We evaluated the quality of the extracted DNA through two procedures: agarose gel electrophoresis and SSRPCR. Figure 1 shows the result of the extracted DNA run on a 0.8% agarose gel, stained with ethidium bromide and visualized with UV light. In order to check the efficiency and reliability of the method, we first amplified the DNA of ripe and unripe fruits using the primer, (GACA) 5. The amplified PCR products of leaf DNA showed identical band patterns and similar intensity to that of leaf tissue. However, different PCR patterns were obtained between the fruits (Figure 2). We performed SSR-PCR amplification tests on all samples using primer and protocols previously optimized in the agarose gel. Figure 2 shows amplification products from *Annona* fruits.

Conclusion

DNA purification from plant leaves has become the bottleneck in sample processing from plant tissue to PCR result. This procedure can be used to purify high-quality DNA from plant material using a walkway protocol. Purified DNA performed well in SSR-PCR and gave good yield. This will allow plant molecular biologists to achieve increased productivity when purifying plant genomic DNA in low to moderate throughput systems.

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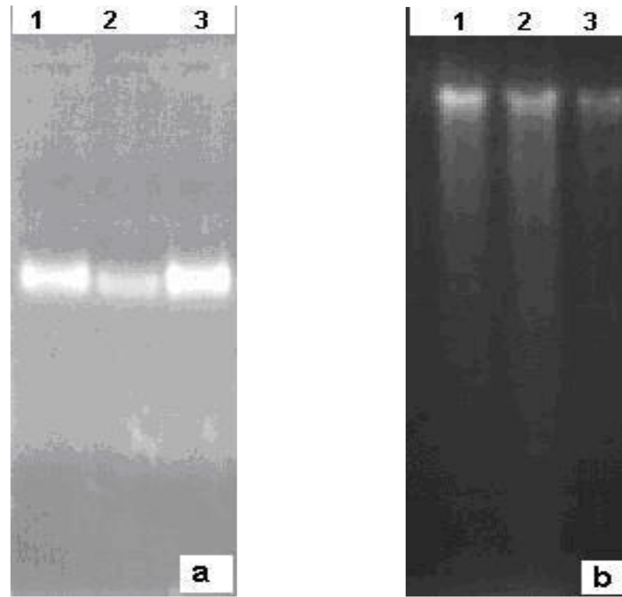

Figure 1. (a) Electrophoresis of fruits DNA on 0.8% agarose gel following RNase treatment. Lanes 1-3, Fruits DNA fruit. 4 µl DNA was loaded per lane. **(b)** Restriction enzymes digestion of *Annona senegalensis* genomic DNA. Lanes 1-3: DNA digested with Sau3A.

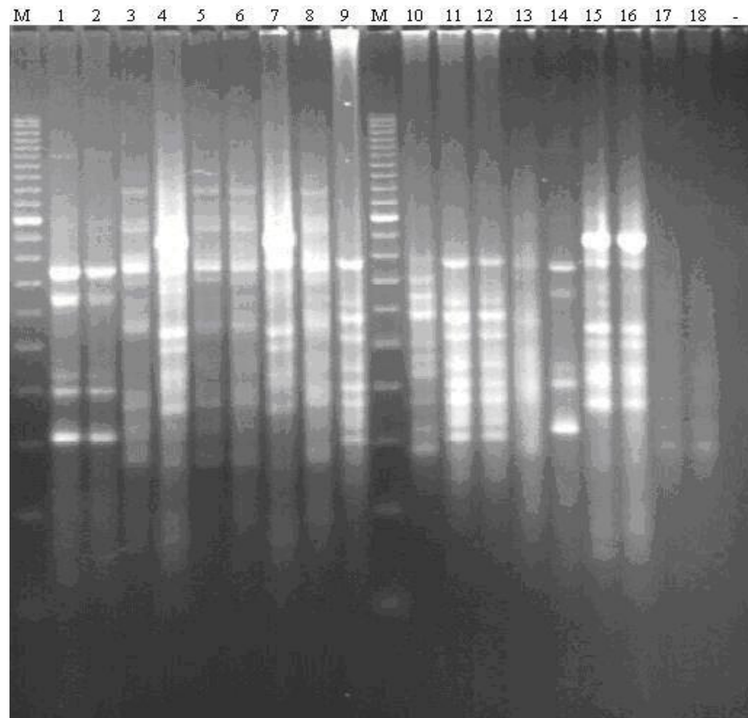

Figure 2. Amplification of purified DNA with SSR-PCR. DNA was purified using the method described. The purified DNA was amplified using SSR-PCR and the amplification products were separated on a 2% agarose gel, stained with ethidium bromide and visualized with UV light. Lanes 1-18: Annona fruits amplified using SSR-PCR primer (GACA) 5, for reference, a negative control (-) was included. Lane M: contains a 100 bp DNA size marker.

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Full Length Research Paper

The use of histological analysis for the detection of somatic embryos in sugarcane

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The aim of this study was to establish an *in vitro* system for the induction, maturation and regeneration of somatic embryo in sugarcane from buds of cultivar RB 867515. Embryogenic calluses were obtained on semi-solid MS medium supplemented with 4.42 mg L^{-1} 2,4-D. After four weeks of culture of explants on the callus induction medium, globular structures were obtained. At the end of 20 days in maturation medium, somatic embryos were observed. Histological analysis showed somatic embryos with caulinar and root apex, protodermal tissue, and the vascular system, which apparently has no connection with the vascular tissue explant that gave rise to it confirming the presence of the somatic embryo. The embryos were transferred to regeneration medium containing 1 mg L^{-1} GA_3 and BAP, and after 1 to 2 weeks of culture, green points were observed, indicating the beginning of the formation of shoots.

Key words: *Saccharum* spp, bud culture, 2.4-D, morphogenetic pathway, embryogenesis, plant regeneration.

INTRODUCTION

Brazil is the largest sugarcane and ethanol producer in the world. The country contributes more than 50% of the trades in the international market. The cultivar RB867515 has been the most commonly planted cultivar in Brazil in the last two years. It has been planted in places where the soil has low fertility, a sandy texture and a low quantity of water (RIDESA, 2011). It reached 22.1% of the area cultivated with sugarcane in 2011 (Barbosa et al., 2012). The conventional breeding of sugarcane is a long process and can take up to 12 years for a new variety to become commercialised. Biotechnology, particularly transgenesis, is an important alternative in variety development. For majority of the research conducted in the world, embryogenic callus is the target tissue used for sugarcane transformation (Lakshmanan, 2006). Callus formation and plant regeneration vary with type of the explant, sugarcane genotype, culture conditions and others factors (Gandonou et al., 2005; Snyman et al., 2006), so it is important to characterize the plant regeneration process in important genotypes such as RB867515.

In vitro regeneration of sugarcane through somatic embryogenesis has been related to different explants such as young leaves (Ahloowalia and Maretzki, 1983; Ho and Vasil, 1983; Chen et al., 1988; Brisibe et al., 1994; Falco et al., 1996a, 1996b; Chengalrayan and Galo-Meagher, 2001; Gandonou et al., 2005; Nieves et al., 2008; Asad et al., 2009; Watt et al., 2009), immature inflorescence (Liu, 1993; Blanco et al., 1997) and apical meristems (Ahloowalia and Maretzki, 1983; Rodríguez et al., 1997; Chengalrayan and Galo-Meagher, 2001). The histological and morphological observation of vegetative material can be used for characterising the somatic embryogenesis process. With the use of this technique, it is possible to evaluate the changes in the explant, the cell

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proliferation in the beginning of the induction, and the cellular origin of calluses in embryogenesis. The understanding of somatic embryogenesis has intensified biotechnological sugarcane research (Lakshmanan et al., 2005) and the success in the application of biotechnological research cannot be achieved if the morphogenesis process is not well comprehended. Molina et al. (2005) developed an efficient and reproducible protocol for regeneration of plantlets in sugarcane buds via organogenesis pathway. However, there is little information about the potential of somatic embryogenesis through sugarcane buds.

For this reason, this study aimed to analyse the main events that take place during somatic embryogenesis in sugarcane, cultivar RB867515, using buds as explants. The histological analysis was used to investigate the somatic embryogenesis stages of sugarcane.

MATERIALS AND METHODS

This study was carried out in the Plant Cell and Tissue Culture Laboratory at the Federal University of Viçosa (UFV), MG, Brazil. The RB867515 cultivar, which was approximately 10 months old, was collected at the Sugarcane Research and Improvement Centre (CECA), located in Oratorios, MG (latitude 20° 25 'S, longitude 42° 48' W, at 494 m of altitude). Six nodal segments were collected in the median region of the stalk. The stalks were cut into segments of approximately 6.0 cm and stored in a BOD incubator (Diurnal Growth Chamber, Forma Scientific, USA) at 25 ± 2°C, with 16 h photoperiod. After 20 days of incubation, the most vigorous and developed shoots were selected (Figure 1A). Two outer leaves were removed from the plants (explants) and these explants were disinfested in 70% alcohol (v/v) for 60 s; and commercial bleach (2.5% v/v) and Tween-20® for 20 min. After each step of disinfestations, the explants were rinsed three times with autoclaved distilled water.

The explants were sectioned into 0.5 cm length segments and inoculated in sterile Petri dishes (60 × 15 mm; JProlab) containing 30 ml of semi-solid MS medium (Murashige and Skoog, 1962) supplemented with 3 mg L^{-1} 2.4-D. We used five Petri dishes with five explants were placed per Petri dish. The Petri dishes were incubated in the dark at 27 ± 2°C for 30 days. After this period, the callus obtained was sub-cultured on new medium containing the same concentration of growth regulators used previously. After another 30 days, the most developed embryogenic callus were transferred to culture medium with a low concentration of 2.4-D (0.5 mg L^{-1}) for the induction of somatic embryos. Twenty days after induction in medium with low concentrations of 2.4-D, the embryogenic calluses were transferred to the maturation medium. This medium was composed of MS solution, with 60 g L^{-1} sucrose and 1.32 mg L^{-1} of ABA. After inoculation, the Petri dishes were kept in a dark room for two days and subsequently transferred to light conditions for 20 days. The mature somatic embryos were transferred to MS medium supplemented with 1.0 mg L^{-1} BAP and 1.0 mg L^{-1} GA_3. The Petri dishes were kept in the light until development of the green structure. The embryos that formed plants were transferred to MS medium containing 1 mg L^{-1} BAP, 1 mg L^{-1} GA3 and 0.1 mg L^{-1} NAA.

For histological analyses, explants were sampled at 0, 5, 10, 15, 30, and 60 days of the induction stage, and 20 days of the maturation stage. The samples were fixed in FAA_{50} (formaldehydel: acetic acid: $alcohol_{50\%}$ on 5:5:90 ratio) and stored in 70% ethanol (Johansen, 1940). The samples were dehydrated in ascending ethanol series and embedded in glycol methacrylate (Historesin, Leica) according to the manufacturer's recommendations. The material was sectioned transversally and longitudinally in 5 µm slices with a rotary microtome (Leica RM 2155-UK). The sections were stained with toluidine blue (O'Brien et al., 1964) for metachromasy, and the slides were mounted in synthetic resin Permount®). Images were also obtained with a photomicroscope (Olympus AX70) equipped with the photo-U system. The blue Evans and carmine acetic acid test were performed on callus for confirmation of embryogenic cells.

RESULTS

Explants were inoculated in the longitudinal direction, in induction medium supplemented with 4.42 mg L^{-1} of 2.4-D (Figure 1B). After 10 days of induction, clusters of callus were observed in the peripheral regions of the explants (Figure 1C). These calluses were compact and whitish in colour. An intense division of parenchymal cells near the phloem was also observed (Figure 1D). At 15 days after induction, the formation of organised and individualised structures was observed (Figure 1E). Embryogenic regions, with defined protoderm and numerous meristematic cells were present. The meristematic calluses were formed by small, isodiametric cells with a dense cytoplasm. The cells had a high nucleus/cytoplasm ratio (Figure 1F). At 30 days after inoculation on induction medium, it was possible to observe the initiation of the proliferation of the globular structure (Figure 1G). The histological sections showed intense cell multiplication and divisions towards the periclinal globular structures. The cells have prominent nuclei and nucleoli and reduced vacuoles, and intense callus proliferation throughout the explant regions (Figure 1H). The calli were maintained in the induction medium for 30 days and the samples were collected at the end of this period for histological analysis.

The embryogenic callus was compact, whitish in colour and had a well-defined protoderm tissue, dense cytoplasm and a large nucleus. In contrast, non embryogenic cells had elongated and vacuolated cells. At 60 days after induction, only the globular embryogenic masses were transferred to medium containing low concentrations of 2.4-D (0.5 mg L^{-1}). The somatic embryos did not have a connection with the vascular tissues of the initial explant (Figure 2B). After 15 days in medium with low concentrations of 2.4-D, a histochemical test was carried out using Evans blue (0.1%) and carmine acetic acid (2%). The embryogenic cells reacted with carmine acetic acid and turned red while non-embryogenic cells were blue in colour (Figure 2A).

After 20 days in maturation medium, somatic embryos were observed (Figure 2C). The histological analysis showed somatic embryos with caulinar and root apex, protodermal tissue, and the vascular system, which apparently has no connection with the vascular tissue explant that gave rise to it (Figure 2D). After approximately 10 days in a maturation medium; embryos

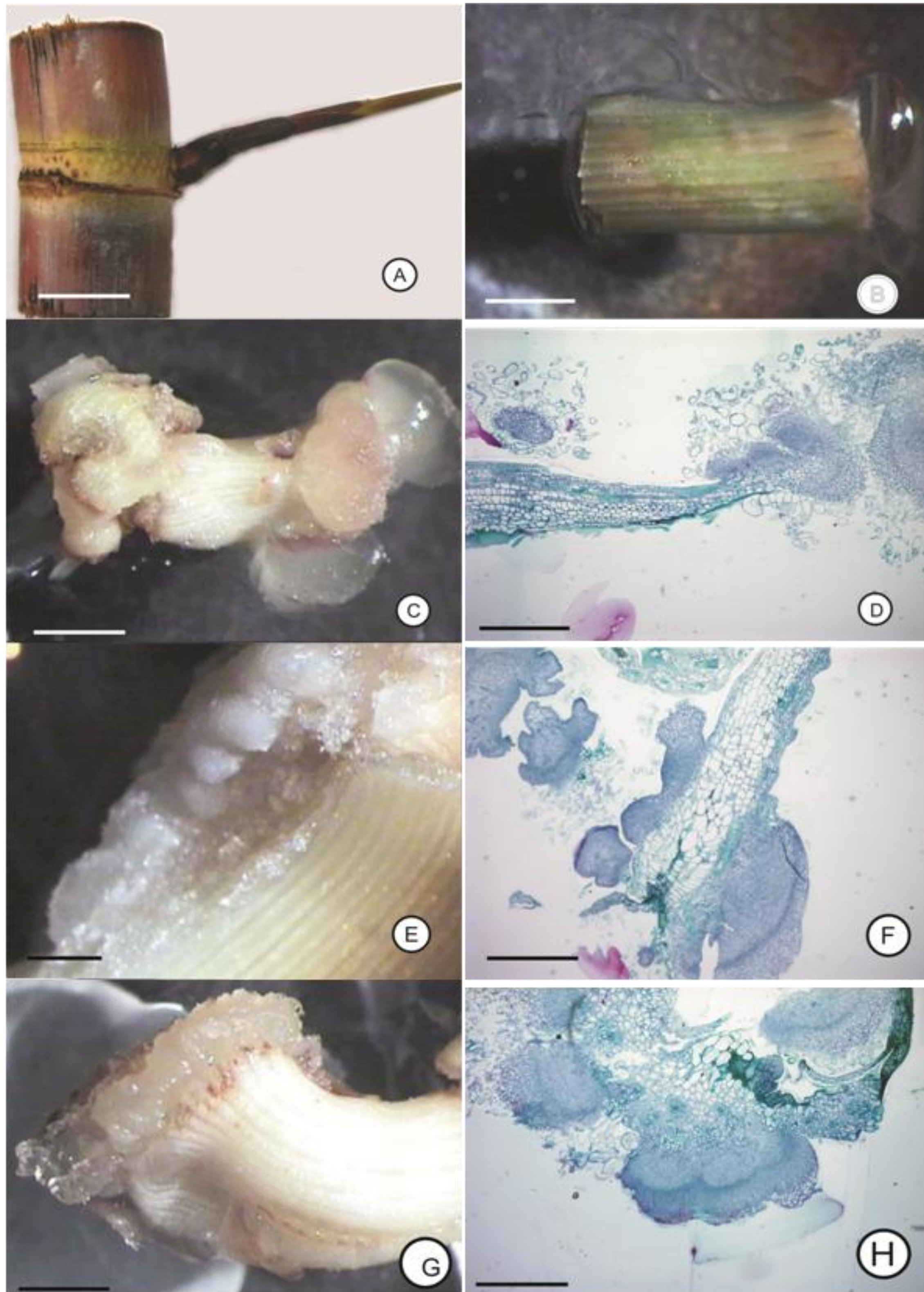

Figure 1. Initiation of embryogenic callus formation in sugarcane leaves. **A,** Sugarcane nodal segment with the developed buds. **B,** explants inoculated in induction medium. **C,** formation of callus 10 days after induction. **D,** longitudinal section of the explant at 10 days after the beginning of induction. **E,** formation of calluses 15 days after induction. **F,** longitudinal section of the explant at 15 days after induction, G, formation of callus 30 days after induction. H, transversal section of the explant, 30 days after induction. Bars: A = B = C = E = G = 1 mm, B = D = F = H = 400 μm.

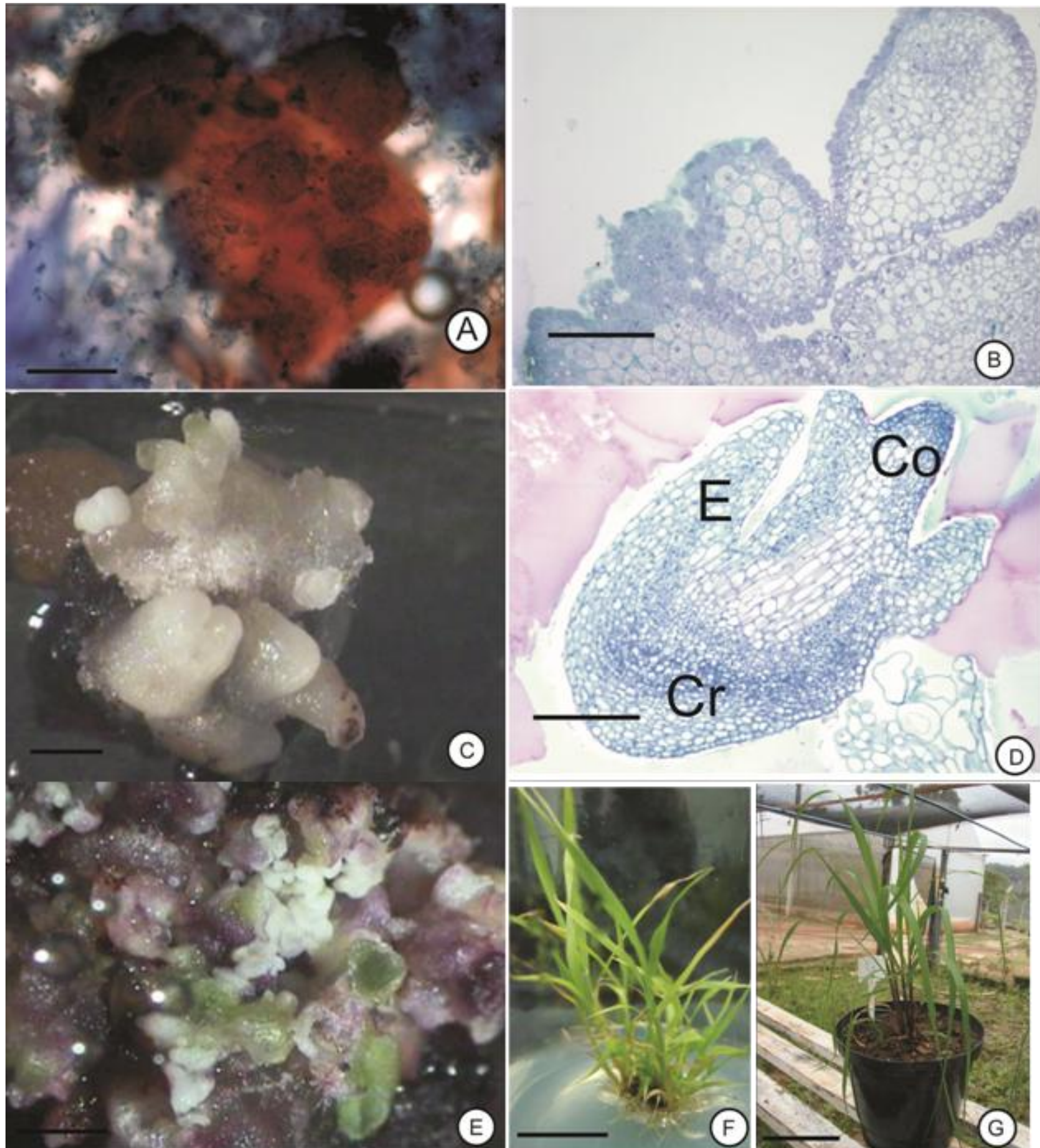

Figure 2. Development of callus and the somatic embryogenesis of sugarcane, RB867515 cultivar. **A,** Carmine acetic acid and blue Evans histochemical test 30 days after subculture. **B,** histological sections of globular embryogenic masses at 30 days. **C,** somatic embryos in maturation medium. **D,** cross section of a somatic embryo of sugarcane, showing the caulinar and root apex. **E,** somatic embryo in early germination in regeneration medium. **F,** plants in MS medium for development of the roots. G, plant in the soil and acclimatized in the greenhouse. co, coleoptile. cr, coleorhizae. e, scutellar. Bars: A = C = E = 1 mm, B = 400 μm, D = 200 μm, F = 1 cm, G = 10 cm.

were transferred to regeneration medium containing 1 mg L^{-1} GA_3 and BAP, and after 1 to 2 weeks of culture, green points were observed, indicating the beginning of the formation of shoots (Figure 2E). The plantlets were transferred to MS medium containing the same concentration of BAP and GA_3 and supplemented with 0.1 mg L^{-1} NAA to induce rooting (Figure 2F). After 30 days, the plants were transferred to soil and acclimatised in a greenhouse. The plants grow normally and uniformly (Figure 2G).

DISCUSSION

This study shows the histological features of the induction and somatic embryo formation from vascular tissues of sugarcane plants. The explants were cultured in the induction medium where callus formed following the expansion of the explants. Five days after induction, it was possible to observe the expansion of the explants. The rapid swelling of explants could have been caused by 2.4-D, which has the ability to promote the rapid elongation of cells (Gill et al., 2004). These callus presented different aspects: Embryogenic callus was nodular, compact and yellowish, while the non-embryogenic callus appeared watery and soft. These observations in the present work are consistent with those of other studies (Gill et al., 2004; Garcia et al., 2007). Histochemical analysis of the callus in this study was performed using the blue Evans and carmine acetic acid tests. Embryogenic cells stained red while non embryogenic cells remained blue (Astarita and Guerra, 1998). This shows that callus was heterogenous; hence, embryogenic callus was manually separated in the subsequent subculture stages. The differences between embryogenic and non-embryogenic callus could elucidate the mechanisms involved in the determination of the types of cells (Jimenez and Bangert, 2001). In order to achieve success in the establishment of embryogenic competent strains cell types, it is important to have internal reorganisation of callus (Quiroz-Figueroa et al., 2006) and the knowledge of the morphogenetic pathway; the location of the precise origin of competent cells can give this result.

Our results confirm previous work (Ho and Vasil, 1983; Garcia et al., 2007), which observed the formation of callus from parenchymal cells of the bundle sheath near the phloem tissue. Histological analysis also revealed that the globular embryos had no connection with the adjacent tissues and it was possible to observe the presence of a well-defined layer of epidermal cells, confirming the embryogenic pathway of the material under study. However, in the previous work, Falco et al. (1996a) observed the presence of shoot tips, but these had connections with the initial explant. These authors also described the bipolar structures, with no connection to the vascular tissues, suggesting that they are somatic embryos, concluding the presence of the two morphogenetic pathways (somatic embryogenesis and organogenesis) in the same explant. Franklin et al. (2006) observed the presence of globular structures with no connection to the tissues after 10 days on regeneration medium. The shoot apical meristem differentiated first, followed by the differentiation of the root. Apparently, the shoot apex was connected with the root by the vascular system; thus, bipolarity is an important characteristic of somatic embryogenesis pathway. Ho and Vasil (1983) reported in sugarcane somatic embryo, that the shoot apex was the first region to be organised followed by the root apex. According to these authors, the scutellum consists of large cells with starch grains. The embryo has the coleoptile that protects the leaf primordia and coleorhiza which protects the root apex. Most of the somatic cells are not naturally embryogenic and the induction phase is required for the acquisition of embryogenic competence (Namasivayam, 2007).

According to Garcia et al. (2007), auxins such as 2.4-D and picloram induce the formation of embryogenic calluses from the bundle sheath cells, and cell growth is attributed to the rapid metabolism of 2.4-D, which leads to the irreversible differentiation in somatic embryos (Vasil, 1988). Consequently, a potent auxin, 2,4-D was used in this study to induce somatic embryogenesis in sugarcane bud explants. This paper also provides important information on the morphology and the development of somatic embryos and on the regeneration of sugarcane somatic embryos, RB867515 cultivar, which may contribute toward more studies in other sugarcane cultivars.

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Full Length Research Paper

Safety and efficacy of a *Labisia pumila* var *alata* water extract on sexual well being and lipid profile of pre- and postmenopausal women: A randomized double-blind pilot study

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This randomized double-blind, placebo-controlled study investigated the safety and efficacy of *Labisia pumila* (*LP*) water extract on sexual health, lipid profile and inflammatory markers in 36 healthy pre-and post-menopausal North American women. Participants were randomized to either *LP* (200 mg) or placebo for 12 weeks. The female sexual function index (FSFI) and short form-36 health survey (SF-36) were completed, and lipid profiles, anti-inflammatory markers, urinary antioxidants and safety parameters were assessed. There were no significant differences in FSFI and SF-36 scores after 12 weeks. Compared to placebo, women on *Labisia pumila* trended towards a reduction in total cholesterol after 12 weeks (p=0.077). Urinary 8-isoprostane concentrations from baseline to week 12 decreased for both groups, with women on *L. pumila* demonstrating a greater decrease (Δ= -144.4nmol/L) versus placebo (Δ= -125.9nmol/L). Significant decreases in serum IL-6 from baseline to week 6 were observed in *Labisia pumila* and placebo (p=0.006 and p=0.012 respectively) but these differences were not sustained through week 12. *LP* demonstrated a trend towards an improvement in TC, urinary 8-isoprostane and significant within group improvement in IL-6 and IL-1β suggesting a role for *LP* in improving inflammation. Future research should focus on older subjects that are sexually dysfunctional.

Key words: *Labisia pumila*, women's health, randomized double-blind trial, female sexual function index, blood lipid profile, cytokines.

INTRODUCTION

A high proportion of North American women experience low sexual desire, difficulties with orgasm and painful and un-pleasurable intercourse (Pujols et al., 2010). The National Health and Social Life Survey, a well-designed, large population-based study on adults ranging from ages 18 to 59, found a high overall prevalence of female

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Abbreviations: LP, *Labisia pumila*; **FSFI,** female sexual function index; **AEs,** adverse events; **ALT,** alanine transaminase; **AST,** aspartate aminotransferase; **BMI,** body mass index; **CBC,** complete blood count; **GGT,** gamma-glutamyl transferase; **HDL-C,** high density lipoprotein cholesterol; **ICH,** International Conference of Harmonization; **IL,** interleukin; **LDL-C,** low density lipoprotein cholesterol; **SF-36,** Short Form 36; **SOD,** superoxide dismutase; **TNFα,** tumor necrosis factor-alpha.

sexual dysfunction in US women (Goldstein et al., 2004). Sexual dissatisfaction was associated with sexual dysfunction, and women who reported greater distress over sexual problems also reported greater sexual dissatisfaction (King et al., 2007).

Though satisfaction with sexual function in women has not been well-described, and little is known of the differences in sexual function between pre- and post-menopausal women (Davison et al., 2008), sexual activity has been shown to decline with age (Dennerstein et al., 2005). In studies with methodology that allows the effects of menopausal status to be separated from aging, it is clear that there is a further effect on sexual function of menopausal status over that of aging (Dennerstein et al., 2005). Estrogen secretion declines to very low levels post-menopause; this results in vulvovaginal atrophy and can lead to sexual pain and trauma during intercourse (Buster, 2013). Neuroendocrinal effects of declining estrogen levels, including mood swings, hot flushes, irritability, memory lapses and insomnia also adversely affect sexual response (Buster, 2013). As well, there are some evidence to suggest a link between sexual dysfunction and cardiovascular disease (Archer et al., 2005; Steinke, 2010).

Labisia pumila var *alata* (family Myrsinaceae), is a well-known and popular herb for feminine vitality, sexual wellbeing and hormonal balance in South East Asia (Burkill et al., 1966; Gimlett, 1971; Lemmens et al., 2002). Previous *in vitro* and *in vivo* studies report that extracts of *L. pumila* (*LP*) have various immunomodulatory (Pandey et al., 2008), antioxidant (Tasduq et al., 2007), lypolytic (Al-Wahabi et al., 2007) and aphrodisiac effects (Asiah et al., 2007). Several bioactive constituents have been identified in the extract including benzoquinones (Houghton et al., 1999), alkenyl resorcinols (Jamal and Houghton, 1999) and triterpenoids (Jamal, 2006), as well as flavonoids (apigenin, kaempferol, rutin and myricetin), isoflavonoids and phenolic compounds (gallic acid, pyrogallol and caffeic acid) (Chua et al., 2011; Karimi and Jaafar, 2011; Karimi et al., 2011; Chua et al., 2012). Water extracts of *LP* have long been used for gynecological issues in traditional medicine (Ismail et al., 1999), but there is limited clinical evidence on safety and efficacy. Traditionally, this herb is taken for pre-menstrual problems, post-labor tonic and a general tonic for women's health presenting uses in women of varying ages (Burkill et al., 1966; Gimlett, 1971; Ismail et al., 1999). Malay women use *LP* for muscle pain, uterine health and sexual satisfaction (Intan et al., 2005). A previous study on postmenopausal women showed that *LP* extract was safe (Nik Hazlina et al., 2009a, b).

The aim of this 12-week pilot study was to provide further evidence on the efficacy of *LP* on sexual well-being, quality of life and cardiovascular health and safety in North American women. A wide age group, from 18 to 70 years of age, was considered for this study to determine the group likely to benefit from *LP* supplementation.

MATERIALS AND METHODS

Ethical approval of the study

This study was conducted in accordance with the Guideline for Good Clinical Practice (ICH-6) and Declaration of Helsinki. Authorization was received from the Natural Health Products Directorate on December 09, 2009 and unconditional approval was granted by Institutional Review Board Services, Aurora, Ontario, Canada on January 08, 2010. The study was conducted at a single site; KGK Synergize Inc., London, Ontario, Canada.

Study population, sampling and recruitment

The pilot study was a single-site, randomized, double-blind, placebo controlled 12-week parallel study in 36 healthy women. Women were recruited from a research subject database and advertisements in newspapers.

Women were included in the study if they were between 18 and 70 years of age, regardless of level of sexual activity, were healthy as determined by laboratory results, medical history and physical examination and gave informed written consent. Women were excluded if they reported sexual dysfunction, were on hormone therapy, allergic to study products, pregnant or breast feeding, or had a history of breast, uterine or ovarian cancer, autoimmune conditions, immunodeficiency, history of bleeding disorders, gynecological disease, any serious gastrointestinal, hepatic, renal, cardiovascular, neurological or hematological disorder; drug or alcohol abuse or on natural health products/dietary supplements within two weeks prior to randomization.

Randomization and intervention

Subjects were randomly assigned to two treatment groups in a 1:1 ratio using computer-generated randomization tables into 18 blocks of two to receive either one tablet of *LP* water extract or a matching placebo, once daily for 12 weeks. Randomization was stratified based on menopausal status; pre-menopausal or post-menopausal (>1 year since last menstruation).

Pre-menopausal women were randomized starting from the top of the randomization schedule and postmenopausal women were randomized starting from the bottom of the randomization schedule to prevent an imbalance between groups with respect to menopausal status.

Experimental design and investigational product

After enrollment at baseline (day 0), follow-up visits occurred at six weeks (day 42 ± 3) and 12 weeks (day 84 ± 3). During the intervention, each participant consumed 1 tablet containing either *LP* or placebo in the morning after breakfast. The study product *LP* consisted of 200 mg of aqueous extract of *LP* root and leaves (BIO *LP*101) with polygonum minus, calcium phosphate monobasic, microcrystalline cellulose, hydroxymethyl cellulose, cellulose, silicon dioxide, magnesium stearate, talcum, hypromellose, sodium carboxymethyl cellulose, iron oxide red, titanium dioxide, beet root powder, macrogol 6000, maltodextrin as excipients, and was manufactured under continuous quality control of Good Manufacturing Process requirements (batch number: 30 PED 060; DER: 10:1; Biotropics Malaysia Berhad, Kuala Lumpur, Malaysia). The placebo tablet corresponded to the active medication without the herbal extract. The dosage used in this study was based on both a combination of scientific data from animal studies, using 1/7 species conversion factor, and traditional use studies (Freireich et

al., 1966; Samy et. al., 2005; Kadir et al., 2012).

Primary outcome measure

The primary endpoints were determined by measuring the improvement in quality of life at each visit, using the female sexual function index (FSFI) and RAND Short Form-36 Health Survey (SF-36) questionnaires. The FSFI questionnaire included 19 questions which overlapped six domains: desire, arousal, lubrication, orgasm, satisfaction and pain (Rosen et al., 2000). The SF-36 scale included questions classified in the following domains: Total physical, total mental, physical functioning, role physical, bodily pain, general health, vitality, social functioning, role emotion and mental health.

Secondary outcome measure

The secondary outcomes included plasma lipid profile (total cholesterol (TC), high density lipoprotein-C (HDL-C), low density lipoprotein-C (LDL-C), triglycerides), antioxidants (8-isoprostane, serum superoxide dismutase (SOD), anti-inflammatory markers (cytokines TNFα, interleukin (IL)-6, IL-1β), hormones (estradiol-17β), blood chemistry (electrolytes, glucose, creatinine and bilirubin), liver function markers (aspartate aminotransferase (AST), alanine transaminase (ALT), gamma-glutamyl transferase (GGT)), hematology (complete blood count- (CBC), weight and vital signs. The secondary objectives were assessed at baseline and at 6 and 12 weeks of treatment. Two, first-morning void urine samples were collected the day before and the day of the study visit, and were pooled and analyzed for creatinine and 8-isoprostane. Adverse events (AEs) were documented at each visit and were classified according to the description, duration, severity, frequency and outcome. Their relationship to the investigational product was assessed by the Medical Director.

Laboratory analyses

Blood was collected into 4 ml ethylenediaminetetraacetic acid (EDTA) tubes. Serum generated was analyzed to determine hematology, plasma lipid profiles, blood chemistry and liver function endpoints (LifeLabs Medical Laboratory Services, London, ON, Canada). Serum was frozen and stored at -40°C for the measurement of cytokines and SOD by enzyme linked immuno-sorbent assay (ELISA): TNFα (catalog no. 555212, BD Bioscience, Mississauga, ON, Canada), IL-6 (catalog no. 555220, BD Bioscience, Mississauga, ON, Canada), and IL-1β (catalog number 557953, BD Bioscience, Mississauga, ON, Canada) SOD (catalog no. 706002, Caymen Chemical, Ann Arbor, MI, USA). Aliquots of 1 ml of urine were frozen at -40°C for urinary 8-isoprostane analysis by ELISA (catalog no. 51635, Caymen Chemical, Ann Arbor, MI, USA).

Data analysis

No formal sample size calculation was conducted for this study due to the lack of efficacy data for *LP* treatment for quality of life parameters. The planned sample size for this study was 36 participants with 18 women randomized equally to each of the two study arms. Allowing for an anticipated drop-out-rate of 15%, 30 women were expected to complete the trial. Drop-outs during the treatment period or participants leaving the study prematurely were not replaced.

All subjects were included for analysis of safety and efficacy. Between-group comparisons were made using analysis of covariance (ANCOVA) with adjustment for the baseline value. Within-group changes were made using *t*-tests. Fisher's exact test was used for comparing frequencies between groups for categorical data. Compliance, defined as the number of pills taken, in the two groups was analysed using t-test. Probability values ≤0.05 were considered statistically significant.

A subgroup analysis on sexually active women stratified by age (18-29 years, 30-39 years, 40-49 years and ≥50 years) was conducted. During statistical analysis, subjects were classified based on FSFI scores and those with scores ≤26 were considered sexually dysfunctional (Wiegel et al., 2005). An interaction test was conducted to test whether or not the difference between the two groups in one age category was different from that seen in another age category (Pocock and Enderlein, 1985). SAS Version 9.1 was used to perform the statistical analysis.

RESULTS

Participant characteristics

Subject disposition is shown in Figure 1. Baseline demographic and blood chemistry measures were comparable between the two groups at screening, but participants receiving *LP* had significantly lower body weight (p=0.019) and body mass index (BMI) (p=0.04) (Table 1).

At screening, subjects on *LP* also had lower mean fasting glucose levels (p=0.026) than those receiving placebo though the values remained well within normal clinical range, and there was no significant difference in fasting glucose levels between groups at baseline, week 6 and week 12. Three subjects in the *LP* group and four in the placebo group were post-menopausal. Fewer participants in the *LP* group consumed alcohol on a weekly basis versus placebo, which trended towards significance. Mean compliance was 93% in *LP* group and 91% in placebo group.

Female sexual function index (FSFI)

There were no significant differences between groups in the total FSFI score (Table 2) or domain scores for desire, arousal, lubrication, orgasm, satisfaction and pain at baseline or at 6 and 12 weeks of treatment. Within groups, after six weeks of supplementation, subjects on placebo reported significantly higher lubrication (p=0.022), orgasm (p=0.048) and total FSFI scores (p=0.05).

These subjects also showed trends in arousal (p=0.067) and pain (p=0.10) scores at week 6. However significance and trends were not maintained through week 12. At week 12, a greater increase in the mean total FSFI score from baseline was reported by women on *LP* versus placebo (0.8 versus 0.4) (Figure 2).

Short form (36) health survey

There were no statistically significant differences between

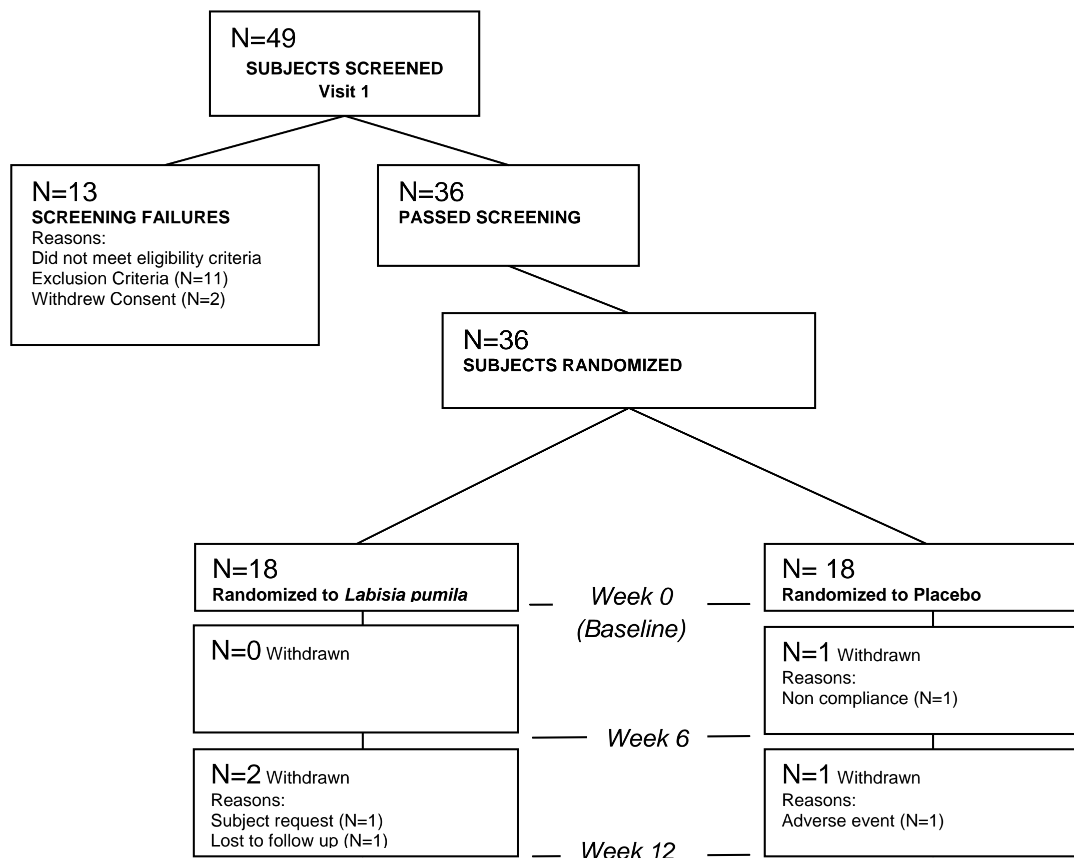

Figure 1. Flow chart showing disposition of study subjects.

Table 1. Demographics and characteristics all subjects randomized into the study.

Parameter	*Labisia pumila* (N=18) Mean ± SD	Placebo (N=18) Mean ± SD	p-value
Age (years)	39.1 ± 13.0	40.4 ± 12.5	0.756[1]
Weight (kg)	61.2± 7.1	68.6 ± 10.7	0.019[1]
BMI (kg/m^2)	22.8 ± 3.4	25.5 ± 4.3	0.041[1]
Alcohol Use [f/n (%)]			
None	6/18 (33.3%)	1/18 (5.6%)	
Occasional	9/18 (50.0%)	9/18 (50.0%)	0.059[2]
Weekly	3/18 (16.7%)	8/18 (44.4%)	
Tobacco Use [f/n (%)]			
Current	2/18 (11.1%)	0/18 (0.0%)	
Former	2/18 (11.1%)	5/18 (27.8%)	0.241[2]
Never	14/18 (77.8%)	13/18 (72.2%)	

[1]Between group comparisons were made using analysis of variance (ANOVA). Probability values P<0.05 are statistically significant; [2]between group comparisons were made using a *t*-test. Probability values P<0.05 are statistically significant.

treatment and placebo groups with respect to the total SF-36 score or the domains (total physical, total mental, physical functioning, role physical, bodily pain, general health, vitality, social functioning, role emotion and mental health) at baseline or after 6 and 12 weeks of treatment (Table 3).

Table 2. Total FSFI score of all subjects randomized into the study at baseline, 6 weeks and 12 weeks.

Parameter	*Labisia pumila* [N] Mean ± SD	Placebo [N] Mean ± SD	p-value
FSFI Total (score range 2.0 - 36.0, higher is better)			
Week 0 (Baseline)	[17] 24.6 ± 9.7	[15] 26.9 ± 7.7	0.473[1]
Week 6	[17] 25.9 ± 7.5	[17] 28.9 ± 6.1	0.320[2]
Week 12	[15] 24.8 ± 10.2	[16] 26.1 ± 11.3	0.863[2]

[1]Between group comparisons were made using a *t*-test. Probability values P<0.05 are statistically significant; [2]Between group comparisons were made using analysis of covariance (ANCOVA) using baseline as a covariate. Probability values P<0.05 are statistically significant.

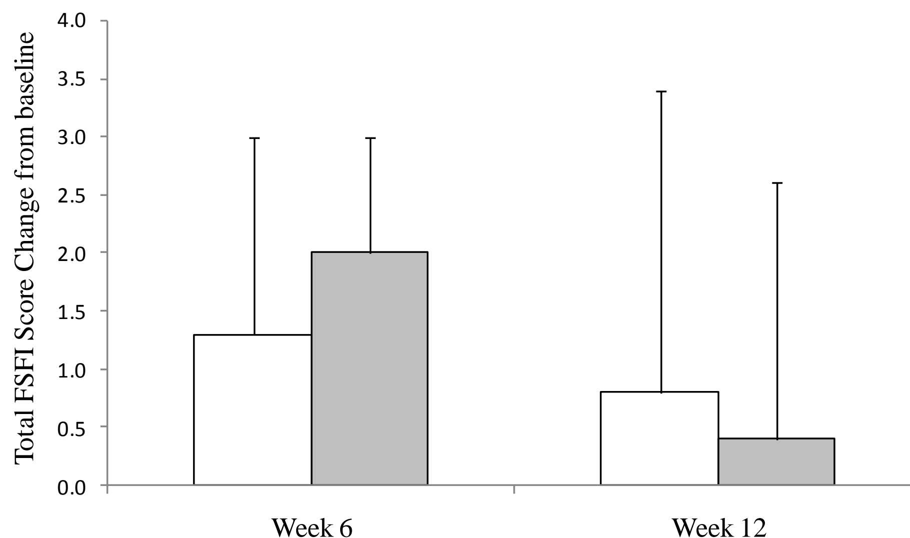

Figure 2. The within-group change of total FSFI score from baseline to week 6 and week 12 for subjects on *Labisia pumila* (white) or placebo (grey).

Table 3. Total SF-36 score of all subjects randomized into the study at baseline, 6 weeks and 12 weeks.

Parameter	*Labisia pumila* [N] Mean ± SD	Placebo [N] Mean ± SD	p-value
SF 36 Total (Score Range 0.0 - 100.0, Higher is better)			
Week 0 (Baseline)	[18] 89.5 ± 5.9	[18] 90.6 ± 4.9	0.520[1]
Week 6	[18] 88.2 ± 5.2	[18] 87.1 ± 9.8	0.442[2]
Week 12	[16] 88.9 ± 6.1	[17] 88.2 ± 8.7	0.676[2]

[1]Between group comparisons were made using a *t*-test. Probability values P<0.05 are statistically significant; [2]between group comparisons were made using analysis of covariance (ANCOVA) using baseline as a covariate. Probability values P<0.05 are statistically significant.

Lipids

For women on *LP*, TC decreased from baseline to week 12 and when compared to placebo, this trended towards a significant difference (Table 4). Compared to placebo, women on *L. pumila* trended towards a reduction in total

Table 4. Total cholesterol values at baseline, week 6 and week 12 for all randomized subjects.

Parameter	*Labisia pumila*	Placebo	p-value
	[N] Mean ± SD	[N] Mean ± SD	
Total cholesterol (mmol/L)			
Week 0 (Baseline)	[18] 5.0 ± 1.0	[18] 4.9 ± 1.1	0.882[1]
Week 6	[18] 4.9 ± 1.1	[18] 4.9 ± 1.0	0.791[2]
Week 12	[16] 4.8 ± 1.0	[17] 5.2 ± 0.9	0.077[2]
LDL-C (mmol/L)			
Week 0 (Baseline)	[18] 3.0 ± 0.9	[18] 2.8 ± 1.0	0.645[1]
Week 6	[18] 2.9 ± 0.9	[18] 2.8 ± 0.9	0.648[2]
Week 12	[16] 2.9 ± 0.8	[17] 3.0 ± 0.8	0.194[2]
HDL-C (mmol/L)			
Week 0 (Baseline)	[18] 1.5 ± 0.3	[18] 1.7 ± 0.3	0.270[1]
Week 6	[18] 1.6 ± 0.3	[18] 1.7 ± 0.3	0.197[2]
Week 12	[16] 1.5 ± 0.3	[17] 1.8 ± 0.3	0.011[2]
Triglyceride (mmol/L)			
Week 0 (Baseline)	[18] 0.9 ± 0.3	[18] 0.9 ± 0.3	0.538[1]
Week 6	[18] 1.1 ± 0.6	[18] 0.9 ± 0.3	0.159[2]
Week 12	[16] 1.0 ± 0.5	[17] 0.9 ± 0.4	0.441[2]
Total cholesterol/HDL-C ratio			
Week 0 (Baseline)	[18] 3.3 ± 0.8	[18] 3.0 ± 0.7	0.276[1]
Week 6	[18] 3.2 ± 0.9	[18] 2.9 ± 0.6	0.326[2]
Week 12	[16] 3.4 ± 0.8	[17] 3.0 ± 0.7	0.435[2]

[1]Between group comparisons were made using a *t*-test. Probability values P<0.05 are statistically significant; [2]between group comparisons were made using analysis of covariance (ANCOVA) using baseline as a covariate. Probability values P<0.05 are statistically significant.

TCC after 12 weeks (p=0.077) (Table 4). HDL-C increased significantly after 12 weeks of treatment in women on placebo, whereas those taking *LP* maintained baseline levels. The TC/HDL-C ratio was not significantly affected in either group (Table 4).

Estradiol-17β

Changes in serum estradiol-17β were not statistically significant between- or within-groups at baseline, week 6 or week 12 for either treatment. Women aged ≥ 50 years showed the lowest estradiol levels during the study period indicating age-related physiological changes associated with menopausal status (< 130 pmol/L). This age category also demonstrated no change from baseline in hormone levels in either treatment group suggesting that *LP* did not adversely affect estradiol levels.

Oxidative stress markers and cytokines

There were no significant differences in the oxidative stress markers 8-isoprostane and SOD at baseline, week 6 or week 12. However, both groups demonstrated a decreasing trend in urinary 8-isoprostane concentrations from baseline to week 12, with women on *LP* having a greater decrease (Δ= -144.4 nmol/L) versus placebo (Δ= -125.9 nmol/L). Compared to baseline, TNF-α decreased in subjects on placebo and increased in subjects on *LP* after 12 weeks of supplementation; however these changes were not significant. There were significant within-group decreases in serum IL-6 from baseline to week 6 in both *LP* (p=0.006) and placebo (p=0.012) but these differences were not sustained through week 12. IL-1β was significantly decreased in women on *LP* from baseline to week 6 and week 12 (p<0.001, p=0.001) (Figure 3). There were no significant differences between groups in serum IL-6 and IL-1β at baseline, week 6 or week 12.

Subgroup analysis

Subgroup analysis of sexually dysfunctional women was classified by baseline FSFI scores ≤26, but sexually active for the duration of the study, showed that women taking *LP* had significantly higher “orgasm” scores versus

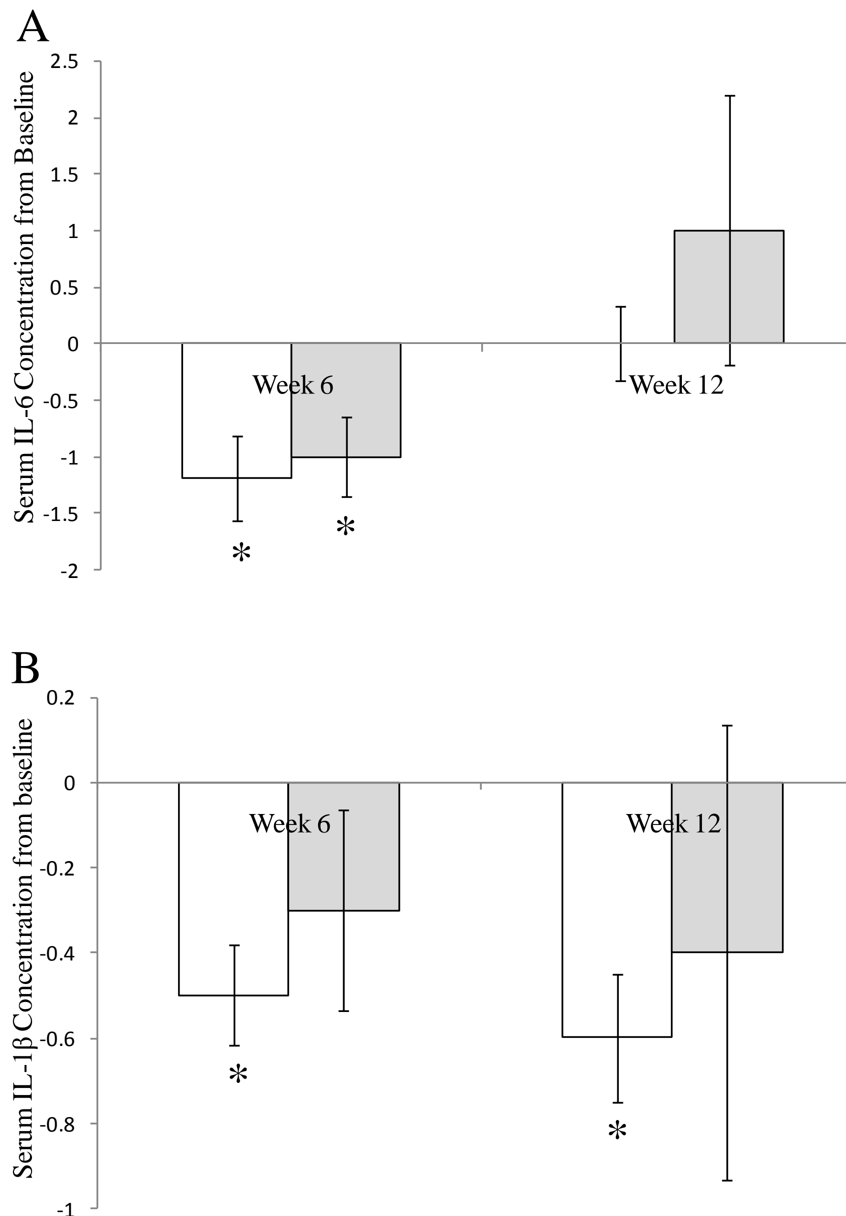

Figure 3. The change from baseline of serum (A) IL-6 and (B) IL-1β concentration at week 6 and 12 for subjects on *Labisia pumila* (white) or placebo (grey). Within-group comparisons were made using a *t*-test and * represents within group significance of P<0.05.

those on placebo (4.1 vs. 2.8, p=0.037). In contrast, women on placebo demonstrated significantly higher scores for "arousal" after 6 weeks of treatment versus women on *LP* (p=0.046); however, these scores were not maintained to week 12. When sexually active women were analyzed to determine whether the difference between *LP* and placebo was different between age groups (18-29, 30-39, 40-49 and ≥50 years), no significant interaction was found for age and total FSFI score, or any individual domain score after 6 and 12 weeks of treatment.

Safety evaluation

No serious AEs were reported during the study. The number of participants reporting AEs was similar in both groups (Table 5). In the placebo group, seven AEs with "possible relation to the treatment" were reported by four participants, while five AEs were reported by four participants in the *LP* group. All AEs except one resolved without any intervention. The other was menstrual cramping and resolved with concomitant medication. Vital signs, biometrics, hematological, clinical chemistry para-

Table 5. Adverse events with "possible" causal relation to the study medication.

Study group	Number of subjects	Adverse events "possibly" related to the medication listed for each subject
Labisia pumila	4	Nausea, menstrual cramps, vaginal spotting, mood alteration, stomach gas
Placebo	4	Breast tenderness, increased vaginal wetness, increased flatulence nausea, headache, mood alteration, constipation

meters and urinalysis were similar in both groups.

DISCUSSION

Sexual desire is a complex phenomenon that involves physiological and psychological influences and is thus difficult to treat (Jha and Thakar, 2010). Therapy options vary depending on the cause of sexual dysfunction, including the use of psychotherapy, prescription of estrogens, progestins or testosterone (Buster, 2013), but the gaps in the body of information related to female sexual dysfunction result in the condition remaining under-reported and poorly managed (Jha and Thakar, 2010). Extracts of *LP* have a history of use in South-East Asian women to maintain reproductive function and enhance sexual function (Melissa et al., 2012). To date, only one pilot human study has been published on the efficacy of water-soluble extracts of *LP* on menopausal symptoms, cardiovascular risk factors and hormonal profiles of Malay postmenopausal women (Kadir et al., 2012). The current study is the first to investigate the safety and efficacy of *LP* in a North American population of pre- and postmenopausal women.

This study recruited healthy women regardless of their level of sexual activity, as FSFI scores were not used as criteria for enrollment. Based on an FSFI cut off score of ≤26, only seven out of 36 enrolled participants were classified as sexually dysfunctional, and two were not sexually active for the duration of the study. As the majority of subjects in this trial were already sexually functional females, highlighting the effectiveness of *LP* in improving FSFI scores may have been confounded. Statistically significant results may have been more easily obtained if recruitment was limited to sexually active but sexually dysfunctional women (FSFI ≤ 26). Further, the absence of differences in FSFI scores between *LP* and placebo may be due to the wide age range of subjects. Subgroup analysis of FSFI scores on sexually active females showed that participants aged 30-39 years, 40-49 years and ≥50 years on herbal treatment performed better than females who were 18-29 years, though no definitive conclusions can be reached due to the small sample size.

Analysis of participants identified as sexually dysfunctional at baseline (FSFI ≤ 26) showed that women on *LP* reported significantly improved scores at week 6 in the "orgasm" domain versus those on the placebo; while higher scores continued to be reported at week 12 in women on *LP*, these did not reach significance. These results are consistent with literature as *LP* is reported to have aphrodisiac properties (Asiah et al., 2007). A bioactive peptide, recognized as an aphrodisiac marker, has also been identified in *L. pumila* (Asiah et al., 2007), perhaps eliciting improvements in FSFI scores. The total FSFI scores were improved in women identified with sexual dysfunction in both *LP* and placebo groups, though no definitive conclusions can be reached due to the small sample size.

The results of the self-reported SF-36 were not influenced by *LP* or placebo. Results from a previous study, in middle aged women, showed that while serious illness, employment and marital status were significant predictors of quality of life, hormone replacement therapy use and menopausal status were not (O'Dea et al., 1999). In a recent placebo-controlled double-blind study in Malaysian women between the ages of 40 and 60 years, a water extract of *LP* reduced anxiety levels by 55% compared to placebo (unpublished data). In the current study, the high baseline SF-36 and FSFI scores may have limited the response to the quality of life measures.

Water extracts of *LP* inhibit estradiol binding to antibodies against estradiol suggesting the presence of estrogen-like or estrogenic compounds (Husniza, 2000). Water extracts of *LP* were found to exhibit high estrogenic activity when tested in an *in vitro* alkaline phosphatase assay using Ishikawa cells and low induction of cell proliferation when compared against a positive estradiol control (Melissa et al., 2013). A study on Wistar rats found that treatment with either *LP* or estrogen replacement therapy had similar efficacy in preventing estrogen deficiency-induced changes from ovariectomy by regulating RANKL, OPG and BMP-2 gene expressions in femoral bones (Fathilah et al., 2013).

In the current study, 44% females on placebo consumed alcohol weekly in comparison to 17% on *LP*. Furthermore, 11% of females on *LP* were current smokers while none were smokers in the placebo group. As alcohol consumption increases circulating estrogen and androgen levels (Purohit, 1998), and smoking has anti-estrogenic effects (Tanko and Christiansen, 2004), these demographic differences may have impacted the results of the current study. Furthermore, participants on placebo had a significantly higher BMI and body weight at baseline in comparison to participants on *LP.* Increased

BMI, waist circumference and hip circumference are associated with increased levels of estrone, estradiol and free estradiol (Purohit, 1998), which may have influenced the results of the current study. Conclusions regarding the interaction between age and estradiol levels are difficult, since these parameters are dependent on the stage of the menstrual cycle at the time of blood sampling as well as the presence or absence of menstruation in the age groups. *LP* may be efficacious in older females deficient in female hormones; however, this was not specifically examined in this study. Previous data did not show significant fluctuations in follicle-stimulating hormone, luteinizing hormone and estradiol during a 280 mg/day intake of *LP* sprayed-dried water extract in 29 postmenopausal women versus placebo (Nik Hazlina et al., 2009b).

The link between cardiovascular disease (CVD) and sexual dysfunction in males is well-established; mild or moderate erectile dysfunction is much more common in patients with CVD (Archer et al., 2005). In contrast, sexual dysfunction in women with CVD has received limited attention (Steinke, 2010). Research has shown that the mechanism of clitoral engorgement is very similar to penile erection, and thus may also be adversely affected by CVD (Steinke, 2010). Neuropathy or vascular disease resulting from CVD risk factors such as hypertension, smoking, diabetes or hyperlipidemia are known organic causes to female sexual dysfunction (Archer et al., 2005).

Studies on rats showed a dose dependent decrease in TC with increasing doses of *LP* extract (unpublished). A significant reduction in trigly-cerides was also reported in post menopausal women on sprayed-dried water extract of *LP* for six months (Nik Hazlina et al., 2009a). 8-isoprostane is regarded as one of the best indices of lipid peroxidation and oxidative stress (Tanko and Christiansen, 2004). The decreasing trend in TC and urinary 8-isoprostane from baseline to week 12 seen in the current study, together with the literature, suggests a role for *LP* in decreasing CVD risk in women.

The analysis of renal and liver function tests, CBC and other clinical chemistry parameters showed that *L. pumila* was safe and well tolerated at a dose of 200 mg/day in the population studied. This is consistent with data from a six-month randomized placebo-controlled trial in postmen-pausal women using dose regimens of up to 560 mg sprayed-dried water extract of *LP* (McTiernan et al., 2006; Nik Hazlina et al., 2009a; Nik Hazlina et al., 2009b).

The randomization schedule was designed to prevent an imbalance between groups with respect to meno-pausal status; therefore it is possible to suggest that the menopausal status of women did not affect the compa-rability of data between groups. As this was a pilot study, the purpose being to explore the effects of *LP* over a wide range of women, recruitment of subjects were not limited to a particular age-group or their sexual function.

An analysis of sexually active women on *LP* or placebo found no significant interaction between age and total FSFI scores, or any individual domain scores after 6 and 12 weeks of treatment. However, this may certainly be due to the small sample sizes of the subgroups. The small sample size limited the ability to stratify the population by age, BMI and menstrual status, and was certainly a limitation of this study and may have impacted the results. Identifying populations by sexual dysfunction defined by FSFI at recruitment as well as those sexually active may have provided better evaluation of the efficacy of *L. pumila* in improving sexual function. Lastly, the improvements in FSFI scores in the placebo group at week 6 that were not sustained through to week 12 could have been expected, as previous reports have suggested a placebo response in the treatment of sexual dysfunction (Bradford and Meston, 2009, 2011). A run-in period prior to enrollment in the study may have improved the response in FSFI scores by controlling for placebo effect.

Conclusions

This study investigates the efficacy of *LP* in healthy women regardless of their level of sexual function. While there were no significant differences between groups in the total FSFI score or domain scores for desire, arousal, lubrication, orgasm, satisfaction and pain at baseline or at 6 and 12 weeks of treatment, *LP* demonstrated a trend towards an improvement in TC, urinary 8-isoprostane and significant within group improvement in IL-6 and IL-1β suggesting a role for *LP* in improving inflammation. Future research of the effectiveness of *LP* on women's sexual well-being should focus on an older population of subjects that are sexually dysfunctional.

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Full Length Research Paper

Resistance-related gene transcription and antioxidant enzyme activity in *Nicotiana* spp. resistant to anthracnose

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The two tobacco relatives of *Nicotiana alata* and *Nicotiana longiflora* display a high level of resistance against *Colletotrichum nicotianae* and the two genes *NTF6* and *NtPAL* related to pathogen defense transcription were higher in *N. alata* and *N. longiflora* than the commercial cv. K326. Inoculation with *C. nicotianae* reduced the abundance of *NTF6* and *NtPAL* transcript during the first 48 h post inoculation (hpi) when only biotrophic hyphae were present, but increased it over the following 24 h as necrotrophic hyphae began to predominate. Activity levels of phenylalanine ammonia lyase, peroxidase and polyphenol oxidase changed markedly at 72 hpi. The conclusion was that the pathogen enters the host leaf within 24 hpi, triggering the up-regulation of various defense-related genes in a resistant host plant. By 72 hpi, the pathogen switched to necrotrophic growth to avoid contact with the increasing presence of host defense compounds.

Key words: *Nicotiana alata*, *Nicotiana longiflora*, tobacco anthracnose resistance-related gene, antioxidant enzyme activity.

INTRODUCTION

Plants have evolved a multifaceted system of defense against pathogens, some of which are highly specific to a particular microbial species (Doehlemann et al., 2008). The host response typically involves a concerted series of events ranging from the rapid generation of reactive oxygen and nitrogen species, through changes in ion flux across the plasma membrane, proteolysis, and the reprogramming of hundreds of genes (Dangl and Jones, 2001). Although, many components of the plant defense signal transduction pathway remain obscure, those responsible for both salicylic acid-dependent systemic acquired resistance and jasmonate- and ethylene-induced resistance are well documented (Hammond-Kosack and Parker, 2003; Bari and Jones, 2009). For biotrophic pathogens, effectors trigger the plant's defense responses and salicylic acid signaling is used to initiate apoptosis at the site of the pathogen's entry. The dying cells can, however, support the growth of necrotrophic pathogens (Doehlemann et al., 2008). The defense response therefore has evolved such that the salicylic acid-dependent pathway is largely activated by biotrophic pathogens, while the jasmonate- and ethylene-dependent pathway operates against necrotrophic pathogens and insects (Qiu and Wang, 2007). Associated with the defense signal transduction network, plants have also evolved a set of antioxidation strategies which are brought into play to miti-

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gate against the deleterious effects of reactive oxygen species. Prominent among the enzymes involved in reactive oxygen species scavenging are phenylalanine ammonia lyase (PAL), various peroxidases (POD) and various polyphenol oxidases (PPO) (Averyanov, 2009).

PAL features in the phenylpropanoid metabolic pathway, which is responsible for lignin synthesis (Galis et al., 2006; Ferrareze et al., 2013). POD enzymes may also participate in lignin and suberin synthesis, as well as acting to underpin the production of various molecules endowed with antibiotic properties (Goodman et al., 1986; Averyanov, 2009). Increased levels of POD activity have been observed in conjunction with both local and systemic disease resistance (Karban et al., 1989; Kombrink and Somssich, 1995). PPO catalyzes the oxygen dependent oxidation of phenolics to quinines (Li and Steffens, 2002). The extent of the damage caused by reactive oxygen species depends to a large extent on the level of coordination among the various scavenging enzymes (Hao et al., 2012). Tobacco anthracnose, the causative agent of which is the hemibiotrophic fungus *Colletotrichum nicotianae* (Lucas, 1965), is a highly destructive pathogen of tobacco seedlings. *C. nicotianae* is classified as a hemibiotroph because it initially establishes a biotrophic interaction with its host before eventually switching to its destructive necrotrophic lifestyle (Shen et al., 2001). Two close relatives of cultivated tobacco, namely *Nicotiana alata* and *Nicotiana longiflora*, which are grown mainly as ornamentals, have been shown to be extremely resistant to anthracnose. Here, we set out to elucidate the mechanisms controlling their resistance by comparing their transcription profiles of known defense-related genes, and the activity levels of their antioxidant enzymes.

A better understanding of the gene network underlying anthracnose resistance in these tobacco relatives may help elaborate resistance breeding strategies in the commercial crop species.

MATERIALS AND METHODS

Fungal inoculation

An isolate of *C. nicotianae* was cultured on potato dextrose agar (PDA; 39 g of PDA, 1 L of water) under continuous fluorescent light at 25°C for about seven days until the surface of the plate was almost covered with mycelia as well as conidia. Each Petri plate of culture was flooded with 10 ml sterilized distilled water and conidia were released by scraping with a metal spatula. The resulting suspension was filtered through two layers of cheesecloth and 0.02% (v/v) Tween 20 was added as a surfactant. The concentration of conidia was adjusted to $\approx 1 \times 10^7$ spores/ml by hemacytometer prior to inoculation. Tobacco seedlings were grown at 24°C and a relative humidity of 30% under a 16 h photoperiod with a photosynthetically active radiation intensity of 200 μmol $s^{-1}m^{-2}$. At the six leaf stage, leaves were rinsed in sterile water and coated with the *C. nicotianae* conidia suspension. Negative control leaves were rinsed in 0.02% Tween 20. After inoculation, the seedlings were enclosed in a plastic bag and held at 26°C for 24 h in the dark, and then immediately transferred into a 24°C 14 h photoperiod, 95% relative humidity regime with the bag removed. Leaf samples were harvested immediately and then after a set number of hours (as below) depending on whether the assay was for gene transcript or gene product.

Resistance to anthracnose diseases

We evaluated resistance of the two tobacco relatives of *N. alata*, *N. longiflora* and cv. K326 to anthracnose. Susceptibility to anthracnose infection was evaluated at 30 days after inoculation based on Chen et al., (1997) disease index. In order to explore the resistance of two tobacco *N. alata* and *N. longiflora* to the anthracnose pathogen. Using the percent disease index as the reference standard, the percent disease index was scored after inoculation in intensity scale of 0 to 5: No infection = 0, 0.1 to 11%; leaf area affected = 1, 11.1, -33%; leaf area affected = 2, 33.1 to 55%; leaf area affected = 3, 55.1 to 77%; leaf area affected = 4, a77.1%; leaf area affected = 5. Percent disease incidence (PDI) was calculated based on the following formula:

$$PDI = \frac{\text{Sum of all numerical grades}}{\text{Total number of leaves counted} \times \text{Maximum grade}} \times 100$$

Primer design, RNA isolation and reverse transcription

Primer pairs of genes (Table 2) were designed using Primer Premier 5.0 software under default parameters and were custom-ordered from a commercial supplier (Invitrigen, Guangzhou). The specifity of each primer was confirmed by melting-curve analysis after amplification for the six genes (Figure 1a). Standard curves using a dilution series of the cDNA (from each species of tobacco and spanning six orders of magnitude) were made to calculate the gene-specific PCR efficiency and regression coefficient (R^2) for each gene (Table 2). Total RNA was extracted from leaf tissue at each time point (0, 1, 2, 3, 6, 12, 24, 48 and 72 hpi) using the TRIzol reagent (Invitrogen) following the manufacturer's protocol. This preparation was treated with RQ1 RNase-Free DNase (Promega) before conversion to cDNA using a M-MLV reverse transcriptase kit (Promega). Two independent reverse transcription (RT) reactions were pooled from each leaf processed (three biological replicates per harvest time). The subsequent quantitative real time (qRT) PCRs were run as three technical replicates per each pooled cDNA. The qRT PCRs were implemented on a TP900 Thermal Cycler DiceTM Real Time System (TakaRa) using a SYBR Premix Ex Taq kit (TaKaRa). Specific primers were developed for five separate defense-related genes based on public domain sequences (Table 2). Each PCR comprised 12.5 μL SYBR®Green Real-time PCR Master Mix (TaKaRa), 1.0 μL of each primer (10 μM), 9.5 μL ddH_2O and 1 μL cDNA. The reactions were subjected to 40 cycles of 95°C/30 s, 58°C/30 s, 72°C/60 s.

The fluorescence signal detected at the end of each extension step was used to generate an amplification profile. The transcription data were normalized against those obtained by monitoring tobacco *β-actin* using the ΔΔCT method (Livak and Schmittgen, 2001).

PAL, PPO and POD extraction and activity measurement

Leaves were harvested at give the sampling hpi's, frozen in liquid nitrogen, and ground to a fine powder, which was stored at -80°C until required. The assay for PAL was derived by extracting 0.25 g leaf powder with 1 ml 0.1 M sodium borate buffer (pH 8.8) for 30 min at 4°C, while those for both PPO and POD were obtained from 0.20 g powder extracted in 1 ml 0.1 M sodium phosphate buffer (pH 6.8) for 30 min at 4°C. The suspensions were centrifuged (10,000 *g*, 15 min, 4°C) and the crude supernatant used to assay for the activity of PAL, PPO and POD. PAL activity was determined by mixing 3.4 ml deionized water, 0.6 ml of a 100 mM solution of L-phenlyalanine, and 2 ml of the solution containing the PAL extract. The mixture was kept in a water bath at 40°C for 45 min and it was read at 290 nm in a spectrophotometer (Shimadzu UV-2550) against a blank made with

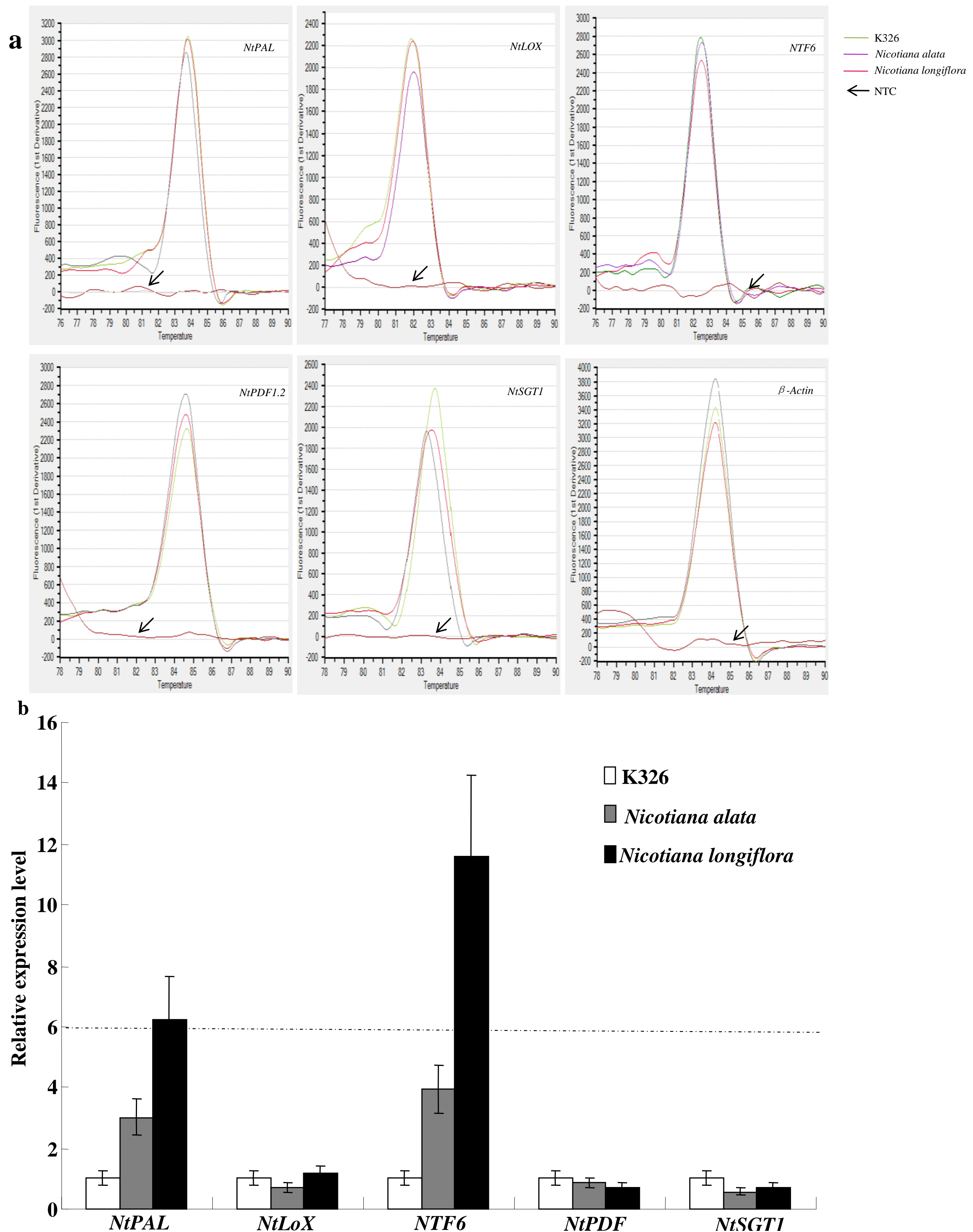

Figure 1. Specificity of primer pairs for RT-qPCR amplification. Melt curve showed a single peak for each selected reference gene and no amplicon was observed in no template control (NTC) indicated by arrows (a), real-time qRT-PCR analysis of the transcription of selected defense-related genes in three *Nicotiana* spp. The *β-actin* gene was used as a reference. Data expressed as transcription abundances relative to that present in cv. K326, and are given in the form mean ± standard error (SD). Dashed lines indicate the 6-fold threshold level (b).

4 ml water and 2 ml of the solution containing the PAL extract. Enzymatic activity is reported as micromoles of cinnamic acid/milligrams of protein (Gonzalez-Aguilar et al., 2004). PPO activity was determined using a spectrophotometric method based on an initial rate of increase in absorbance at 410 nm. Phosphate buffer solution pH 7 (0.1 M, 1.95 ml), 1 ml of 0.1 M catechol as a substrate and 50 µL of the enzyme extract were pipetted into a test tube and mixed thoroughly. Then, the mixture was rapidly transferred to a 1-cm path length cuvette. The absorbance at 410 nm was recorded continuously at 25°C for 5 min using ultraviolet-visible spectrophotometer, Shimadzu UV-2550 (Flurkey, 1985). POD activity was assayed spectrophotometrically at 470 nm (Shimadzu UV-2550) using guaiacol as a phenolic substrate with hydrogen peroxide.

0.1 ml of leaf extract was mixed with 2.9 ml of 25 mm sodium acetate buffer, pH 4.5, and 0.05 ml of 20 mm guaiacol. The reaction was started by addition of 0.01 ml of 40 mm H_20_2, and the initial rate of increase of absorbance at 470 nm was measured. One milli-unit of enzyme is the amount causing an absorbance increase of 1.0 per min at 24°C (Rathmell and Sequeira, 1974).

Statistical analysis

Means were compared using the Student's *t* test implemented in SPSS v11.5 for Windows (Microsoft). Data are given in the form of mean ± standard error (SD).

RESULTS

The resistance to anthracnose diseases

The results show that the disease index of cv. k326 was 74.17%, and the two tobacco relatives of *N. alata* and *N. longiflora* were 23.33 and 26.67%, which displayed high levels of resistance against the anthracnose pathogen (Table 1).

The transcription of defense-related genes

The transcription of all five target genes (*NtPAL*, *NtLox*, *NTF6*, *NtPDF* and *NtSGT1*) was detectable in mock-inoculated *N. alata*, *N. longiflora* and cv. K326. The abundance of both *NtPAL* and *NTF6* transcript was higher in *N. alata* and *N. longiflora* than in cv. K326, whereas that of the other three genes was comparable across the three hosts (Figure 1b). *NtPAL* and *NTF6* were therefore targeted for a temporal analysis of transcription following inoculation with *C. nicotianae*. In *N. alata*, *NTF6* transcription was induced by about 6.9 fold by 1 hpi; it decreased over the period 2 to 12 hpi, peaked again to 13.3 fold of background around 24 hpi, fell to its minimum level at 48 hpi and finally recovered to 0.8 fold of background by 72 hpi. In *N. longiflora*, the same gene was induced by 6.6 fold at 3 hpi, fell to 1.7 fold by 6 hpi and peaked to 81.2 fold of background at 12 hpi. In cv. K326, *NTF6* transcription was also induced by inoculation, peaking at 12 hpi but to a level substantially below that reached in either *N. alata* or *N. longiflora* (Figure 2a). The transcription response of *NtPAL* in *N. alata* and *N. longiflora* was also induced by the infection of pathogen (Figure 2b). The abundance of *NtPAL* transcript in *N. alata* was 21.0 fold above background at 6 hpi, less than background in the period 12 to 48 hpi, but had recovered by 72 hpi. In *N. longiflora*, transcription peaked at 3.1 fold of background at 12 hpi, fell to a minimum level at 48 hpi, and had recovered partially by 72 hpi. The level of *NtPAL* transcription in cv. K326 was substantially lower than in either *N. alata* or *N. longiflora*, but peaked to 2.2 fold of background at 6 hpi.

Antioxidant enzyme activity

PAL activity in *N. alata* reduced over the period 24 to 72 hpi, but then increased rapidly up to the activity peak of PAL was 16.18 $U.g^{-1}Fw.min^{-1}$ in the 168 dpi, higher 0.6 fold the level detected in the inoculated cv. K326. At all time points (except for 72 hpi, where the difference was significant), the activity level in the inoculated plants exceed that in the inoculated cv. K326 (Figure 3a). Both PPO and POD activity rose over the first 72 hpi, peaking at, respectively, 2.1 and 2.6 fold the level detected in the inoculated cv. K326 (Figure 3b and 3c). PAL activity in *N. longiflora* behaved in a similar fashion. Its level reached a minimum at 120 hpi, and was lower than in the inoculated cv. K326 by the end of the measurement period. The activity peak of PAL was 18.18 $U.g^{-1}Fw.min^{-1}$ in the 168 dpi, higher 0.8 fold the level detected in the inoculated cv. K326 (Figure 3a). PPO activity had fallen by 4.1 fold at 72 hpi and remained below that of the inoculated cv. K326 until 168 hpi (Figure 3b). Meanwhile, POD activity increased sharply over the first 24 hpi and then declined up to 72 hpi, thereafter recovering somewhat (Figure 3c).

DISCUSSION

The MAP kinase encoding gene *NTF6* is a key regulator of SAR, since its silencing has been recorded as attenuating both the resistance of tobacco to tobacco mosaic virus and of tomato to *Pseudomonas syringae* (Liu et al., 2004; Ekengren et al., 2003). Here, *NTF6* transcription was found to be induced by infection with *C. nicotianae*, which suggests that similar, if not the identical MAP kinases are involved in the host's response to virus, bacterial and fungal pathogens, as proposed by Liu et al. (2004) and Bartels et al. (2009). The double peak of *NTF6* induction observed during the *C. nicotianae* infection process was remarkable. The first coincided with the fungal penetration stage (1 to 3 hpi) and a second, much stronger one with the formation of an infection vesicle in the host cell (12 to 24 hpi) (Shen et al. 2001). In contrast, in the susceptible host cv. K326, there was little or no evidence of induction at either of these time points. The inference is that resistant types such as *N. alata* and *N. longiflora*, but not susceptible ones such as cv. K326, are able to sense the occurrence of developmental changes in the pathogen and use this information to mount a successful defense. Such a qualitative difference between resistance and susceptibility is consistent with the consensus view that in a susceptible host, the defense response tends to be delayed and not very intense (Métraux et al.,

Table 1. Resistance of different cultivars tobacco to anthracnose.

Cultivar	Number of treatment	Disease index 0	1	2	3	4	5	Percent disease incidence (%)	Significant difference
N. longiflora		12	8	7	2	1	0	26.67	b
N. alata	30	13	9	6	1	1	0	23.33	c
K326		1	2	5	11	11	0	74.17	a

Table 2. Primers sequences and amplicon characteristics of RT-qPCR.

Gene name	Primer pair	GenBank	*Nicotiana alata*		*Nicotiana longiflora*		K326	
			Regression coefficient (R^2)	Amplification efficiency (%)	Regression coefficient (R^2)	Amplification efficiency (%)	Regression coefficient (R^2)	Amplification efficiency (%)
NtPAL	5'-TCGGGCTTTCCATTCATCACC-3' 5'-AAGAAGCGTTCCGTGTTGCTG-3'	AB289452	0.993	109.4	0.994	112.3	0.998	113.5
NtLOX	5'-GCCTATGCAGCAGTGAATGA-3' 5′-TGCCTATTTGTTGCGATCA-3′	X84040.1	0.995	103.5	0.997	105.2	0.999	102.1
NTF6	5'-GCTATTAGGTTCGCCAGAG-3' 5'-CCTCAACAGTTATGCGTTTAGC-3'	AY547494.1	0.998	107.8	0.991	101.9	0.994	103.9
NtPDF1.2	5'-GGAAATGGCAAACTCCATGCG-3' 5'-ATCCTTCGGTCAGACAAACG-3'	X99403.1	0.999	99.3	0.996	99.8	0.998	99.5
NtSGT1	5'-TCCACACTCGCCATCCTGAA-3' 5'-GTGCCGCAAATTCAACTCTG-3'	AF516180	0.994	105.7	0.995	103.6	0.997	105.9
β-actin	5'-ATGCCTATGTGGGTGACGAAG-3' 5'-TCTGTTGGCCTTAGGGTTGAG-3'	U60495	0.998	104.7	0.996	105.7	0.994	105.9

2009). *NtPAL* encodes the first committed enzyme in the phenylpropanoid pathway, which is responsible for the synthesis of both phytoalexins and lignin. Both these compounds are implicated in preventing the pathogen from penetrating the cell wall (Shadle et al., 2003; Sun et al., 2013). Here, *NtPAL* transcription was substantially induced at an early stage in the infection process in *N. alata*, but was not induced in either *N. longiflora* or cv. K326. The differential response between the two resistant hosts implies that there is more than one specific mechanism underpinning resistance against *C. nicotianae* even within the same genus. Biotrophs such as *Uromyces vignae* and *Uromyces maydis*, as well as hemibiotrophs such as *Mycosphaerella graminicola* during their biotrophic phase, need to suppress or at least attenuate the host's defense resposne if they are to successfully parasitize the host (Panstruga, 2003; Caldo et al., 2006; Jones and Dangl, 2006). In maize infected with *U. maydis*, the initial response involves the activation

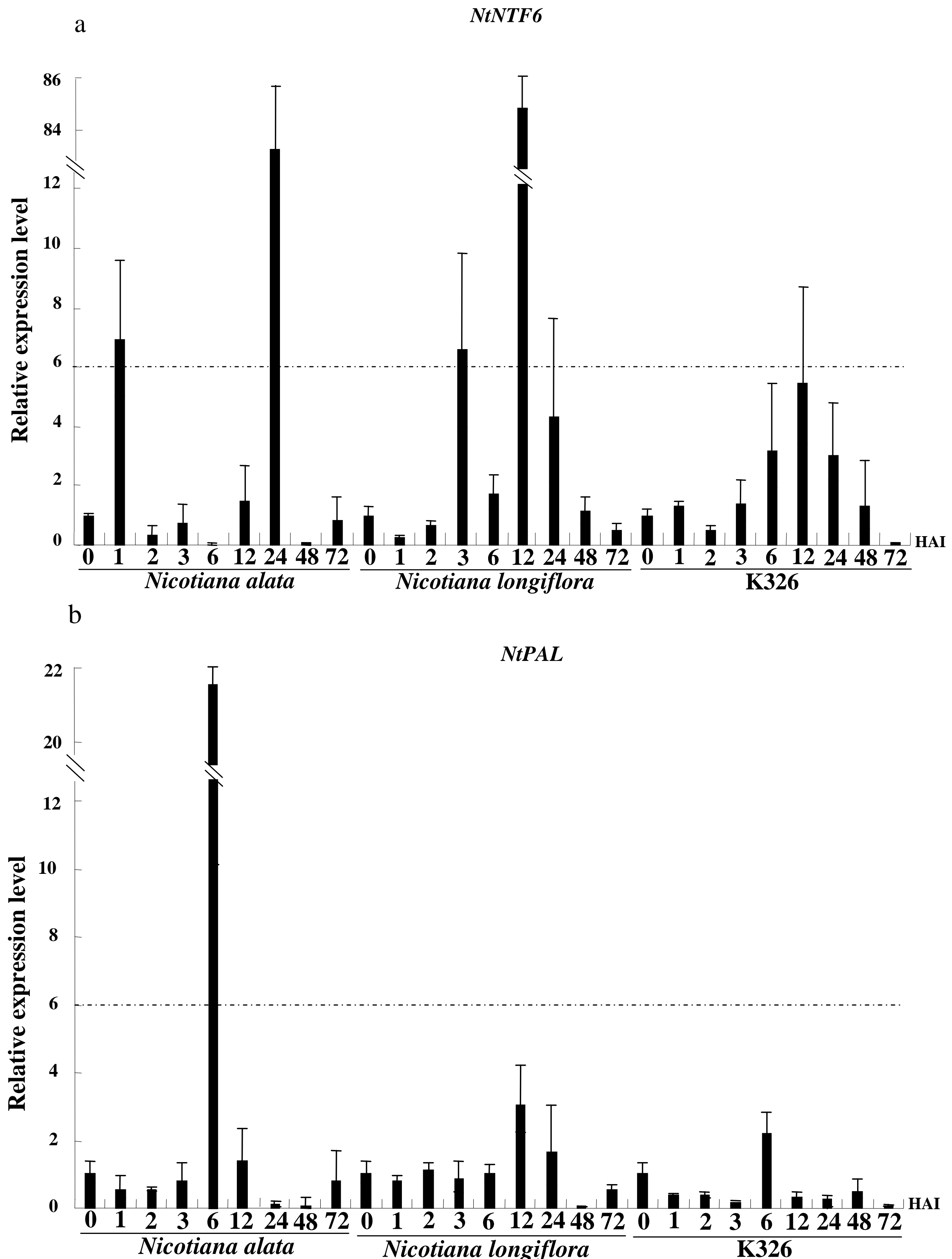

Figure 2. Transcription of **(a)** *NTF6* and **(b)** *NtPAL* following infection by *C. nicotianae*. Transcript abundance was quantified using real time qRT-PCR at a number of time points after inoculation. Transcript abundances were normalized with respect to that of the reference gene *β-actin*, and expressed relative to the level present in mock-inoculated plants for each time point. Values given in the form mean ± SD. Dashed lines indicate the sixfold threshold level.

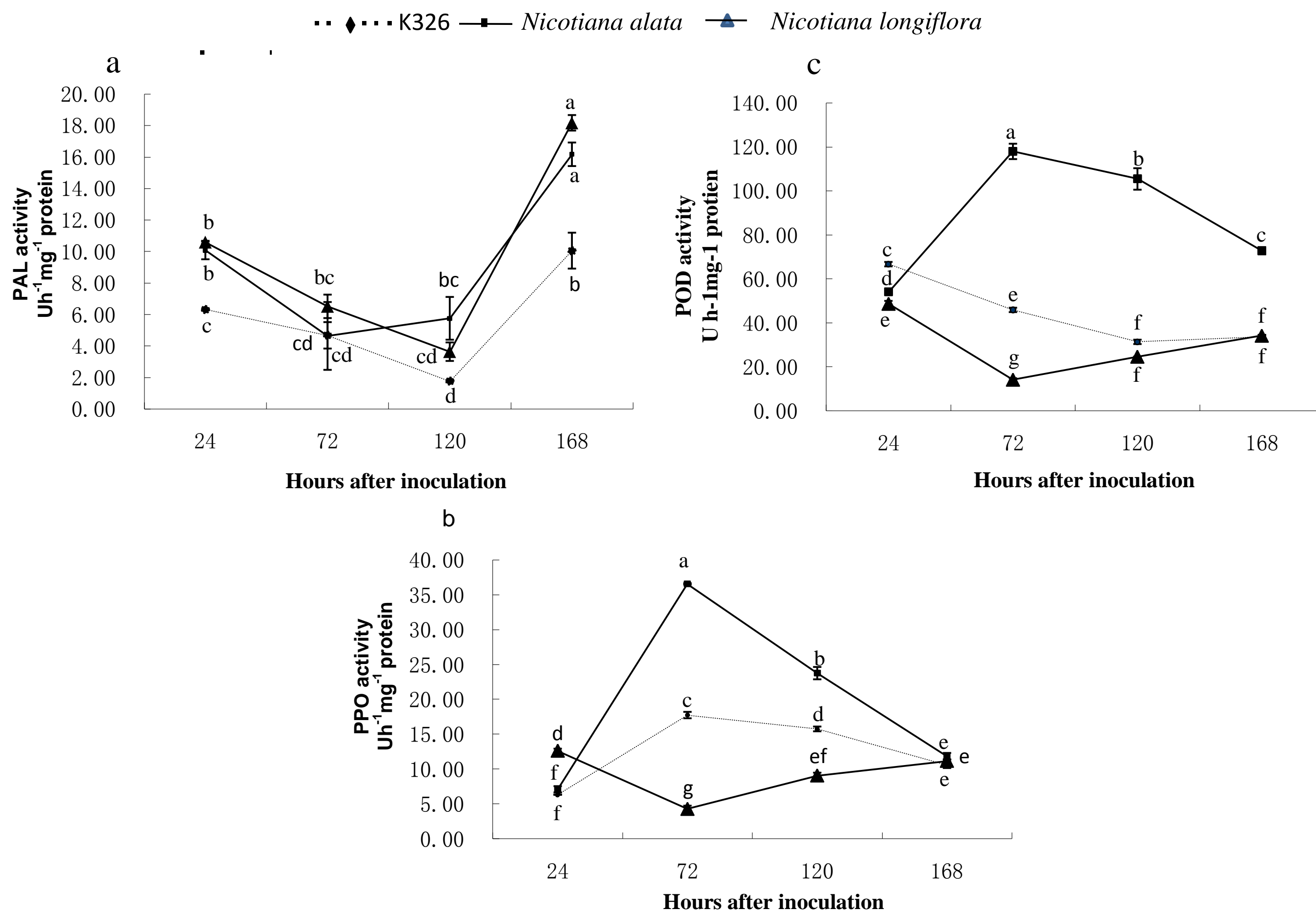

Figure 3. Defense response-related enzyme activity in *N. alata* and *N. longiflora* infected by *C. nicotianae*. **(a)** PAL, **(b)** PPO and **(c)** POD. Values given in the form mean ± SD based on $n = 3$ (two technical replicates). Different lower case letters at each point indicate statistically significant differences ($P < 0.05$).

of a number of defense-related genes, but the effect is only transient, thereby allowing the establishment of the pathogen's biotrophic growth (Doehlemann et al., 2008). Here, we have shown similarly that in the tobacco *C. nicotianae* interaction, there was evidence for the transient activation of defense-related genes. Once the biotrophic growth of the pathogen became established (between 48 and 72 hpi), the transcription of both *NTF6* and *NtPAL* was substantially suppressed, and after this time point, necrotrophic hyphae began to predominate; thus, the developmental change in the pathogen was readily recognized by the host. The 72 hpi time point featured too in the antioxidant enzyme response. The probability is that during the first 24 hpi, the pathogen locates the stomatal cavities on the tobacco leaf and penetrates the leaf via the stomata. In a resistant host, this event stimulates the induction of a whole spectrum of defense-related genes. By 72 hpi, the pathogen has switched from biotrophic to necrotrphic growth, and thereby succeeds in avoiding exposure to an increasing concentration of defense compounds.

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Full Length Research Paper

Potentiality of benthic dinoflagellate cultures and screening of their bioactivities in Jeju Island, Korea

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Eleven strains of benthic dinoflagellates (*Amphidinium carterae* (D1), *Prorocentrum rhathymum* (D2), *Symbiodinium* sp. (D3), *Coolia malayensis* 1 (D4), *Ostreopsis ovata* 1 (D5), *Ostreopsis ovata* 2 (D6), *Coolia malayensis* 2 (D7), *Amphidinium operculatum* 1 (D8), *Heterocapsa psammophila* (D9), *Coolia malayensis* 3 (D10) and *Amphidinium operculatum* 2 (D11)) were collected in Jeju Island, Korea and cultured in 20 L carboys after establishing unialgal cultures. Their growth potential and biomass productivity were evaluated using two different culture media (IMK and f/2 medium); it was found that IMK medium has the potential to culture benthic dinoflagellates compared to commonly used f/2 medium. Among the benthic dinoflagellates, *A. carterae* (D1) had the maximum cell density (148.6×10^3 cells mL^{-1}), growth rate (0.317 ± 0.01 divisions day^{-1}) and biomass (0.260 ± 0.03 g L^{-1} dry weight) in IMK medium at 20 days of culture. Also, screened bioactivities among the methanolic extracts of cultured dinoflagellates showed *A. carterae* (D1) to have the highest antioxidant and anti-inflammatory effect and *O. ovata* 1 (D5) had the highest anticancer activity compared to the other strains. Taken together, this is the first report on the growth potential and biomass production of benthic dinoflagellate strains isolated from Jeju Island in appropriate culture medium as well as their importance in potential pharmacological applications.

Key words: *Amphidinium carterae*, benthic dinoflagellates, biomass, bioactivities, culture conditions, Jeju Island.

INTRODUCTION

Dinoflagellates are the largest group of eukaryotic microalgae with approximately 2000 living species (Taylor et al., 2008). Marine benthic dinoflagellates are unicellular organisms that have received a great attraction due to their importance as primary producers in the marine ecosystem and also due to their encounter with potentially harmful species, which causes economic losses (particularly in the aquaculture, recreation and tourism industries) as follows: death of fish/shellfish through toxicity, human health problems (for example, paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), azaspiracid poisoning (AZP), diarrheic

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Abbreviations: PSP, paralytic shellfish poisoning; **NSP,** neurotoxic shellfish poisoning; **AZP,** azaspiracid poisoning; **DSP,** diarrheic shellfish poisoning; **CFP,** ciguatera fish poisoning; **DPPH,** 1,1-diphenyl-2-pricrylhydrazyl; **ESR,** electro spin resonance; **DMEM,** Dulbecco's modified eagle medium; **RPMI,** Roswell park memorial institute; **FBS,** fetal bovine serum; **NO,** nitric oxide; **ELISA,** enzyme-linked immunosorbent assay; **MTT,** 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide; **DMSO,** dimethylsulfoxide.

shellfish poisoning (DSP) and ciguatera fish poisoning (CFP) in subtropical to tropical coasts (Godhe et al., 2002; Gilbert et al., 2005; Hallegraeff, 1993; Lehane and Lewis, 2000). Some of the most common benthic dinoflagellate species in tropical and subtropical marine environments belong to the genera, *Amphidinium, Coolia, Gambierdiscus, Ostreopsis, Prorocentrum,* among others. Benthic dinoflagellates possess unique structures with powerful bioactive secondary metabolites (Kita et al., 2005; Kobayashi et al., 2003; Onodera et al., 2005) and bio-toxins (Daranas et al., 2001). In particular, different types of toxins have been detected in different species of benthic dinoflagellates such as *Amphidinium carterae* (Haemolysins) (Nakajima et al., 1981; Yasumoto et al., 1987), *Prorocentum lima* (okaidic acid and dinophysistoxins) (Murakami et al., 1982), *Prorocentrum rhathymum* (water-soluble fast-acting toxins and hemolytic effects) (Nakajima et al., 1981; Tindall et al. 1989), *Coolia monotis* (cooliatoxin) (Holmes et al., 1995) and *Ostreopsis* sp. (toxic butanol-soluble compound, palytoxin analogue) (Elbrächter and Faust, 2002; Nakajima et al., 1981; Usami et al., 1995).

Over the years, only a few studies have been done in evaluating the specific bioactivities of cultured dinoflagellates (Camacho et al., 2007; Nakagawa et al., 1998). They have been recognized as potential sources bearing novel compounds for appraisal as pharmaceuticals (Camacho et al., 2007; Dragunow et al., 2005). Moreover, bioactive components are gaining the interest of researchers because of their potential usefulness in many applications. Toxic metabolites have been reported from cultured dinoflagellates and are considered as valuable laboratory tools in the case of drug discovery (Elbrächter and Faust, 2002; Nakajima et al., 1981). Hence, bioactive components can be isolated from benthic dinoflagellates and rendered in a range of biological activities, including cytotoxic, antitumor, antibiotic, antifungal, immunosuppressant and neurotoxic (Camacho et al., 2007; Wright and Cembella, 1998).

Dinoflagellates' bioactive molecules are inaccessible in large quantities and this severely hampers research in potential applications of these compounds (Camacho et al., 2007). Some of the toxins which can be extracted from dinoflagellates are quite expensive, even in small amounts (Belarbi et al., 2003; Kobayashi and Tsuda, 2004). In order to utilize the toxins and other bioactive molecules produced by mass scale cultivated dinoflagellates, research on biomedical, toxicological, chemical, pharmacological and therapeutic potential is essential. In fact, the study of their growth, high cell density, high productivity and physiology in cultures is considered a worthwhile.

Biomass production and utilization of microalgae culture has been quite successful recently (Sevilla et al., 2010); however, little effort has been devoted to mass culture of dinoflagellates in bioreactors (Beuzenberg et al., 2012; Grünewald et al., 2013; Rodríguez et al., 2010b; Walid et al., 2011). The effectiveness of the quantity of extracted bioactive components depends on the culturing systems and productivity of dinoflagellate strains. For the optimization of growth conditions and toxin production of benthic dinoflagellate, various kinds of research have been conducted in different parts of the world (Dixon and Syrett, 1988; Kitaya et al., 2008; Morton et al., 1992; Nascimento et al., 2012; Pistocchi et al., 2011; Rhodes and Thomas, 1997; Tanimoto et al., 2013; Tosteson et al., 1989; Vidyarathna and Granéli, 2012; Yamaguchi et al., 2012; Zimmermann, 2006). However, most studies were only done for small-scale cultures. The maximum biomass concentration was attained below 1 g L^{-1} in a typical photosynthetic culture of dinoflagellate (Rodríguez et al., 2010b). Due to slow growth rates of dinoflagellates, large volume of culture is necessary to produce sufficient material for characterization of novel compounds and toxin production (Beuzenberg et al., 2012; Rodríguez et al., 2010a). In fact, there is no previous research on culturing of marine dinoflagellates and exploration of their bioactive components from Jeju Island, Korea. Hence, a special effort must be done in developing stable and reliable culture systems for the desirable culture strains. Therefore, the aim of this study was to evaluate the potentiality of growth and biomass productivity of benthic dinoflagellates using two culture media, which were collected from different coastal sites of the Jeju Island in Korea. Then, the biomasses of the 11 selected strains of benthic dinoflagellates were screened to evaluate the anti-inflammatory, anticancer and antioxidants effects using chemical and *in vitro* assays.

MATERIALS AND METHODS

Dinoflagellates isolation and culture

Dinoflagellate strains were collected from sand and macroalgae from the Coast of Jeju Island, Korea in 2011. The species were identified by morphological feature using light microscope (Zeiss Axioplan 2, Germany) with digital camera (Axiocam ERc5s) and epifluorescence microscope (violate excitation ca 430 nm, blue emission ca 490 nm; Zeiss Axioplan 2, Germany; Axiocam ICm1 digital camera) with Calcofluor White M2R (Fritz and Triemer 1985). Cell size was measured for the preliminary identification of the dinoflagellates. Taxonomic identification was done based on our previous research (Shah et al., 2013). Identification was also confirmed by molecular analysis (genomic DNA extraction, LSU rDNA D1D3 region sequenced and phylogenetic analysis) (unpublished data). Summary of the benthic dinoflagellates used for this study is presented in Table 1.

Single cells were isolated by the capillary pipette washing method under an inverted microscope (Olympus 1X71, Olympus, Tokyo, Japan) and transferred to a 24-multiwell plate containing 2 mL of IMK culture medium (Nihon Pharmaceutical Co., Ltd., Japan) (Yamaguchi et al., 2012). IMK medium contained the following components: 2.35 mM $NaNO_3$, 50.1 μM NH_4Cl, 9.86 μM Na_2HPO_4, 28.7 μM K_2HPO_4, 12.3 μM Fe-ethylenediaminetetraacetic acid (Fe-EDTA), 0.871 μM Mn-EDTA, 111 μM Na_2EDTA, 0.01 μM $CuSO_4{\cdot}5H_2O$, 0.03 μM $Na_2MoO_4{\cdot}2H_2O$, 0.08 μM $ZnSO_4{\cdot}7H2O$, 49.8 nM $CoSO_4{\cdot}7H_2O$, 0.91 μM $MnCl_2{\cdot}4H_2O$, 0.0132 μM H_2SeO_3, 0.001

Table 1. List of benthic dinoflagellates from Jeju island, Korea used for 20 L culture.

Sample no.	Species/strains	Collected location		Isolation date	Cell size	
		Name	Latitude/ longitude		Length (μm)	Width (μm)
D1	*Amphidinium carterae* (JHWAC)	Hwasun	33° 14' 22.38'' N 126° 19' 55.67''E	15.08.2011	13.4 ± 1.6	9.4 ± 1.1
D2	*Prorocentrum rhathymum* (JHWPMX1)	Hwasun	33° 14' 22.38'' N 126° 19' 55.67''E	27.11.2011	34.5 ± 1.3	22.7 ± 0.8
D3	*Symbiodinium* sp. (JHLSD1)	Hyupjae	33° 23' 38.88'' N 126° 14' 23.02''E	29.09.2011	10.5 ± 1.3	8.2 ± 0.6
D4	*Coolia malayensis* 1 (JHACO6)	Hamo	33° 12' 39.86'' N 126° 15' 38.23'' E	14.05.2011	32.2 ± 1.4	34.8 ± 0.8
D5	*Ostreopsis ovata* 1 (JHAOS5)	Hamo	33° 12' 39.86'' N 126° 15' 38.23'' E	11.06.2011	51.46 ± 2.1	34.5 ± 1.7
D6	*Ostreopsis ovata* 2 (JHWOS13)	Hwasun	33° 14' 22.38'' N 126° 19' 55.67''E	15.08.2011	53.46 ± 1.1	36.5 ± 1.3
D7	*Coolia malayensis* 2 (JHWCO1)	Hwasun	33° 14' 22.38'' N 126° 19' 55.67''E	26.10.2011	33.5 ± 0.7	36.8 ± 0.9
D8	*Amphidinium operculatum* 1 (HLAM2)	Hyupjae	33° 23' 38.88'' N 126° 14' 23.02''E	13.07.2011	33.6 ± 2.5	22.4 ± 2.9
D9	*Heterocapsa psammophila* (JHLHET1)	Hyupjae	33° 23' 38.88'' N 126° 14' 23.02''E	29.12.2011	26.1 ± 1.1	19.8 ± 0.8
D10	*Coolia malayensis* 3 (JHLCO6)	Hyupjae	33° 23' 38.88'' N 126° 14' 23.02''E	13.07.2011	33.2 ± 1.1	37.8 ± 0.5
D11	*Amphidinium operculatum* 2 (SIAM1)	Sinyang	33° 31' 29.86'' N 126° 51' 40.50'' E	17.06.2011	32.6 ± 2.1	24.4 ± 2.2

μM vitamin B_{12}, 0.006 μM biotin, and 0.593 μM thiamine-HCl. After two weeks, the isolated dinoflagellates were confirmed to be growing and then were sub-cultured in screw capped test tubes (15 mL) with a round bottom containing 10 mL ¼ IMK medium as stock culture. Natural seawater (35 psu of salinity) collected from Jeju Coast was filtered through GF/F (47 mm, Whatman) filter, diluted with distilled water to adjust salinity to 30 psu and used for preparation of culture media after autoclaving at 121°C for 40 min and filtering (0.21 μm, Millipore). The stock cultures were then incubated in duplicate at 20°C under approximately 180 μmol photons m^{-2} s^{-1} of cool-white fluorescent illumination on a 12:12 h L:D cycle.

Stock cultures of dinoflagellate strains were gradually scaled up to 30 mL, 300 mL, 1 L and 3 L flasks under the above laboratory conditions. For the 20 L carboys, the culture conditions had small changes, as culture water was treated with sodium hypo chloride solution containing 9% active chlorine (at the rate of 1.1 ml L^{-1} seawater) for 30 min for chemical sterilization, and after that, sodium thiosulphate (at the rate of 0.12 g L^{-1} seawater) was added to neutralize chlorine in the water; also medium level of aeration was provided. From all the scale up stages, inoculums of exponential growing phase were used to start the following cultures. Cultures grown in glass flasks (1 and 3 L) were initiated by inoculation of 10 to 20% volume of 300 mL culture stock (maintained by monthly transfer) and cultures grown in carboys (20 L) were initiated by inoculation of 10 to 20% volume of 3 L cultures.

Experimental design

To evaluate the growth potential and biomass production of the total 11 benthic dinoflagellate strains (Table 1) cultured in IMK medium (Nihon Pharmaceutical Co., Ltd., Japan) (Yamaguchi et al., 2012) and f/2 medium (Guillard, 1979), the batch cultures were run in duplicate for 40 days. The experimental procedure started with the inoculation of late exponential phase cultures from 3 L with an initial cell density of approximately 1 - 3 × 10^3 cells mL^{-1} into 20 L carboy.

Growth rates

Growth rate was measured every five days from the cultured strains. Cells in 50 mL aliquots were fixed with 1 % formalin solution and then direct counts were made with a light microscope using the Sedgwick-Rafter (S-R) cell. Specific growth rate (SGR; μ, day^{-1}) was defined as the increase in cell density per time (Pirt, 1975); it was formulated as follows

$\mu\ (day^{-1}) = \ln N_1 - \ln N_0 / t_1 - t_0$ (1)

N_0 and N_1 are the cell density at the beginning (t_0) and end (t_1) of the selected time interval between inoculation and maximum cell density, respectively. Growth rate as divisions per day was calculated using the following equation (Guillard, 1979).

Divisions per day, $Div.\ day^{-1} = \mu / Ln2$ (2)

Dinoflagellate cell harvest

For biochemical analysis, 11 strains of dinoflagellate were harvested

separately from the cultures (20 L of cultures at exponential growing phase) by centrifuging (5000 rpm for 10 min in 250 mL centrifuge bottle) with VS-24SMTi high speed refrigerated centrifuge (Vision Scientific Co. Ltd, Daejeon Si, Korea). Cells were prepared for dry weight biomass following Zhu and Lee (1997) and biomass was expressed as g/L. The cultures were kept at -80°C and subjected to dry freeze using dry freeze system (Samwon Freezing Engineering Co. Busan, Korea).

Solvent extraction and sample preparation

The lyophilized benthic dinoflagellate strains were grounded separately into fine powder and homogenized. Then the homogenized samples were sonicated (ultra sound-assisted extraction) at 25°C for 90 min for three times, using (80%) methanol. Crude methanol extracts were concentrated by evaporating the solvent under reduced pressure, using rotary evaporator (Fisher Scientific, Loughborough, UK**)** and each of the samples was prepared into 100 mg mL^{-1} concentration. For the determination of antioxidant activity, dilution was done using deionized water and for the *in vitro* assays, dilution was done using Dulbecco's phosphate-buffered saline (DPBS).

DPPH radical scavenging assay

Spin trapping is the most direct method for the detection of highly reactive free radicals, which can overcome the sensitivity problem inherent for the detection of endogenous radicals in biological systems. 1,1 diphenyl-2-pricrylhydrazyl (DPPH) is a free radical donor which can be detected via electro spin resonance (ESR) spectrometer. DPPH radical scavenging activity was measured using an ESR spectrometer (JES-FA machine, JOEL, Japan) by the technique described by Nanjo et al. (1996). A 60 µL of each sample was added into 60 µL of DPPH (60 µmol L^{-1}) in ethanol. After 10 s of vigorous mixing, the solutions were transferred into 100 µL teflon capillary tubes and fitted into the cavity of the ESR spectrometer. The spin adduct was determined on an ESR spectrometer exactly 2 min later. The measurement conditions were as follows: Central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain, 6.3×105; temperature, 298 K.

Cell culture

The murine macrophage cell line (RAW 264.7) and a human promyelocytic leukemia tumor cell line (HL-60) were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). RAW 264.7 cell line was cultured in Dulbecco's modified eagle medium (DMEM) and HL-60 cell line was grown in Roswell Park Memorial Institute (RPMI-1640) medium. Both media were supplemented with 100 U mL^{-1} of penicillin, 100 µg mL^{-1} of streptomycin and 10% fetal bovine serum (FBS). The cells were incubated and maintained in an atmosphere of 5% CO_2 at 37°C. These cells were sub-cultured every two days and cells in exponential phase were used throughout the experiments.

Determination of nitric oxide (NO) production

RAW 264.7 cells ($1×10^5$ cell mL^{-1}) were placed in a 24-well plate and after 24 h the cells were pre-incubated with concentrations (25 and 50 µg mL^{-1}) of the sample at 37°C for 1 h. Then further incubation was done for another 24 h with LPS (1 µg mL^{-1}) at the same temperature. After the incubation, quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production (Lee et al., 2007). Briefly, 100 µL of cell culture medium was mixed with 100 µL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid), the mixture was incubated at room temperature for 10 min and the optical density at 540 nm was measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Sunrise, Tecan Co. Ltd., Australia). The fresh culture medium was used as a blank in every experiment.

Cytotoxicity assessment using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide (MTT) assay

The cytotoxicity of methanol extracts of unialgal cultures against the RAW 264.7 cells and the cancer cells (HL-60) was determined using a colorimetric MTT assay. Cells were seeded in a 96-well plate at a concentration of 1×10^5 cells mL^{-1}. 24 h after seeding, the cells were treated with the extracts. Cells were then incubated for an additional 24 h at 37°C. MTT stock solution (50 µL; 2 mg mL^{-1} in PBS) was then added to each well to a total reaction volume of 250 µL. After 3 h of incubation, the plates were centrifuged (800 × *g*, 5 min) and the supernatants were aspirated using an aspirating pipette attached to the vacuum. The formazan crystals from each well were dissolved in 350 µL of dimethylsulfoxide (DMSO), and the absorbance was measured with an ELISA plate reader at 540 nm.

Statistical analysis

Statistical significance between the growth rates was determined by analysis of variance using the software program Graph Pad InStat ver.3, Microsoft Excel 2007 and Duncan's multiple range tests (DMRT).

RESULTS

Dinoflagellates' growth and biomass production

A total of eleven benthic dinoflagellate strains were cultured in two different media *viz*., IMK and f/2 to estimate their growth characteristics and biomass production. In the present study, maximum cell density, maximum growth rate and biomass production of the strains varied as 12 to 148×10^3 cells mL^{-1}, 0.15 to 0.31 divisions/day and 0.06 to 0.26 g/L, respectively. After 10-15 days of inoculation, dinoflagellate strains started to grow rapidly until they reached their maximum cell numbers (Figure 1). All strains reached their maximum cell density on the 20th day of culturing period except D11 (*A. operculatum* 2) and D4 (*C. malayensis* 1), which reached their maximum on the 25th day, in f/2 medium.

Amphidinium species including *A. carterae* (D1), *A. operculatum* 1 (D8) and *A. operculatum* 2 (D11) showed maximum cell density ($P < 0.05$), growth rates (divisions day^{-1}) ($P > 0.05$) and biomass production ($P > 0.05$) in IMK medium compared to f/2 medium. Among these three strains, the highest cell density (148×10^3 cells mL^{-1}), growth rate (0.317 ± 0.01 div.day^{-1}) and biomass (0.26 ± 0.03 g/L) were recorded in *A. carterae* (D1) (Figures 1, 2 and 3). *Symbiodinium* sp. (D3) got maximum cell density ($P < 0.05$), growth rate ($P > 0.05$) and biomass production ($P > 0.05$) faster in IMK medium than in f/2

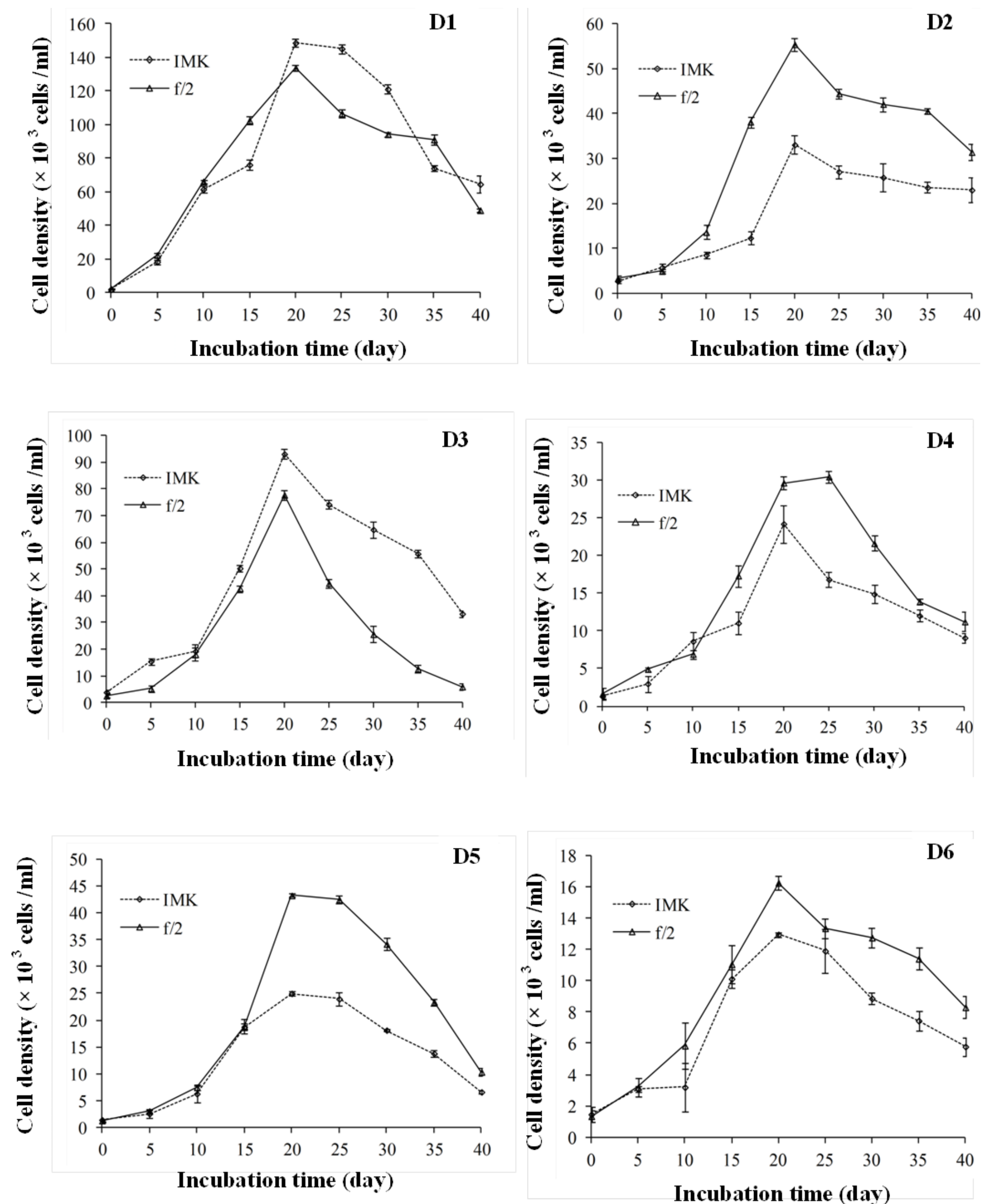

Figure 1. Growth curve (cell density) of benthic dinoflagellate strains cultured in 20 L culture. D1, *A. carterae*; D2, *P. rhathymum*; D3, *Symbiodinium* sp.; D4, *C. malayensis* 1; D5, *O. ovata* 1; D6, *O. ovata* 2; D7, *C. malayensis* 2; D8, *A. operculatum* 1; D9 *H. psammophila*; D10, *C. malayensis* 3; D11 *A. operculatum* 2.

medium (Figures 1, 2 and 3). On the contrary, *P. rhathymum* (D2), *H. psammophila* (D9) and two *Ostreopsis ovata* strains (D5 and D6) showed maximum cell density ($P < 0.05$), growth rate ($P > 0.05$) and biomass production ($P > 0.05$) faster in f/2 medium than in IMK medium (Figures 1, 2 and 3).

Among the three *C. malayensis* strains, *C. malayensis* 1 (D4) and *C. malayensis* 3 (D10) had maximum cell

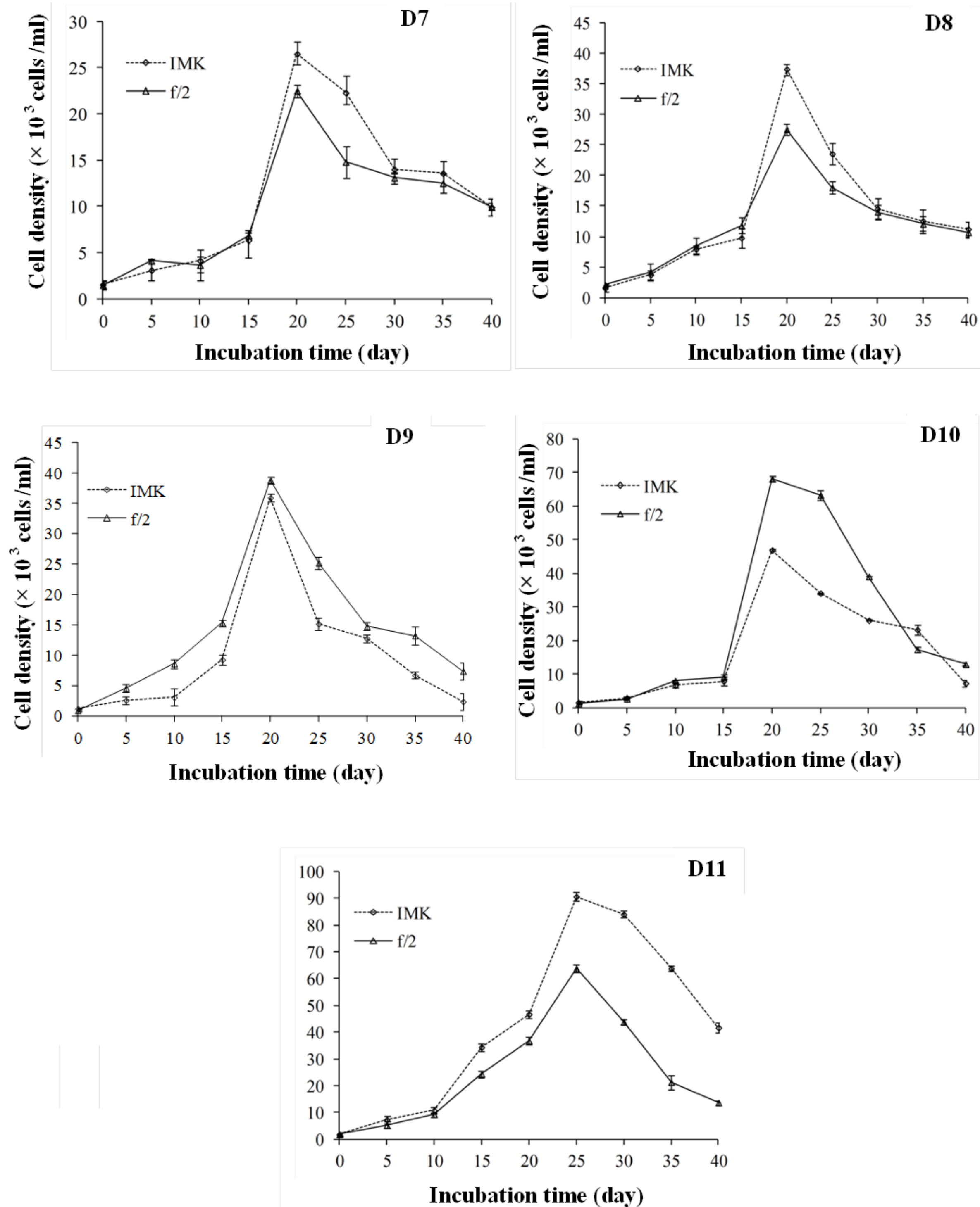

Figure 1. Contd.

density ($P < 0.05$), growth rates ($P > 0.05$) and biomass production ($P > 0.05$) in f/2 medium than in IMK medium, whereas *C. malayensis* 2 (D7) had better performances only in IMK medium (Figures 1, 2 and 3). When ANOVA was performed for IMK and f/2 medium separately, maximum cell density, maximum growth rate and biomass production of *A. carterae* (D1) were statistically significant ($P < 0.05$) compared to all the eleven cultured strains. The other ten strains were not significant ($P > 0.05$).

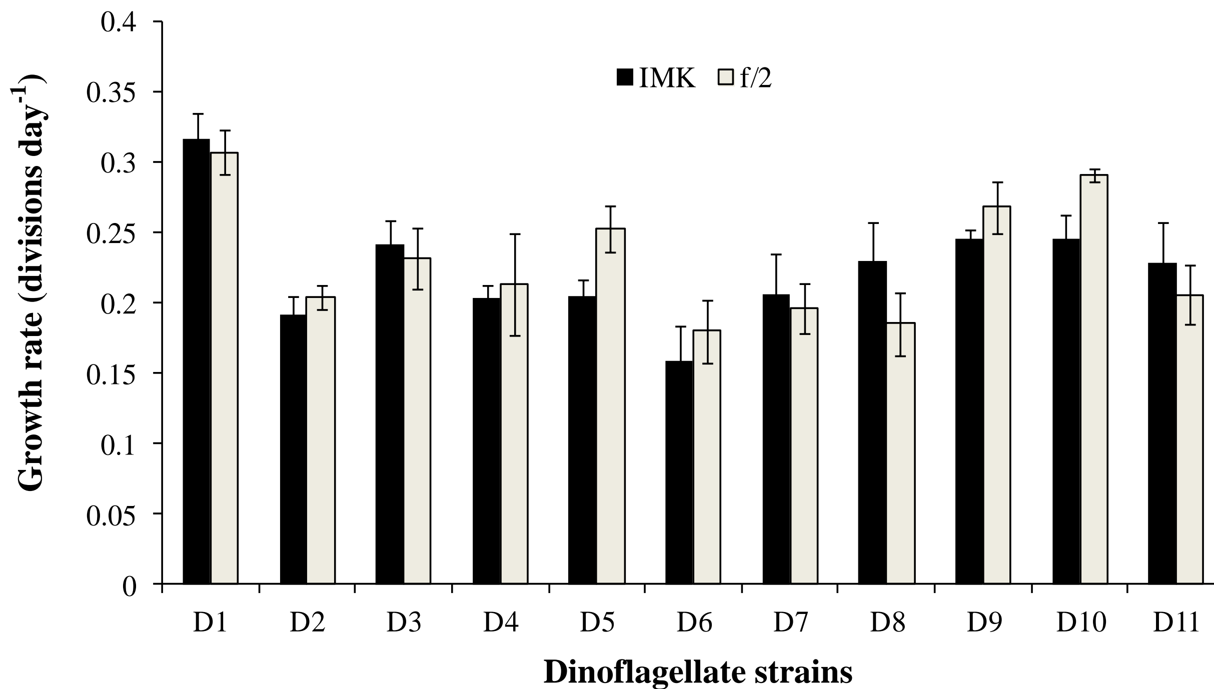

Figure 2. Growth rate (divisions day $^{-1}$) of benthic dinoflagellate strains cultured in 20 L culture. Strains are denoted as D1 *(A. carterae*), D2 (*P. rhathymum*), D3 (*Symbiodinium* sp.), D4 (*C. malayensis* 1), D5 (*O. ovata* 1), D6 (*O. ovata* 2), D7 (*C. malayensis* 2), D8 (*A.operculatum*1), D9 (*H. psammophila*), D10 (*C. malayensis* 3), and D11 (*A. operculatum* 2). Values are expressed as means ± SD in triplicate experiments.

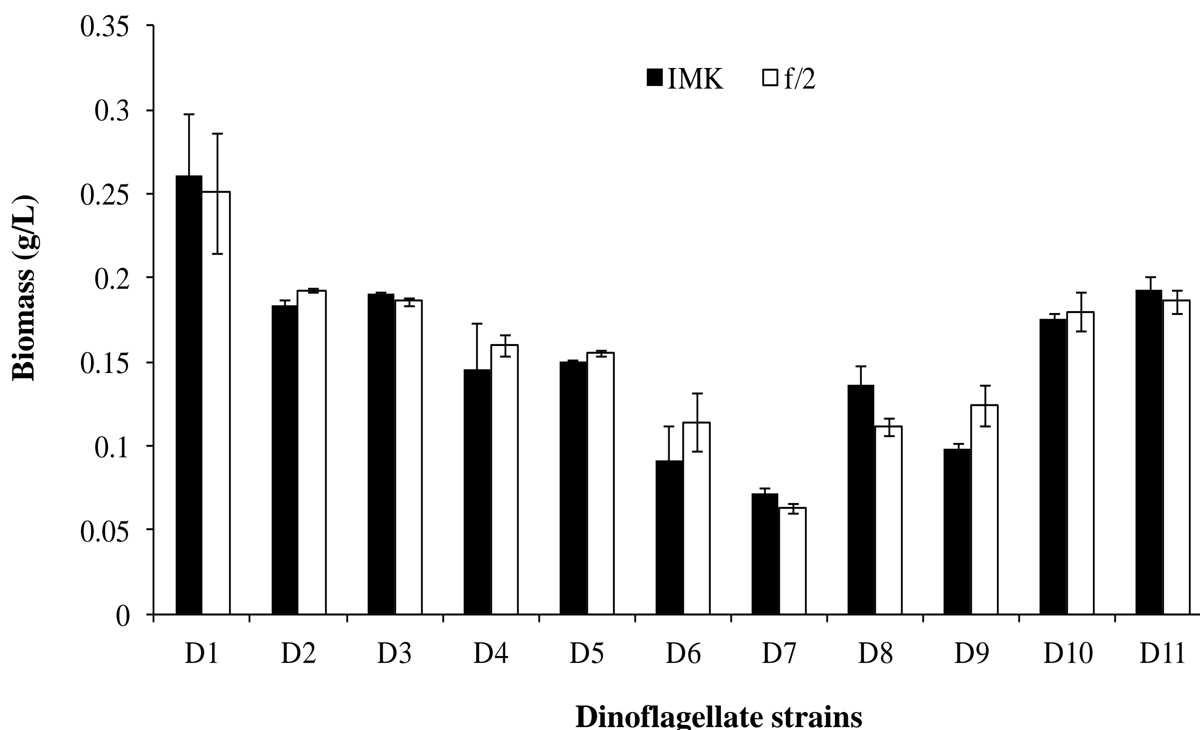

Figure 3. Biomass (g/L) production of benthic dinoflagellate strains cultured in 20 L culture. Strains are denoted as D1 *(A. carterae*), D2 (*P. rhathymum*), D3 (*Symbiodinium* sp.), D4 (*C. malayensis* 1), D5 (*O. ovata* 1), D6 (*O. ovata* 2), D7 (*C. malayensis* 2), D8 (*A. operculatum*1), D9 (*H. psammophila*), D10 (*C. malayensis* 3), and D11 (*A. operculatum* 2). Values are expressed as means ± SD in triplicate experiments.

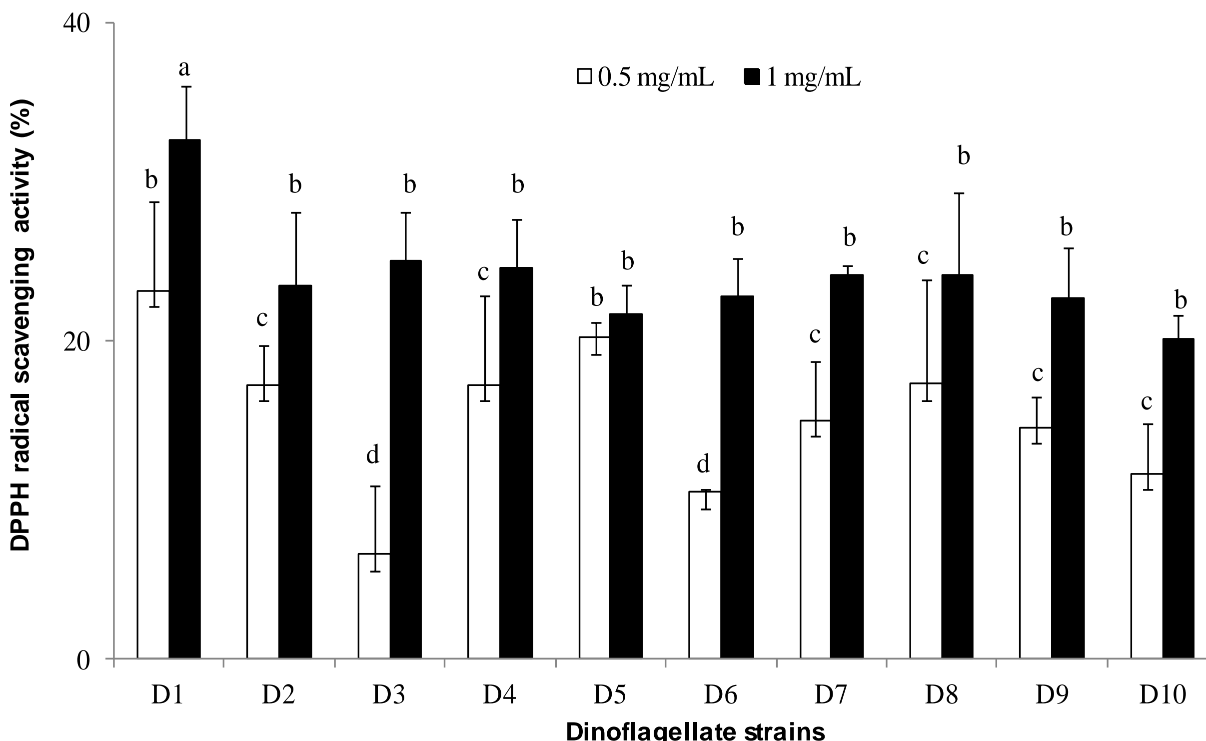

Figure 4. DPPH radical scavenging activity of 80% methanol extracts of dinoflagellate cultured strains by Electron Spin Resonance Spectroscopy. Strains are denoted as D1 *(A. carterae*), D2 (*P. rhathymum*), D3 (*Symbiodinium* sp.), D4 (*C. malayensis* 1), D5 (*O. ovata* 1), D6 (*O. ovata* 2), D7 (*C. malayensis* 2), D8 (*A. operculatum*1), D9 (*H. psammophila*), D10 (*C. malayensis* 3), and D11 (*A. operculatum* 2). Values are expressed as means ± SD in triplicate experiments. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

Screening of antioxidant, anticancer and anti-inflammatory activities

For the screening of bioactivity, the cultured benthic dinoflagellates were extracted in 80% methanol. Figure 4 shows the DPPH radical scavenging activities of the methanolic extracts from the eleven strains determined using ESR spectrometer. Among the extracts, only *A. carterae* (D1) had a significant ($P < 0.05$) DPPH radical scavenging activity (32.57 ± 3.44 %) at 1 mg mL^{-1}, compared to the other extracts. However, all the other methanolic extracts were not significant ($P > 0.05$) in terms of DPPH scavenging activity.

The inhibitory growth activity of the HL-60 cell was determined against methanolic extracts of the cultured benthic dinoflagellates as anticancer activity. Among the incubated methanolic extracts of the dinoflagellate strains at 25 and 50 µg mL^{-1} concentrations, the growth of HL-60 cells was suppressed significantly ($P < 0.05$) by D5 (*O. ovata* 1) and D8 (*A. operculatumi* 1). Moreover, the determined cytotoxicity on HL-60 cells at 50 µg mL^{-1} concentration was reported as 52 and 42% against D5 (*O. ovata* 1) and D8 (*A. operculatumi* 1), respectively (Figure 5).

Anti-inflammatory activity was performed as the inhibitory effect of NO production (%) on LPS-induced RAW 264.7 macrophages. According to the results, the strongest inhibitory effect of NO production (app. 97%) was reported by D5 (*O. ovata* 1) at 50 µg mL^{-1} concentration. However, MTT assay showed that D5 (*O. ovata* 1) methanol extract increased the cytotoxicity of the RAW 264.7 macrophages by 73.5% at 50 µg mL^{-1} concentration compared to the control. Therefore, the examined activity did not comply with the anti-inflammatory activity. In addition, a significant inhibitory effect of NO production (%) was inferred from *A. carterae* (D1), *P. rhathymum* (D2) and *A. operculatum* 1 (D8) in comparison to the other strains. The MTT assay confirmed that there was no significant cytotoxicity at all in the treated concentrations and more than 100% cell viability was observed. Hence, these three dinoflagellate strains are described as having the potential to isolate anti-inflammatory active compounds (Figure 6).

DISCUSSION

This is the first attempt to evaluate and characterize the growth potential of benthic dinoflagellate species collected from Jeju Island, Korea. The maximum cell

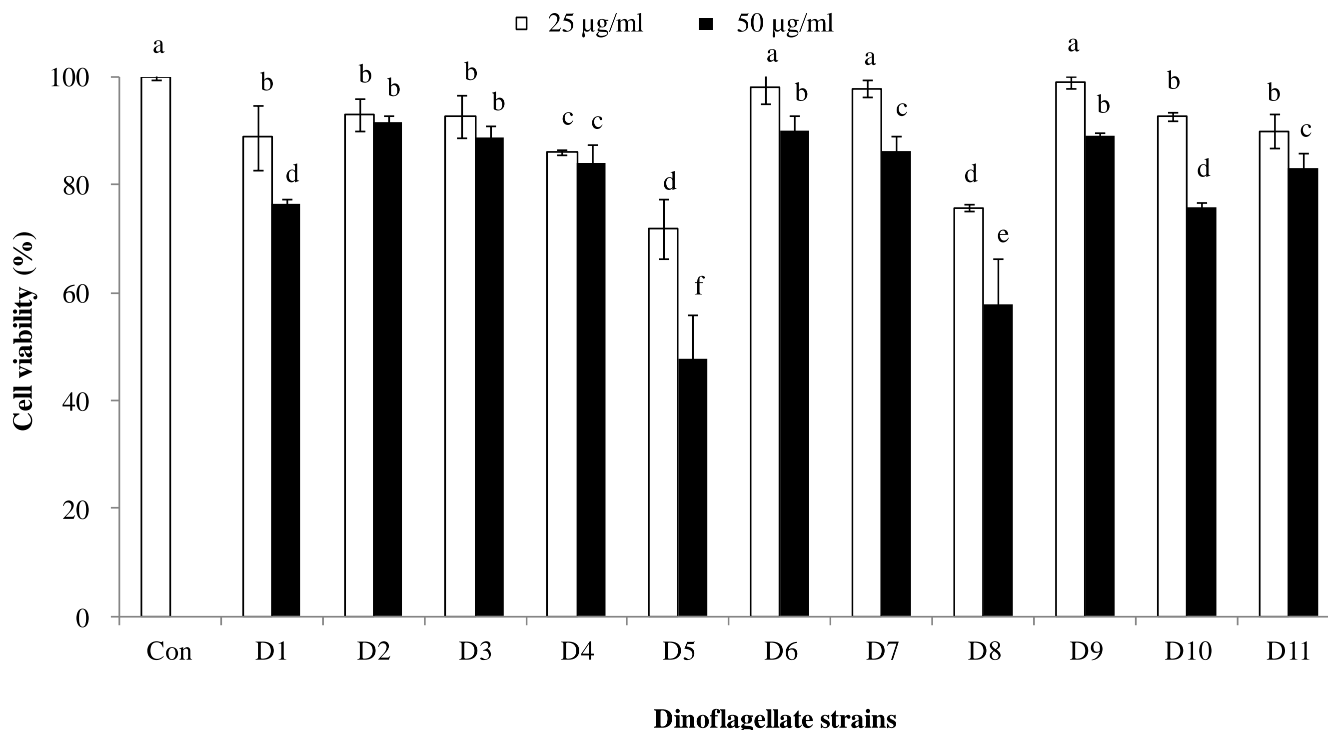

Figure 5. Inhibitory effect of the growth of cancer cells against 80% methanol extracts of dinoflagellate cultured strains on HL-60 cell lines. Cells were treated with the extracts at the indicated concentrations (25 and 50 µg mL^{-1}). After 24 h to treat the extracts cell viability was assessed by MTT assay. Values are expressed as means ± SD in triplicate experiments. Strains are denoted as D1 *(A. carterae*), D2 (*P. rhathymum*), D3 (*Symbiodinium* sp.), D4 (*C. malayensis* 1), D5 (*O. ovata* 1), D6 (*O. ovata* 2), D7 (*C. malayensis* 2), D8 (*A. operculatum*1), D9 (*H. psammophila*), D10 (*C. malayensis* 3), and D11 (*A. operculatum* 2). Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

number of *A. carterae* (D1) obtained in the present study (148.60×10^3 cells mL^{-1} in IMK medium) was closer to the cell density ($1 - 6 \times 10^5$ cells mL^{-1}) reported by Thomas and Carr (1985). However, it was lower than other observations (23.3×10^5 cells mL^{-1}) for *A. carterae* cultured in f/2 medium with the modification of nitrogen and phosphorus concentrations at 33 psu, 25°C and 300 µmol quanta m^{-2} s^{-1} light intensity in 250 mL lab scale culture by Espinoza et al. (2011).

In our study, the maximum growth shown by *A. carterae*, (0.317 divisions day^{-1} in IMK medium) was similar with the growth rate (0.32 to 0.71 divisions day^{-1}) reported by Tomas et al. (1989). In addition, higher growth rate of *A. carterae* (D1) compared to other two *Amhidinium* strains, *A. operculatum* 1 (D8) and *A. operculatum* 2 (D11) in IMK medium indicates that *A. carterae* (D1) could be grown at its highest potential with the described culturing system. The maximum growth rate of *P. rhathymum* (D2) (0.204 divisions day^{-1} in IMK medium) was lower than the growth rate (app. 0.3 to <0.6 divisions day^{-1}) of closely related species, *P. mexicanum* collected from Knight key, Florida, USA and cultured in K medium (Keller and Guillard, 1985) under small scale stock cultures by Morton et al. (1992). *Symbiodinium* sp. (D3) cultured in this study (0.242 divisions day^{-1} in f/2 medium) showed almost similar growth rate of 0.30 d^{-1} in f/2 medium observed during the study of mixotrophic growth rate from *Symbiodinium sp.* (Jeong et al., 2012). Strains of *Coolia* spp. (D4, D7 and D10) showed the growth rate ranges from 0.19 to 0.29 divisions day^{-1}. This complies with the observations of Morton et al. (1992) who reported maximum growth rate of app. 0.2 to 0.6 divisions day^{-1} of *Coolia monotis* at 29°C, 33 psu in K medium (Keller and Guillard, 1985). Moreover, Rhodes and Thomas (1997) found the growth rate of 0.25 divisions per day when *C. monotis* was grown in GP medium (Loeblich and Smith, 1968). In our study, *O. ovata* strains (D 5 and D 6) showed maximum cell density of 12 to 43×10^3 cells mL^{-1}, which is higher than that of other studies: for example, 10.1×10^3 cells mL^{-1} by Nascimento et al. (2012) and 4×10^3 cells mL^{-1} by Vidyarathna and Graneli (2012). In addition, growth rate of 0.15 to 0.25 divisions day^{-1} is similar to growth rate of 0.22 divisions day^{-1} mentioned by Nascimento et al. (2012). However, it was lower than 0.53 divisions day^{-1} (Guerrini et al., 2010), 1.07 divisions day^{-1} (Granéli et al., 2011) and 1.03 divisions day^{-1} (Yamaguchi et al., 2012). Dason and Colman (2004) cultured *Heterocapsa oceanica* in f/2 medium and found a growth rate of 0.344 divisions day^{-1} (calculated from doubling time) which was

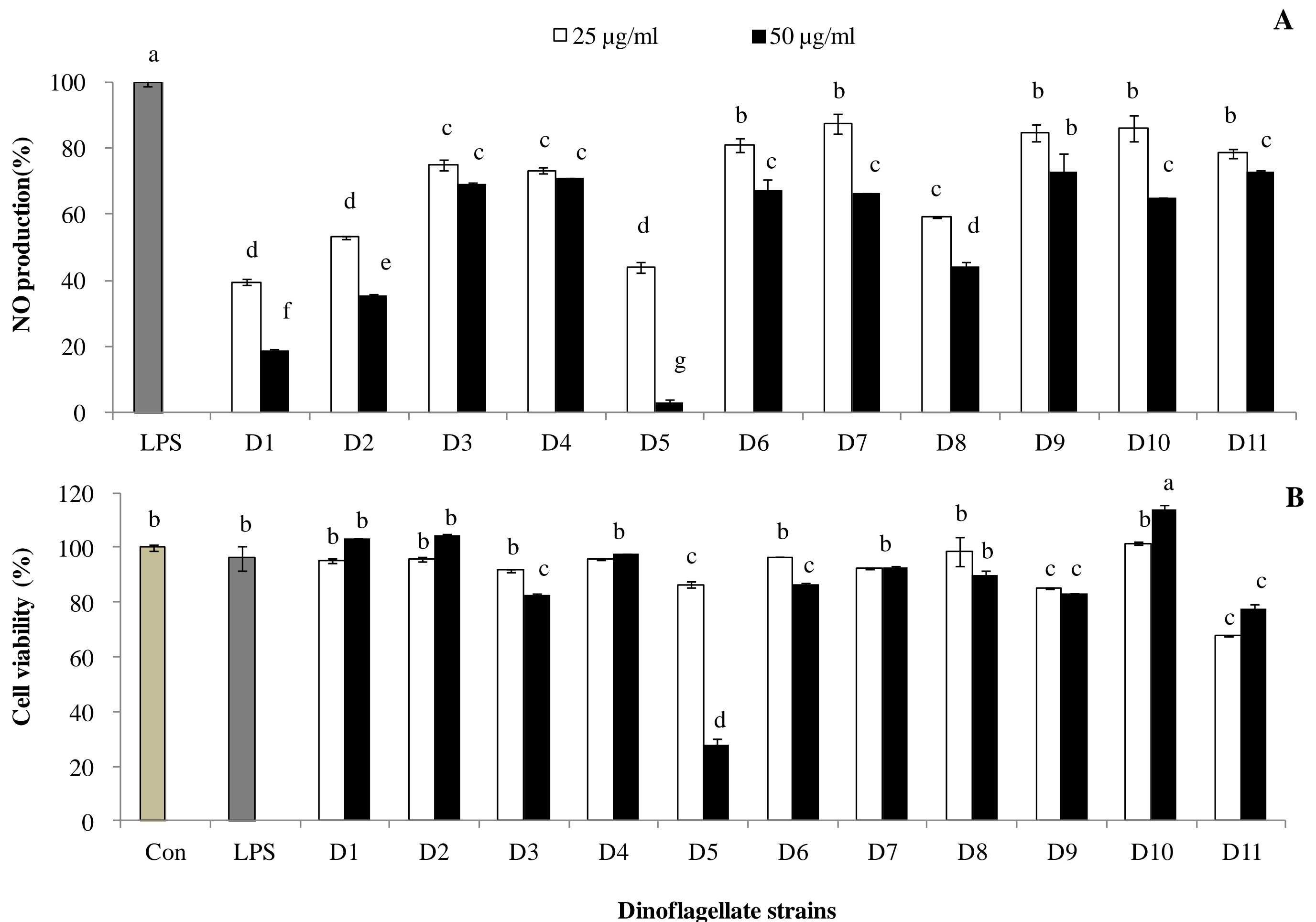

Figure 6. Inhibitory effect of 80% methanol extracts of dinoflagellate cultured strains. **(A)** on LPS-induced NO production in RAW 264.7 macrophages and **(B)** cell viability (%) in RAW 264.7 macrophages, respectively. Incubation of the extract concentrations (25 and 50 µg mL^{-1}) with cells in response to LPS (1 µg mL^{-1}) for 24 h, the NO levels in the medium was measured. Values are mean ± SD of three determinations. Strains are denoted as D1 *(A. carterae*), D2 (*P. rhathymum*), D3 (*Symbiodinium* sp.), D4 (*C. malayensis* 1), D5 (*O. ovata* 1), D6 (*O. ovata* 2), D7 (*C. malayensis* 2), D8 (*A. operculatum*1), D9 (*H. psammophila*), D10 (*C. malayensis* 3), and D11 (*A. operculatum* 2). Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

slightly higher than the growth rate obtained by our strain of *Heterocapsa psammophila* (D9).

Dinoflagellates have commonly low growth rates, a complicated metabolism and low toxin productivity. This lower growth rate reflects in the lower chlorophyll *a* to carbon ratio (Chl *a* : C) as speculated by Tang (1996). The growth rates of dinoflagellates rarely double one per day (Thomas and Carr, 1985). Most of other economically important microalgae species have shown growth rates much higher than 1.0 d^{-1}; for example, *Dunaliella tertiolecta* (1.4 d^{-1}), *Thalassiosira pseudonana* (1.8 d^{-1}) and *Chaetoceros calcitrans* (2.0 d^{-1}) (Thompson et al., 1990). Different chemicals have been experimented for maximizing the biomass production and CO_2 bio-fixation of microalgae (Nayak et al., 2013).

In particular, *Amphidinium, Prorocentrum* and two *Coolia* strains grew better and showed a higher biomass production in IMK culture medium; this may indicate the potentiality of this medium for benthic dinoflagellate culture. Growth rates and cell yields of benthic dinoflagellate strains presented in this study are presumably attributed to the difference of the kind of medium, size of the culture and culture conditions used in other studies.

As a consequence, IMK culture medium can be suggested for the growth performances of benthic dinoflagellates and for their culturing compared to the commonly used f/2 culture medium. Moreover, maximum cell yield, growth rate and biomass yield of D1 (*A. carterae*) were comparatively better than that of the other dinoflagellate strains, which suggests that this species might be the most efficiently cultured in the present culture system.

Benthic dinoflagellates cultured in our study are commonly found associating with sea grasses, macro algae, dead corals, rocks, soft sediments and inverte-

brates in tropical, subtropical and temperate marine environment (Totti et al., 2010). Among the cultured strains, only *Ostreopsis* sp. is known to produce bloom mainly in tropical waters or in temperate areas during summer (Pistocchi et al., 2011; Parsons et al., 2012). High water temperatures, high irradiance and high remineralisation are factors that create an environment favouring the blossom growth of benthic harmful algal (Fraga et al., 2012). In the benthic environment, as cells are linked to the substrate, their relative movement to water surrounding them depends more on water motion than on swimming. The efficiency of benthic species in nutrient uptake depends not only on their own physiological characteristics but also on water velocity according to the mass-transfer theory (Atkinson, 2001). A variety of factors other than wave action and temperature may also be important in controlling and promoting the occurrence and intensity of the blossom of benthic dinoflagellates, for example, availability of macroalgal substrates, light intensity, precipitation and nutrients (Tindall and Morton, 1998).

Nutrient availability is to be considered as an important environmental factor for controlling and promoting the occurrence and intensity of the blossomy of benthic dinoflagellates (Pistocchi et al., 2011), whereas the relationship between benthic/epiphytic dinoflagellates and nutrient conditions is less clearer (Pistocchi et al., 2011). Vila et al. (2001) found no significant correlations between epiphytic dinoflagellate and nutrients, and the authors stated that mechanisms that trigger species abundance remain unclear (Vila et al., 2001). In the Veracruz Reef Zone (Gulf of Mexico), a lack of correlation was recorded between nutrient concentrations and the abundant benthic/epiphytic dinoflagellate assemblage (Okolodkov et al., 2007).

Therefore, the role of nutrients in supporting elevated dinoflagellate biomass is still uncertain. In fact, as reported by Tindall and Morton (1998), epiphytic/benthic dinoflagellates do not appear to be unique in their requirements for the two major limiting macronutrients, nitrogen and phosphorus.

In our culture system, temperature, salinity, light intensity and aeration were not in variable conditions and growth performance of eleven strains differed with the variation of culture medium. Growth performance of these strains could be different by the variation of other factors (for example, temperature) in the present culture system. Further research with varying temperature, salinity, light intensity, aeration and nutrient concentration is necessary to clarify the growth physiology such as temperature-salinity tolerance and nutrition of these cultured strains with the present system as well as to understand the mechanisms of dynamics of benthic dinoflagellates in coastal environments.

DPPH chemicals generate stable free radicals and are widely used to test the ability of antioxidant activity of compounds or extracts of marine sources as free radical scavenging properties or hydrogen donor capacity. In this study, we attempted to assess the antioxidant effects of the methanol extracts from dinoflagellate strains by ESR method. In addition, inflammation is initiated due to the pathogenic invasion or injury to cells and tissues as a physiological process (Newton and Dixit, 2012). In fact, inflammatory mediators, such as NO, play an important role as the signaling molecule that is induced in macrophages. LPS acts as endotoxins for mammals and stimulate the RAW cells in terms of enhancing the NO concentration in the medium (Wadleigh et al., 2000). Hence, as a screening technique, we used to measure the NO accumulation inhibitory percentage in the RAW macrophages *in vi*tro with the treated methanol extracts of dinoflagellates. Furthermore, anticancer activity was determined as the inhibitory effects of cancer cell growth *in vitro* followed by pre-treated cultured dinoflagellates methanol extracts. MTT assay was performed to determine the cell viability of cancer cells.

As a consequence, methanol extracts from the cultured dinoflagellates were screened against DPPH radical scavenging, inhibitory effect of NO production (%) on LPS-induced RAW macrophages and inhibitory effect of the growth of human leucamia (HL-60) cells; it showed the possible potentials needed to isolate secondary metabolites. Importantly, among the cultured benthic dinoflagellates, *A. carterae* (D1) and *O. ovata* 1 (D5) performed profound bioactivities against the determined activity assays. It also showed the best culture conditions in IMK media and obtained the highest biomass yield compared to the f/2 media. This is further furnished by separating its active components through the bioassay guided fractionations. In fact, Echigoya et al. (2005) have shown the potentiality of isolation of novel bioactive compounds from the cultured *A. carterae.* Moreover, a potent hemolytic and antifungal active compound (amphidinol 2) was isolated from the cultured *A. klebsii* (Paul et al., 1995). However, biochemical analysis proved that D5 (*O. ovata* 1) strain showed the highest anticancer activity among the cultured dinoflagellats strains and was successfully cultured in f/2 media. Previous studies reported that cultured dinoflagelates are prolific sources for lipid or fatty acid isolation (Rodriguez et al., 2010a). The screening results of our studies emphasized that available lipids including stanols, steroids and polyunsaturated fatty acids are potential metabolites for anti-inflammatory effects. On the other hand, available polyhydrox metabolites can be responsible for the antioxidant and anticancer activity of the cultured dinoflagellates.

More than 21,000 bioactive metabolites have been isolated from marine species over the years (Blunt et al., 2009). In fact, dinoflagellates have rendered many natural compounds useful for the field of drug discovery. Therefore, benthic dinoflagellates are described as alternative candidates for the isolation of bioactive metabolites with the pharmacological value for future

therapeutic applications (Estrada et al., 2007; Kobayashi and Tsuda, 2004; Mydlarz et al., 2003). However, many have failed to produce and culture dinoflagelallates in laboratory scale to gain the desired bioactivities under the artificial culture media (Rodriguez et al., 2012). Despite that, in this study, 11 stains of the cultured benthic dinoflagellates were screened for antioxidant, anticancer and anti-inflammatory assays to figure out the available potentiality for isolation of bioactive metabolites. A few studies have been done on the isolation of new secondary metabolites from dinoflagellates; for example, Wu et al. (2005) have isolated a new unsaturated glycoglycerolipids from a cultured marine, *A. carterae*. In addition, a novel polyhydroxy metabolite, zooxanthellamide A was isolated from *Symbiodinium* sp. (Onodera et al., 2002), and another polyhydroxy compound with potent hemolytic activity, Amphidinol 2 was identified from *Amphidinium klebsii* (Paul et al., 1995). Therefore, in this study, cultured dinoflagellates would be the key sources for novel finding with respect to chemical, pharmacological and toxicological research.

Conclusions

This study reveals a useful culture system for scaling up benthic dinoflagellate using IMK and f/2 media successfully by demonstrating the growth potential of the strains collected from Jeju Island, Korea. Besides the common f/2 medium, IMK medium was proven to have the potential to culture some of benthic dinoflagellates in 20 L significantly. Among the cultured strains, *A. carterae* showed the highest antioxidant and anti-inflammatory effect *in vitro* assays. In addition, deemed bioactive potentials from the cultured strains in the respective media can be considered to extract novel secondary metabolites for future pharmacological and commercial applications. Furthermore, research can be carried out to explore the effects of salinity, light and temperature on the growth characteristics of benthic dinoflagellates by batch and continuous culture systems.

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