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A close-up photograph of a microscope, showing the objective lens, eyepiece, and stage. The image is overlaid with a blue gradient that fades from the top to the bottom. The text 'African Journal of Biotechnology' is centered over this gradient.

# African Journal of **Biotechnology**

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## Review

# Actinomycetes bioactive compounds: Biological control of fungi and phytopathogenic insect

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**Actinomycetes are aerobic and gram-positive spore forming bacteria. They belong to the order actinomycetales and are characterized by substrates and aerial mycelium growth. They are the most abundant microorganisms in soil. They play important roles in the cycling of organic matter and inhibit the growth of several plant pathogens in the rhizosphere. Due to the presence of enzymes such as proteases and chitinase, actinomycetes have been studied as a natural controller of insects and phytopathogenic fungi that cause considerable losses in agriculture. Additionally, the facilities for the industrial manipulation of cultures, and the diversity of metabolites produced make actinomycetes preferred for the control of pests. Furthermore, actinomycetes constitute a “green” alternative for controlling insects and fungi, since they do not contaminate the environment, and are natural members of the soil. They also contribute to the sustainability of soil by formation and stabilization of compost piles, due to their degrading capabilities, and ability to form stable humus. Moreover, they can be associated with other soil microorganisms to degrade recalcitrant residues like celluloses to maintain biotic soil equilibrium.**

**Key words:** Actinomycetes, compounds, agriculture, fungi, insect.

## INTRODUCTION

Historically, discoveries and developments of a class of new bioactive compounds with antimicrobial and anti-parasitic activities have frequently emerged from natural sources (Chin et al., 2006; Ganesan, 2008). These natural bioactive compounds produced serve as a model for the synthesis of synthetic and semi-synthetic drugs. These developments are directly linked to the screening of natural producers such as microorganisms and plants,

which require employment of biotechnological techniques (Marinelli and Marconi, 2011; Newman and Cragg, 2012). Particularly, the use of microbial sources for the investigations of novel natural bioactive compounds has proved to be productive during the last two decades and was emphasized extensively in review articles (Berdy, 2005; Balts, 2007; Naine, 2011; Raja and Prabakarana, 2011). Microbial natural products are

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biotechnologically preferable due to their remarkable appropriate pharmacological activities and facility for controlling variables in bioprocess (Sanchez and Demain 2002).

Well-known as the source of several drugs such as antibiotics, antitumor, immunosuppressants, antiviral and antiparasitic agents, microorganisms are responsible for the production of about 23,000 bioactive secondary metabolites (dos Reis Feitosa et al., 2014; Subbanna et al., 2018). Despite this large amount, only 150 of these compounds have been employed in pharmacology, agriculture or other fields (Brzezinska et al., 2014). The filamentous bacteria group actinomycetes alone are the main producers responsible for the production of over 10,000 of these compounds, representing 45% of all bioactive microbial metabolites discovered (Brzezinska et al., 2014). When considering only these compounds in practical use, it may represent about 80% (Olano et al., 2008). This bacterial group represents the most economically and biotechnologically worthwhile microorganisms (Baltz, 2007; Naine et al., 2011; Raja and Prabakarana, 2011).

Actinomycetes are a group of gram-positive branching unicellular filamentous bacteria belonging to the order actinomycetales; it is so called, because it has a fancied similitude with the radiating rays of the sun when seen in tissue ruptures. It is characterized by high content of G+C in DNA, presence of LL-Diaminopimelic acid (LL-DAP) and the presence or absence of characteristic sugars in the cell wall. Members of this group are ubiquitous and the discovery of new actinomycete taxa from diverse habitat with unique metabolic activity implies, generally, discovery of novel bioactive compound. Their reproduction is based on fission of hyphae or by means of special spores (conidia). They may also form branching threads or rods, and their hyphae are generally nonseptate. Septa, when existing, may be observed in some forms (Manulis et al., 1994). The chemical composition of cell wall is similar to that of Gram-positive bacteria but because of their well-developed morphological (hyphae) and cultural characteristics, actinomycetes have been considered as a group, well separated from other bacteria. Cell wall maintains cell shape, preventing bursting due to osmotic pressure. This wall, as distinctive in prokaryotic organisms, consists of a thick layer of peptidoglycan, a structure composed of glycan (polysaccharides) chains of alternating N-acetyl-d-glucosamine (NAG) and N-acetyl-d-muramic acid (NAM) and diaminopimelic acid (DAP). Teichoic and teichuronic acids are chemically bonded to peptidoglycan (Bhatti et al., 2017; De Schrijver and De Mot, 1999).

Although historically referred to as the ray fungi due to the mycelia of branching filaments (hyphae), actinomycetes, unlike the true fungi, have thin hyphae (0.5–1.5 mm in diameter) with genetic material coiled inside as free DNA (Bhatti et al., 2017). Linked polymers

containing short chains of amino acids and long chains of amino sugars are found in cell wall of the hyphae. The actinomycetes cell wall composition is of considerable taxonomic significance, varying considerably among different groups. Therefore, there are at least, four major cell wall types based on the three features of peptidoglycan composition and structure: (i) diaminopimelic acid isomer on tetrapeptide side chain position 3, (ii) sugar content of peptidoglycan and (iii) the presence of glycine in interpeptide bridges (Davenport et al., 2000). Interactions between actinomycetes and plants in soil rhizosphere make bacterial species essential for the micro-environment, characterizing them as plant growth-promoting rhizobacteria.

Increase in population and food prices concomitant with the reduction in agricultural activities has become a global food security concern. On the other hand, losses of US\$ 120 billion (representing 20 to 40%) have been attributed to insect pests and fungal attack (FAO, 2010). Approximately, 70,000 different insect species damage food crops across the world (Vijayabharathi, 2013). Among them, the species belonging to Lepidoptera order are the major cause of crop losses (Qin et al., 2009). Bacteria belonging to actinomycetes group also present several mechanisms especially useful in the development of potential anti-fungal drugs based on anti-fungal bioactive metabolites, due to their versatility in the production of extracellular enzymes and a variety of these secondary metabolites. Many of these anti-fungal bioactive compounds have been characterized and employed in agriculture (Arasu et al., 2008).

The aim of the present study is to show the potentialities of actinomycetes for producing biofungicide and bioinsecticide important in agriculture. This study also describes the general mechanisms of action and status of main commercial products derived from bioactive compounds.

## ACTINOMYCETES AND BIOCONTROL OF INSECTS IN AGRICULTURE

Since the application of chemical insecticides on crops for controlling deleterious insects has become hazardous to environment and human health, many efforts have been oriented in order to amend their use for a more ecofriendly and safe alternative control methods (Bream et al., 2001). The bacterial species, *Bacillus thuringiensis*, for example, is the most successful microorganism employed as a commercial insecticide for biological control, replacing conventional chemical insecticides in some areas of application. Several other varieties of microorganisms including fungi and nematodes have been reported as strategies to biologically control insect pests, but, actinomycetes especially, play an important role in the biological control of insects through the production of a large variety of

**Table 1.** Some bioactive compounds produced by actinomycetes and their related biological activities.

| Bioactive compound | Actinomycete                          | Activity         |
|--------------------|---------------------------------------|------------------|
| Lomofungin         | <i>Streptomyces lomondensis</i>       | Antifungal       |
| Sclerothricin      | <i>Streptomyces scleogranulatus</i>   | Antifungal       |
| Spoaxamicin        | <i>Streptosporangium oxazolanicum</i> | Antitrypanosomal |
| Avermectin         | <i>S. avermitilis</i>                 | Antiparasitic    |
| Antimycin          | <i>Streptomyces lucitanus</i>         | Antifungal       |
| Rosamicin          | <i>Micromonospora rosaria</i>         | Antibacterial    |
| Validamycin        | <i>Streptomyces hygroscopicus</i>     | Antifungal       |
| Azalomycin         | <i>Streptomyces malaysiensis</i>      | Antifungal       |
| Roseoflavin        | <i>Streptomyces davawensis</i>        | Antibacterial    |
| Rifamycin          | <i>Micromonospora rifamycinica</i>    | Antibacterial    |

insecticidally active compounds against different order of insects (Hokkanen and Lynch, 1995).

Hussain et al. (2002) confirmed the very high mortality of larval and pupal stages of *Musca domestica* reaching up to 90% of mortality, after actinomycetes treatments, and Sundarapandian et al. (2002) verified actinomycetes effectiveness against *Culex quinquefasciatus*. The effective action of actinomycetes against insects is not only attributed to the production of bioactive compounds, but especially attributed to their capacity to produce chitinase enzyme, which degrades the insect chitin surface, allowing penetration of bioactive toxic lethal compound in the insect body (Brzezinska et al., 2014). Therefore, the action of actinomycetes in insects is combined, depending on the action of two or more mechanisms (Gadelhak et al., 2005). A large variety of compounds that act in isolation or generally in combinations with other molecules is related to actinomycetes and it justifies the ability or even the potentiality of several of them to be employed as insect plagues controllers. Some typical actinomycetes and their major role as biocontrol agent are depicted in Table 1.

Cotton bollworms represent a highly polyphagous insect species (Lepdoptera order), attacking crops such as tomato, cotton, pigeon pea, chickpea, rice, sorghum and cowpea. Other vegetal hosts include groundnut, okra, peas, field beans and soybeans. The high mobility, high fecundity, and facultative diapause contribute to the dispersion of these species and, therefore, over 181 plant species of economically important crops are potentially infested (Shad et al., 2012). The second most important polyphagous lepidopteran pest, *Spodoptera litura*, causes 25 to 100% yield loss on economically important crops such as cotton, groundnut, chilli, tobacco, castor and okra.

In the last two decades, actinomycetes have been shown to be a vast source of novel agents having considerable potential for the biocontrol of insect pests. Many secondary metabolites produced by the members of this group of bacteria show insecticidal activity.

Hence, the possibility of using these metabolites in controlling insects had been achieved by several researchers (Dutton and Gueguen, 1999; Sazonova et al., 1993; Dindo, 1993; Spindler and Spindler-Barth, 1994; Lasota and Dybas 1991; Scholl et al., 1992; Campbell et al., 1983; Vijayan and Balaraman 1991; Takahashi et al., 1989; Mishra et al., 1987).

#### ACTINOMYCETES' MAIN BIOACTIVE COMPOUNDS USED AGAINST PHYTOPATHOGENIC INSECTS

As discussed, a large variety of metabolites found in actinomycetes species may contribute to the control of insects in plants. As a rule, most of them act based on combined effects.

##### Chitinase as a coadjuvant

Chitinase is an enzyme used by insects to degrade the structural polysaccharide "chitin" during the molting process (Gupta et al., 1995; Zhang et al., 2002). The largest chitinase activity among bacteria has been observed in species of *Streptomyces*. Gadelhak et al. (2005) isolated streptomycete and non-streptomycete actinomycetes and tested their capacity to produce chitinase enzyme on colloidal chitin agar (CCA). These experiments confirmed the high ability of *Streptomyces* to produce chitinase. Species of *Streptomyces* show high multiplicity of chitinase genes.

##### Spinosyn as the first specific insect pest controller

The tobacco budworm, *Heliothis virescens* (F.) is a well-known tobacco plague in United States of America. As reported by Sparks and co-workers (1981, 1982, 1983), this species and others belonging to the same genus developed resistance to a wide variety of insecticides including DDT, methyl-parathion and the pyrethroids. In some areas, the resistance spectrum of *H. virescens*

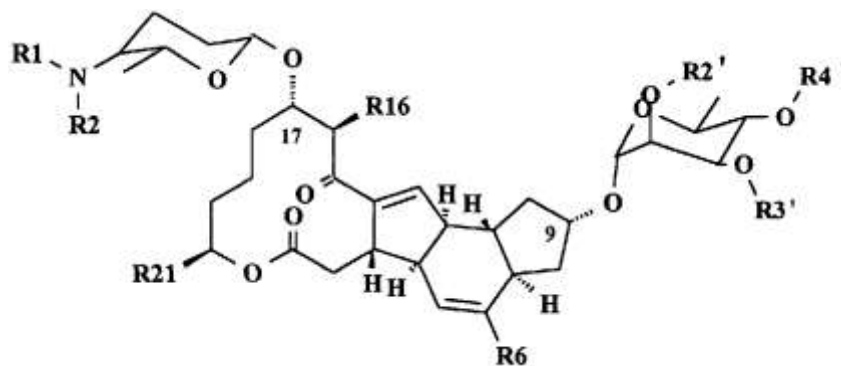


Figure 1. Main structure of spinosyn (Sparks et al., 1998).

expands and incorporates many of the newer organophosphorus and carbamate insecticides (Sparks et al., 1993). The search on new insect control agents includes an assortment of random or directed screening scenarios, sometimes referred to as the trial and error approach (Sparks and Hammock, 1983; Hammock, 1985; Hammock et al., 1986).

Discovery of new insect control agents is still a common and hard effective development, particularly when the focus is the natural product source as well as synthetic organic chemistry. In this context, in 1985, a program to screen fermentation broths from soil microorganisms for pharmaceutically and agriculturally useful compounds was conducted by scientists at Lilly Research Laboratories (Indianapolis, IN, USA). Those scientists discovered a new species of Actinomycete, *Saccharopolyspora spinosa* (Mertz and Yao 1990) isolated from soil sample. Extracts of this fermentation broth were active against mosquitos (Kirst et al., 1992) and southern armyworm, *Spodoptera eridania* (Cramer), larvae in early screening assays. Consequently, they discovered this activity was associated with a new bioactive compound classified in the group of macrocyclic lactones (Kirst et al., 1992) called the spinosyns (Sparks et al., 1998). Spinosyn A (1 H-as-Indaceno [3,2-d] oxacyclododecin-7,15-dione, 2-[(6-deoxy-2,3,4-tri-O- methyl- $\alpha$ - L- man nopyranosyl)oxy]-13-[[5-(dimethyl-amino)tetrahydro-6-methyl-2H-pyran-2-yl]oxy]-9-ethyl-2,3,3a,5a,5b,6,9,10,11,12,13,14,16a,16b-tetradecahydro-14-methyl-, [2R\*,3aS\*,5aR\*,5bS\*,9S\*,13S\*(2R\*,5S\*,6R\*),14R\*,16aS\*,16bR\*]]-[9CI]) are the principal components of spinosad™ (Tracer Naturalyte Insect Control, Indianapolis, IN), and other spinosyns active against larvae of *H. virescens*.

Spinosyns are a macrolide insecticide belonging to the family of tetracyclic lactones to which are attached an amino-sugar (D-forosamine) and a neutral sugar (L-rhamnose). Members of the family differ in the extent of N- and O-methylation on the sugars, or C-methylation on the polyketide nucleus (Ichikawa et al., 2013; Kirst, 2010; Sparks et al., 1998). Their activity against

mosquitos (mosquito larvicidal activity) was evidenced. In complementary works, Strobel and Nakatsukasa (1993) identified a strain A83543, as *S. spinosa*, and confirmed it as a new species of actinomycete which produces a large family of macrolide compounds. Particularly, spinosyns A and D are the two active ingredients. Most of the genes involved in spinosyn biosynthesis are clustered in an 74 kb region of the *S. spinosa* genome. This region has been characterized by DNA sequence analysis and targeted gene disruptions. The spinosyn biosynthetic gene cluster contains five large genes encoding a type I polyketide synthase, and 14 genes involved in modification of the macrolactone, or in the synthesis, modification and attachment of the deoxysugars. Four genes required for rhamnose biosynthesis (two of which are also required for forosamine biosynthesis) are not present in the cluster. A pathway for the biosynthesis of spinosyns was proposed by Waldron et al. (2000). Figure 1 shows the main structure of spinosyn.

## FUNGI IN AGRICULTURE

The economic significance of fungal biotechnology cannot be overstated; indeed, fungi have been exploited to yield a range of valuable products, some of which have proved invaluable to mankind. Since ancient times, fungi have been utilized for simple food processing. In the last century, the development of fungal biotechnology for the subsequent production of valuable commodities such as antibiotics, enzymes, vitamins, pharmaceutical compounds, fungicides, plant growth regulators, hormones, and proteins were seen (Wiley, 2018); however, fungi can be detrimental to agriculture, especially foods of largest commercial importance.

Fungi represent one of the major threats for biodeterioration of cereals and pulses during storage, causing economic losses to growers by increasing the free fatty acid content of seeds and decreasing germination ability of the plants (Dhingra et al., 2001;



Kedia et al., 2014). In addition, the mycotoxins secreted by different food borne molds cause qualitative losses of commodities, potentially inducing various health problems in consumers. Some species of *Aspergillus* are highly aflatoxigenic, particularly in tropical and subtropical countries, secreting high level of aflatoxins. Furthermore, aflatoxin is classified as group 1 human carcinogen by the International Agency for Research on Cancer (Mishra et al., 2013).

Food commodities are frequently contaminated by fungi, and the associated toxins generated by some of them during storage, transportation and post-harvest processing cause significant losses in quality, quantity, nutrient composition, and thereby reduce market value. According to the Food and Agriculture Organization (FAO), about 1000 million metric tons of food is spoiled globally each year due to mycotoxins produced by fungi (Bhat et al., 2010; Prakash et al., 2005; Prakash et al., 2014).

Commodities are intensive products in natural state (primary) or with a small degree of industrialization. This category involves agricultural products (raw and/or processed), minerals (raw and/or industrialized) and energy (Veríssimo and Xavier, 2015). In the food sector of Brazil, among the main commodities, crops such as soybean, corn and cotton can be mentioned.

Among the most consumed grains is soy, which is considered as a functional food; it provides nutrients to the body, prevents chronic degenerative diseases and is also an excellent source of minerals. Corn, in turn, is also of great importance for human consumption, as it is an energetic, digestible food with high starch content. It is the raw material of many industrialized products including animal feed, in which corn and soybeans complement each other (Ferrarini, 2004; Balini et al., 2015). Corn has high energy content and soy has rich protein value (Oliveira et al., 2004; Balini et al., 2015). Corn is one of the oldest food grains considered as one of the three major cereal crops in the world, together with rice and wheat. Brazil produced 56.3 million tons of corn in the 2011-2012 harvest, and exported 8.5 million tons (Godfray and Garnet, 2014; De Rossi, 2015).

From the process of cultivation till distribution, the seeds of these grains are conditioned to fungus contamination. This is because the grains present food components of these microorganisms, in addition to several other factors. The most frequently found genera are *Aspergillus* and *Penicillium*; the fungi are so-called because they grow in seeds and store grains with moisture contents within the range of 8 to 18%. Among these fungi, the genus *Aspergillus* is a frequent contaminant of soybean and corn (Balini et al., 2015).

Cotton (*Gossypium hirsutum* L.) is among the most important fiber crops in the world. Each year, approximately 35 million hectares of cotton are planted worldwide. World cotton trade moves about \$ 12 billion annually and involves more than 350 million people in its

production, from farms to logistics, ginning, processing and packaging. Currently, cotton is produced by more than 60 countries, where the five countries that produce the most are: China, India, the United States, Pakistan and Brazil (USDA, 2017). Throughout the world, there are reductions in cotton productivity due to outbreaks of disease. In Brazil, with the introduction of cultivars adapted to the *Cerrado* and with higher yield of fiber, cotton cultivation has intensified. In this ecosystem, climatic conditions are favorable to the development of various diseases caused by fungi, resulting in increased production costs (Borém and Freire, 2014).

### ACTINOMYCETES' ACTION AGAINST PHYTOPATHOGENIC FUNGUS

Actinomycetes are important enzyme producers, such as chitinases, proteases, peptidases and cellulases. Chitinases are the most important in the process of phytopathogenic fungi control. Similar to insect control mechanisms, the control of actinomycetes in fungi is a result of combined and complementary action. Firstly, the cell wall chitin is degraded by enzymes, and consequently the cell is assessed and antibiotics and others active compounds are released in the cell.

Fungal plant diseases management by *Streptomyces* has been well documented, but few commercial products are in the market using specific strains of the microorganism or its metabolites. While it is unmanageable for massive production, the potential microorganisms like *S. plicatus* used in biocontrol programmes connecting their enzymatic properties seems to be practical to develop methods for production and extraction of secondary metabolites, or the use of extract of broth directly (Sinha et al., 2014).

Investigations on the potential of actinomycetes on biological control have been reported since the 80s, as shown in the classical work of Tahvonen (1982b), who tested strains of *Streptomyces* isolated from peat for control of soil and seedborne disease in peat culture.

A key illustration of *Streptomyces* biocontrol agent is the action of a strain of *Streptomyces griseoviridis*, reported by Tahvonen (1982a). In that paper, the author described a strain originally isolated from light coloured *Sphagnum* peat as antagonistic to a variety of plant pathogens together with *Alternaria brassicola* (Schw.) Wiltsh., *Botrytis cinerea* Pers., *Fusarium avenaceum* Sacc and *Fusarium culmorum*. The species, *S. griseoviridis* has been used in root dipping or growth nutrient treatment of cut flowers, potted plants, greenhouse cucumbers, and different alternative vegetables (Bhatti et al., 2017).

Fungal pathogens pose serious problem worldwide and cause a number of plant diseases including rusts, smuts, rots, wilt, anthracnose causing severe damage to crops (Pakdeevaporn et al., 2005; Ashokvardhan et

al., 2014); in this context and in the natural world, no microorganism can survive independently, and microorganisms can interact with each other by a number of ways. Interactions between microorganisms can be divided into mutually beneficial, neutral and harmful such as mutualism, neutralism, amensalism, antagonism, parasitism, etc. So, interactions between microorganisms can be positive, negative or no effects (Moëne-Loccoz, 2014).

Biological control of postharvest diseases by antagonistic microorganisms seems to be a promising alternative to fungicides. Understanding the methods of action of antagonisms is essential to allow the use of antagonists under partial conditions and to enhance their biological control while protecting human health and the environment. Several modes of action have been documented for the antagonistic activity of biological control agents: they act by multiplying on the fruit surface or within wounds on the fruit; this is done by competing for space and nutrient at an infection court on the product, by antibiosis, by restricting the action of hydrolytic enzymes produced by pathogen, by producing enzyme to degrade pathogen cell walls, and/or by direct parasitism of the pathogen (Long et al., 2005; Shojaee et al., 2014). Soil actinomycetes have revealed their wide antifungal activity (Ventura et al., 2007; Sharma et al., 2014; Tinatin and Nuzrat, 2006). They have been shown to protect several plants to various degree of soil borne fungal pathogens. Actinomycetes as biocontrol agent produce Urauchimycins which is a member of antimycin class, a set of well-identified antifungals, that act by inhibiting the electron flow in the mitochondrial respiratory chain of a phytopathogenic fungus and have been identified in *Streptomyces* isolated from the integument of attine ants (Schoenian et al., 2014). More recently, Dias et al. (2017) isolated strains of *Streptomyces* sp. (a very promising genus of the order *Acinomicetales*) of sediment from an urban mangrove located at the city of São Luís MA and verified antimicrobial activity of these bacteria in front of organisms of clinical and agricultural interest. Costa et al. (2017) isolated species of actinomycetes of biotechnological interest from soil contaminated with agrochemicals and verified, already in isolation, the antibiosis potential of these bacteria. Furthermore, biofungicides such as MYCOSTOP® are produced using actinomycetes for the control of seed- and soil-borne plant pathogens (*Fusarium*, *Alternaria*, *Phytophthora* and *Pythium*) which cause damping-off and root diseases, Actinovate® isolated from streptomycetes species. *Streptomyces lydicus* WYEC108 is a strain of this species which has been formulated to control fungal plant pathogens effectively for fresh market tomatoes, PRESTOP® is used for controlling damping-off and root diseases (*Pythium*, *Fusarium*, *Phytophthora* and *Rhizoctonia*) as well as for the control of Botrytis grey mould and Didymella (*Mycosphaerella*) gummy stem blight in cucumber (Kamara and Gangwar, 2015), which

are all available commercially. After undergoing various biological processes, *Streptomyces* are able to successfully control plant pathogenic fungi including *P. oryzae* by hydrolyzing their cell walls (Kavitha et al., 2010; Awla et al., 2016).

## CONCLUSION

It is of great importance to know the diversity of bioactive compounds produced by soil microorganisms. Understanding the mechanisms of action of these bioactive is crucial for its efficient application in agriculture. In this sense, actinomycetes are preferable due to their predominance in soil, and enormous potential to adapt and produce a variety of bioactive metabolites with activity against important phytopathogenic fungi and insects. Although many biofungicides and bioinsecticides have been produced and successfully applied to cultivars, many other potentially useful molecules with bioactivities against such organisms need more investigations to confirm their efficiency. Additionally, many other compounds need to be commercially available, and the production must be intensified. The status of research worldwide, points out to the necessity of tests involving new metabolites and further investigations, to find new species and optimize production process conditions.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# Expression and purification of human IL-2 protein from *Escherichia coli*

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Human interleukin 2 protein (IL-2) is an important cytokine found to be elevated in several types of cancer. A synthetic DNA sequence of the cDNA of mature IL-2 protein was cloned into the pRSET-B expression vector. The expressed IL-2 protein in *Escherichia coli* [BL21] was associated with the formation of insoluble inclusion bodies (IBs). The effect of different cultivation conditions (temperature, isopropyl- $\beta$ -D-thiogalactoside concentration, and early harvest of cells) together with the incorporation of single or dual His-tag on the formation of IBs of the expressed protein was investigated. Yet, expression of soluble IL-2 was not achieved under any of the investigated conditions. A simple protocol for rapid and effective solubilization of these IBs was optimized. Using this protocol, together with subsequent purification using ion metal affinity chromatography, a purified His-IL2 protein was obtained in a yield of 5.1 mg/cell pellet of 1 L culture. In conclusion, the effect of different expression conditions on the solubility behavior of an expressed eukaryotic protein in *E. coli* was investigated using human IL-2 as a model protein. Moreover, the purified expressed protein could be used as a positive control in early diagnosis of tumors and in cancer research in Egypt.

**Key words:** Human interleukin-2, protein expression, inclusion bodies.

## INTRODUCTION

Interleukins are proteins that play a vital role in intercellular communication among leukocytes. Such proteins are cytokines that are classified into different classes II-1 to II-38 (Akdis et al., 2016), in addition to IL-39 (Wang et al., 2016). Human interleukin-2 (IL-2) is a protein produced by human lymphocytes that have been stimulated by mitogens or antigens (Ju et al., 1987). IL-2 is first synthesized as a precursor polypeptide of 153 amino acids followed by cleavage of the first 20-amino

acids signal peptide resulting in the production of the mature secreted IL-2 protein (133 amino acids, 15 kDa) (Schoner et al., 1992). The mature protein is a potent cytokine that regulates innate lymphoid cells, acts as a B-cell growth factor, promotes antibody synthesis, and induces proliferation and differentiation of natural killer cells (NK) to enhance their cytolytic activities (Roediger et al., 2015). Additionally, it has been used in anti-tumor therapy (Sengupta et al., 2008; Janik et al., 1993), and in

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**Table 1.** Primers used in this study.

| Primer | Sequence (5'-3')  |
|--------|---|
| IL2-F1 | AGAAGAGGATCCggcacctacttcaagttctaca                          |
| IL2-R1 | TCTTCTAAGCTTcaagtcagtggtgagatgatgct                         |
| IL2-R2 | TCTTCTAAGCTTcaatggtgatggtgatggtgtcctgcagtcagtggtgagatgatgct |

treatment of patients with disseminated cancers (Schoner et al., 1992). Several studies showed the increased expression levels of IL-2 in different types of cancer e.g. prostate cancer (Royuela et al., 2000), stomach and renal cancer (Lin et al., 1995), squamous cell carcinomas of the head and neck (Reichert et al., 1998), and neuroblastic tumors (Ridings et al., 1995). Therefore, the elevated expression levels of such cytokine could be used as a diagnostic marker for detection of actively proliferating tumors (Garcia-Tunon et al., 2004).

Recombinant protein expression in *Escherichia coli* is useful for different purposes. (i) The production of vaccines such as the expression of chicken anemia virus capsid protein VP1 (Lee et al., 2011). (ii) The production of protein that could be used in serodiagnosis of infections e.g. poliovirus (Uma et al., 2016), and *Brucella melitensis* (Cloekaert et al., 2001). (iii) Production of purified proteins that are sold by research companies to be used as positive control in different research experiments e.g. recombinant IL-2 in Western blot and ELISA (Gehman and Robb, 1984).

The use of *E. coli* as an expression system has many advantages e.g. rapid transformation process, high yield of expression, and the whole expression is less expensive in comparison with other hosts (Rosano and Ceccarelli, 2014). However, the higher expression rates of recombinant proteins in *E. coli* are often accompanied with the formation of insoluble aggregates of the target protein called inclusion bodies (IBs) (Singh et al., 2015). The aim of this study was to express the recombinant IL-2 protein in considerable amounts that could be used as a positive control for early diagnosis of tumors and in cancer research in Egypt. Another aim was to investigate the effect of different expression conditions on the solubility behavior of eukaryotic protein expression in *E. coli* using human IL-2 as a model protein.

## MATERIALS AND METHODS

### Bacterial strains and construction of recombinant plasmids harboring IL-2 gene

The *E. coli* strain Top10 (Thermo Fisher Scientific) was used for cloning, propagation and maintenance of the constructed plasmids; while the *E. coli* strain BL21 (DE3) [Invitrogen] was used for recombinant protein expression. Both strains were transformed using CaCl<sub>2</sub>-heat shock method (El-Mowafy et al., 2013). Selection of positive transformants was performed on Luria-Bertani (LB) medium agar plates containing ampicillin (100 µg/ml). Positive

clones were further cultivated in LB medium containing ampicillin (100 µg/ml). Glycerol stocks of all strains were prepared and stored at -80°C till further use (El-Mowafy et al., 2013).

The cDNA of human IL-2 precursor (462 bases, 153 amino acids) (Devos et al., 1983) was synthesized and provided by Macrogen Inc (Korea) in a bacterial cloning plasmid (pIL2) with ampicillin as a selection marker.

The primers IL2-F1 and IL2-R1 (Table 1) were used for cloning of mature IL-2 (402 bp, 133 amino acids) from pIL2 into pRSET-B expression vector (Invitrogen). The amplified IL-2 gene and pRSET-B vector were digested with BamHI (NEB, UK) and HindIII (NEB, UK) followed by ligation with T4 DNA ligase enzyme (NEB) to form the plasmid pIL2. The cloned IL-2 gene was in frame with the nucleotides encoding the N-terminal 6 His-tag, which is already introduced by Invitrogen in the pRSET-B vector, resulting in the expression of His-IL2 protein. Similarly, another version of IL-2 was also cloned into pRSET-B vector as a BamHI/HindIII fragment from pIL2 using the primers IL2-F1 and IL2-R2 (Table 1) into pRSET-B expression vector to obtain the plasmid pIL2-His. The expressed IL-2 protein (His-IL2-His) from the latter plasmid was dual His-tagged at both terminals. This was achieved by introducing the nucleotides encoding the C-terminal 6 His-tag at the primer IL2-R2, in addition to the cloning of the BamHI/HindIII-digested polymerase chain reaction (PCR) fragment of IL-2 in frame with the nucleotides encoding the N-terminal 6 His-tag of the pRSET-B vector. Positive clones were confirmed by restriction digestion with BamHI and HindIII. All the cloning steps were performed using Phusion High-Fidelity DNA Polymerase (NEB, UK). All PCR reactions were performed in MultiGene™ Mini Personal Thermal Cycler (Labnet, Multigene gradient, Foster City, CA). The PCR products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. The size of DNA fragments was confirmed using Hyperladder™ 100 bp (Bioline, England) and 1 kb DNA ladder (NEB, UK).

### Expression of IL-2 in *E. coli* BL21 at different conditions

LB medium containing ampicillin (100 µg/ml) was inoculated from the glycerol stock of *E. coli* BL21 transformed with pRSET-B, pIL2 or pIL2-His plasmids and incubated at 37°C with shaking (200 rpm). After overnight cultivation, subculturing into flasks containing 50 ml fresh medium supplemented with the antibiotic was performed so that the starting OD<sub>600nm</sub> was 0.5 (Ma et al., 2006). The expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Preliminary time course screening of expression was performed by harvesting 1.5 ml culture after 3, 4, and 5 h of induction followed by centrifugation at 878 × g for 5 min. The cell pellet was suspended in 30 µl 5x SDS gel loading buffer (0.25 M Tris-HCl, pH=6.8, 40% Glycerol, 8% SDS, 2.9 mM β-mercaptoethanol and 0.1% bromophenol blue) followed by heating at 95°C for 10 min (Laemmli UK, 1970). The resulting denatured protein samples were analyzed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), before staining with Coomassie Brilliant Blue (Laemmli UK, 1970). The size of protein bands was confirmed using chromatein prestained protein marker (Biosepes, China) or Roti®-Mark standard protein marker

(Roth, Germany).

For efficient lysis of the cell pellet after IPTG induction, sonication followed by enzymatic lysis was performed. Briefly, the whole cell pellet after IPTG induction was suspended in 3x volume lysis buffer (10 mM sodium phosphate buffer pH 7, 5 mM NaCl, 5 mM KCl, and protease inhibitor [Complete Ultra, mini, EDTA free, Roche]) (El-Mowafy et al., 2013). The bacterial suspension was subjected to sonication using a Soniprep (150 Sonicator TM-182; UK) in intervals of 30 s sonication and 30 s without sonication for a total of 5 min, while immersed in ice. To allow for enzymatic lysis, lysozyme solution (2 mg/ml) in 20 mM Tris-HCl (pH 8) was added to the sonicated cell suspension in a ratio of 3x volume of the initial cell pellet followed by immersion in ice for 30 min. Benzonase enzyme (EMD Millipore, USA) was added to the mixture to decrease the viscosity of the lysate by degradation of nucleic acids (Final concentration 25 U/ml). The soluble cell lysate (soluble protein extract) was obtained by centrifugation of the whole cell lysate at 11200xg for 10 min, at 4°C, from which an aliquot was denatured by heating in 5x SDS gel loading. Another aliquot was taken for the total protein extract (soluble and insoluble protein extract) before centrifugation, as a control, and was denatured by heating in 5x SDS gel loading (Laemmli UK, 1970).

The expression of the recombinant IL-2 protein in the total protein extract and the soluble cell lysate was investigated at different cultivation conditions by SDS-PAGE Coomassie staining. Such conditions included induction of expression at lower concentration of IPTG (0.25 mM), harvesting at earlier time of expression (3 h after IPTG induction), and expression of the protein at lower incubation temperature (35 or 30°C) (Sorensen and Mortensen, 2005a).

#### Recovery, purification and solubilization of IBs to obtain soluble purified IL-2 protein

The cell pellet from 100 ml culture was collected after IPTG induction and subjected to lysis by sonication and lysozyme enzyme before separation of the soluble protein extract as mentioned earlier. The protocol was carried out as described previously (Schoner et al., 1992) with few modifications. Briefly, the pellet (containing the disrupted cells, insoluble cellular proteins and IL-2 inclusions bodies) was suspended in 100 mM Tris-HCl (pH 8.5) containing triton 1% and urea 1 M. The mixture was left on ice for 10 min followed by centrifugation at 11200 xg for 10 min at 4°C. Washing of the pellet with triton and urea was repeated for additional 2 times, before washing with 100 mM Tris-HCl, pH 8.5. For solubilization of IBs, the pellet was resuspended in equal volume of 8 M guanidine HCl in 100 mM Tris-HCl (pH 8.5) and left in ice for 1 h with gentle shaking. The mixture was centrifuged at 11200 x g for 10 min at 4°C. The supernatant containing the solubilized IBs was subjected to Protino™ Ni-NTA agarose beads (Macherey-Nagel, Germany) to fish the His-tagged-IL2 protein according to the manufacturer's instructions. After elution of the Ni<sup>2+</sup> bound His-tagged IL2 protein with imidazole (500 mM), excess imidazole concentration was removed by ultrafiltration with 100 mM Tris-HCl, pH 7 using Roti®-Spin MINI-3 centrifugal device (Roth, Germany). The purified IL-2 protein was stored at -80°C till further use (El-Mowafy et al., 2013).

#### Western blot

Western blot analysis of the expressed IL-2 proteins was performed using diluted (1:1000) 6-Histidine Epitope Tag Antibody [HRP] (Novus Biologicals, USA) as previously mentioned in El-Mowafy et al. (2013), except that the His-tagged protein bands were visualized by incubation of the membrane with tetramethylbenzidine (TMB) substrate solution (Sigma Aldrich, USA) at room temperature for

few minutes before capturing of photos (Shaaban et al., 2015).

#### Determination of protein content

The concentration of the purified IL-2 protein was measured using Bradford protein assay kit (Biospes, China) according to the manufacturer's instructions. The protein concentration was calculated from the standard curve of bovine serum albumin (positive control) after measurement of the absorbance at 595 nm using ELx808™ Absorbance Microplate Reader (Biotek Instruments Inc., Winooski, VT) (Shaaban et al., 2015). The buffer containing the purified IL-2 protein (100 mM Tris-HCl, pH 7) was used as blank (negative control).

## RESULTS

### PCR and cloning

The DNA sequence of mature IL-2 (402 bp) was successfully amplified from pl-IL2 plasmid (Figure 1A) and cloned into the pRSET-B vector, to allow for the bacterial expression of either the N-terminal His-tagged IL-2 (His-IL-2 protein from pl-His-IL2 plasmid) or the dually His-tagged IL-2 (His-IL2-His protein from pl-His-IL2-His plasmid). Cloning of both versions of IL-2 was confirmed by restriction digestion of the constructed plasmids (Figure 1B).

### Recombinant expression of His-IL2 and His-IL2-His purified proteins

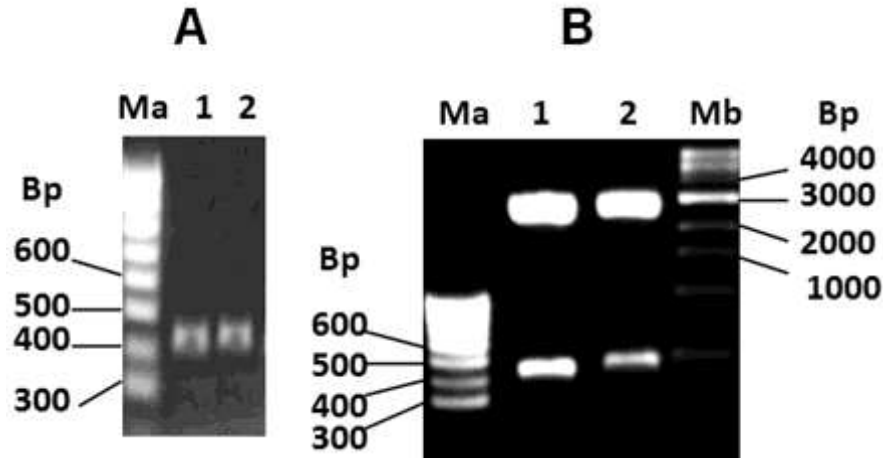
Bacterial expression of both versions of IL-2 protein was successfully detected at different time points following IPTG induction (Figure 2). Maximum production of the recombinant protein was observed after 4 h of induction (Figure 2). Therefore, further expression experiments were performed after 4 h of induction for both types of expressed IL-2 protein, unless otherwise specified.

Formation of IBs was detected in both versions of the expressed IL-2 by SDS-PAGE of the total protein extract and the soluble lysate of the transformed *E. coli* BL21 cells. The presence of the target protein band in the total protein extract, but not in the soluble lysate, confirmed the formation of insoluble IBs (lanes T and S in Figure 3A and B).

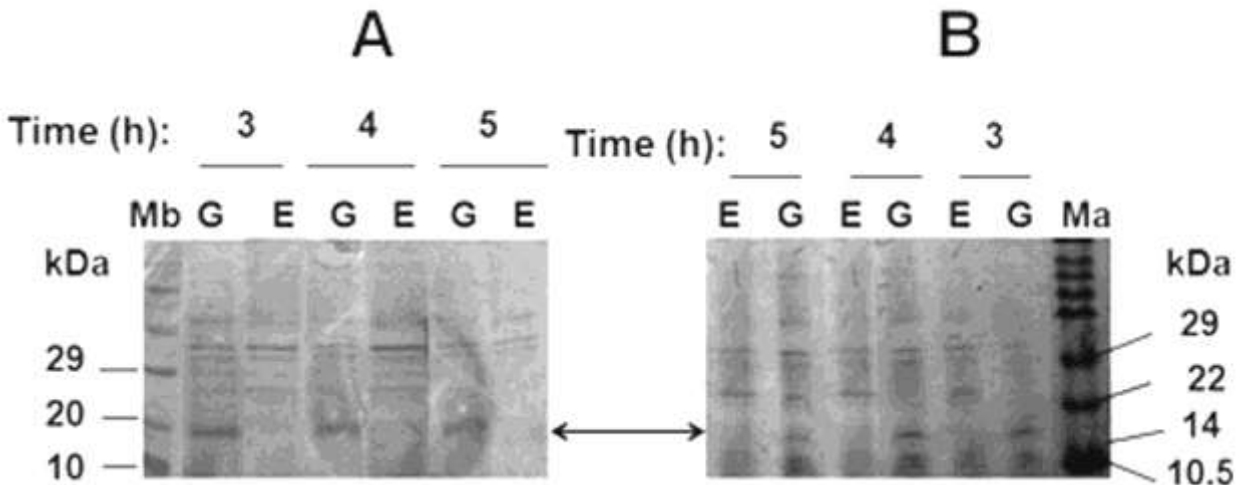
Purification and solubilization of IBs of His-IL2 and His-IL2-His proteins was successfully performed as indicated by SDS-PAGE Coomassie staining (Figure 3A and B) and Western blot detection using anti-histidine tag monoclonal antibody (Figure 3C).

### Investigation of the effect of different expression conditions on the formation of insoluble IBs

Different expression conditions were used to avoid the formation of insoluble IBs. Decreasing the concentration



**Figure 1.** PCR amplification of IL-2 gene (A) and confirmation of successful cloning via restriction enzyme digestion (B). (A) PCR Amplification of mature IL-2 gene from the plasmid pl-IL2 using the primer pairs (IL2-F1, and IL2-R1) and (IL2-F1, and IL2-R2) as demonstrated in lane 1 (Expected size 427 bp) and lane 2 (Expected size 445 bp) respectively. Ma: Hyperladder 100 bp. (B) Enzymatic digestion of the plasmids pl-His-IL2 and pl-His-IL2-His via the enzymes BamHI and HindIII as shown in lane 1 (Expected fragments 2846 and 415 bp) and lane 2 (Expected fragments 2846 and 433 bp), respectively. Ma: Hyperladder 100 bp and Mb: 1 kb DNA ladder.



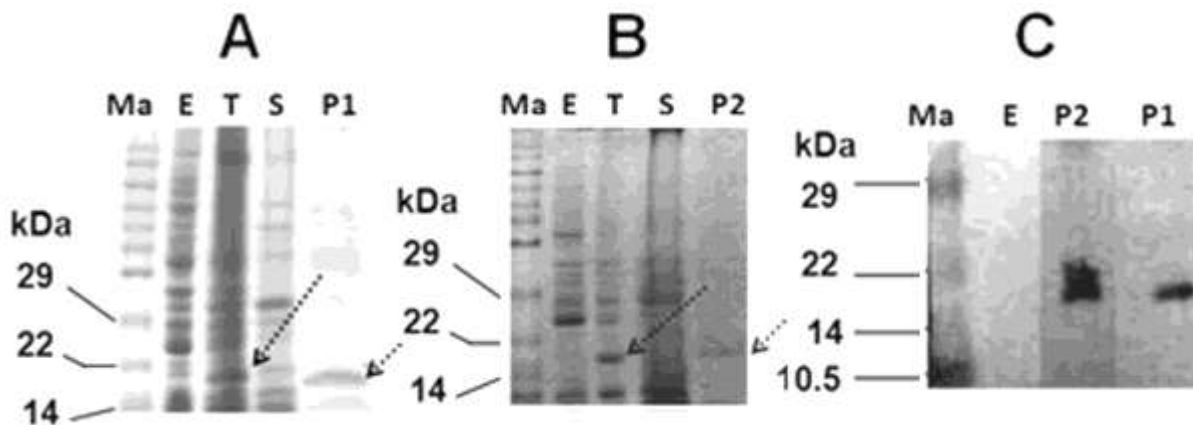
**Figure 2.** Time course screening of expression of His-IL2 protein (A) and His-IL2-His protein (B). SDS-PAGE analysis of total protein extract of *E. coli* BL21 cells transformed with empty pRSET-B vector (E), pl-His-IL2 (G in panel A), and pl-His-IL2-His (G in panel B) after 3, 4, and 5 h induction with IPTG (0.5 mM). Mb: Roti-Mark standard protein marker. Ma: Chromatein prestained protein marker. Expressed His-IL2 and His-IL2-His proteins are indicated by an arrow.

of IPTG to 0.25 mM (Figure 4A), earlier harvest of the induced cells (3 h of induction, Figure 4B), and decreasing the incubation temperature to 35 (Figure 4C) or 30°C (Figure 4D) did not help to avoid the formation of the IBs. This was indicated by the presence of the expressed His-IL-2 in the total protein extract, but not in the soluble lysate (lanes T and S in Figure 4).

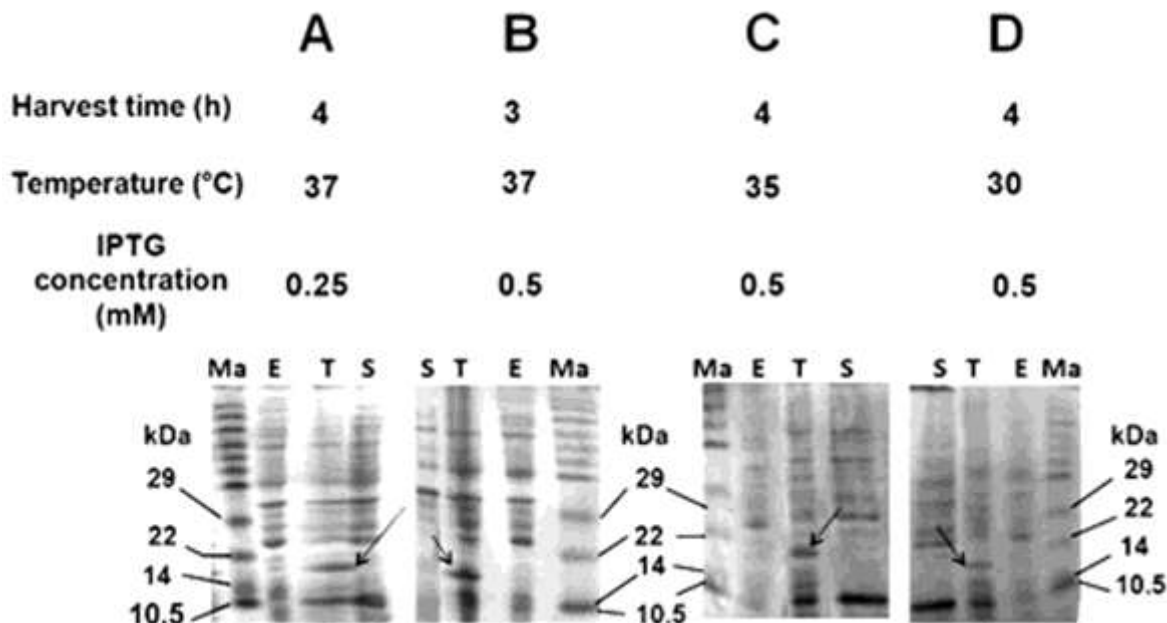
#### Quantification of the yield of the purified recombinant IL-2 proteins

After purification of both versions of IL-2 protein via immobilized metal affinity chromatography of the solubilized inclusion bodies, the concentrations of purified proteins were determined. The yield of His-IL2 and His-





**Figure 3.** Coomassie staining of the purified His-IL2 and His-IL2-His proteins and their detection by Western Blot. SDS-PAGE analysis of total protein extract (T) or soluble lysate (S) of *E. coli* BL21 cells transformed with pI-His-IL2 (Panel A) or pI-His-IL2-His (Panel B) after 4 h induction with IPTG (0.5 mM). Panel C: Western blot detection of purified His-IL2 protein and His-IL2-His proteins. E: Total protein extract of BL21 cells transformed with empty pRSET-B vector, P1: Purified His-IL-2 protein, P2: Purified His-IL-2-His protein, Ma: Chromatin prestained protein marker. Expressed and purified His-IL2 and His-IL2-His proteins are indicated by arrows.



**Figure 4.** Coomassie staining of expressed His-IL2 protein under different cultivation conditions. SDS-PAGE analysis of total protein extract (T) or soluble lysate (S) of BL21 cells transformed with pI-His-IL2 after expression at different conditions. E: Total protein extract of BL21 cells transformed with empty pRSET-B vector, Ma: Chromatin prestained protein marker. Expressed His-IL2 protein is indicated by arrows.

IL-2-His purified proteins was 5.1 and 4.5 mg/cell pellet of 1 L culture, respectively.

## DISCUSSION

Early studies on human IL-2 protein were restricted to the

protein purified from human T-cell leukemia cell line (Jurkat) (Robb et al., 1983; Stern et al., 1984). After recombinant protein technology, IL-2 was expressed after isolation of IL-2 mRNA from human leukemic T-cell line, followed by cloning of the complementary cDNA into a suitable vector. The cloned IL-2 was expressed in different hosts such as *E. coli* (Ju et al., 1987; Devos et

al., 1983; Rosenberg et al., 1984), insect cells (Smith et al., 1985), and monkey COS cells (Taniguchi et al., 1983). Most of the expressed IL-2 in *E. coli* was reported to be insoluble (forms IBs), however, the recombinant IL-2 was still retaining its biological activity even after extraction from SDS polyacrylamide gel (Devos et al., 1983).

In this study, the DNA sequence of the cloned IL-2 gene was derived from a synthesized DNA sequence rather than extraction of mRNA from human leukemic T-cell line and subsequent conversion into cDNA. An N-terminal His-tagged IL-2 (His-IL-2) protein was expressed in *E. coli* BL21 cells. Insoluble IBs of the recombinant His-IL-2 protein was formed, which was indicated by the absence of the target protein in the soluble lysate of the cells, despite its presence in the total protein extract (Figure 3A). A simple protocol was optimized for the solubilization of the insoluble aggregates of both versions of the expressed IL-2 protein (Figure 3). Such protocol depends on washing of the IBs with low concentrations of Tritone (1%) and urea (1 M) to get rid of cell wall and cell membrane components without solubilization of the IBs. The detergent guanidine hydrochloride (8 M) was used for solubilization of the IBs, followed by fishing of the His-tagged IL2 protein using Ni<sup>+2</sup> agarose beads to obtain the purified recombinant proteins (Figure 3) (Palmer and Wingfield 2004).

Different strategies were followed to prevent formation of IBs of the expressed recombinant His-IL2 protein. Incorporation of fusion tags was reported to increase the solubility of the expressed protein and subsequently could prevent formation of IBs (Costa et al., 2014; Sorensen and Mortensen, 2005b). Therefore, a dual His-tagged IL-2 protein (His-IL-2-His) was expressed to decrease the chance of IBs formation. However, the expression of this version of IL-2 protein (His-IL-2-His) was also associated with the formation of IBs (Figure 3B).

High expression rates of recombinant proteins in *E. coli* contribute greatly in IBs formation as described previously by Gatti-Lafranconi et al. (2011). Additionally, the use of high incubation temperature and high IPTG concentration often leads to a high expression rate and subsequent formation of IBs (Singh et al., 2015). Therefore, attempt was made to prevent the formation of IBs by decreasing the cultivation temperature to 35 or 30°C, and by decreasing the concentration of the expression-inducing agent to 0.25 mM. Additionally, the cells were harvested after 3 h of IPTG induction before they enter in the stage of high rate of protein expression. Nevertheless, none of these trials were useful to avoid the formation of IBs (Figure 4).

## Conclusion

The impact of the different expression conditions of IL-2 protein on the formation of IBs was determined. Yet, IBs were still forming under all the investigated conditions.

This study provides an inexpensive protocol for the production of purified human IL-2 protein. The purified human IL-2 protein produced by this work could be used as a positive control for early diagnosis of tumors and in cancer research in Egypt. Additionally, the biological activity of the purified protein, after trying different refolding conditions, will be investigated in a future project.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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## Full Length Research Paper

# Vitamin C, iron and zinc levels of selected African green leafy vegetables at different stages of maturity

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Nutrient level at harvest of vegetables is an important aspect in the efforts to combat food and nutrition insecurity in developing countries. However information on the nutrient levels at different maturity stages of most indigenous African green leafy vegetables is scanty. This study was undertaken to determine the levels of vitamin C, iron and zinc in *Amaranthus cruentus*, *Cleome gynandra* and *Solanum villosum* at different stages of maturity. The vegetables were planted on plots and harvested at 21, 28 and 35 days. At each stage, about 500 to 600 g of the edible part was harvested and standard chemical analyses procedures were followed to determine the levels of vitamin C, iron and zinc. Vitamin C increased significantly ( $p < 0.05$ ) with maturity in all vegetables except *S. villosum* Nduruma BG 16 which had similar values for stage I and II. Vitamin C content was highest ( $163.4 \pm 2.3$  mg/100 g) in *C. gynandra* stage III and lowest ( $27.7 \pm 3.9$  mg/100 g) in *S. villosum* SS 49 stage I. Iron content increased significantly ( $p < 0.05$ ) at all maturity stages. *Amaranthus cruentus* Madiira Ex zim had the highest iron concentration ( $99.9 \pm 3.7$  mg/100 g) while *S. villosum* Olevolosi SS 49 had the lowest value ( $23.1 \pm 1.5$  mg/100 g). Zinc content decreased with plant age, highest values ( $7.8 \pm 0.4$  mg/100 g) were observed in *C. gynandra* at the first stage of maturity which was reduced to about half at the third stage of maturity ( $3.9 \pm 0.0$  mg/100 g). There were marked differences in nutrient content among the various varieties and cultivars involved in this investigation. Iron and vitamin C concentrations increased with plant age whereas levels of zinc decreased with plant maturity. Consuming the different vegetables varieties would be the best approach to get all the essential nutrients in adequate amounts. Further studies are needed to investigate organoleptic acceptability of these vegetables at the different stages of maturity.

**Key words:** Iron, zinc, vitamin C, maturity stage, green leafy vegetables.

## INTRODUCTION

Indigenous African Green Leafy Vegetables (GLVs) are valuable sources of nutrients especially minerals and vitamins (Uusiku et al., 2010). They also contain non-

nutrient bioactive phytochemicals that have been linked to protection against cardiovascular and other degenerative diseases and play an important role in

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income generation and subsistence for most households (Moyo et al., 2013; Schippers, 2000). In Tropical Africa where the daily diet is dominated by starchy staples, indigenous GLVs are the cheapest and most readily available sources of important nutrients. They are often easier to grow, resistant to pests and diseases and hence do not require intensive management (Dzomeku et al., 2011; Moyo et al., 2013; Cordeiro, 2013). In Tanzania and many other African countries, consumption of vegetables including indigenous ones, differ according to season. It was reported that consumption of vegetables among four regions in the rural households in Tanzania varied from once to several times per week (Kinabo et al., 2016; Weinberger and Msuya, 2004); and about 64% of the households consumed vegetables at least three times per week in Morogoro, Kilosa and Gairo districts (Kinabo et al., 2016).

Studies show that among other nutrients, GLVs are generally rich in vitamin C, iron and zinc (Uusiku et al., 2010; Weinberger and Msuya, 2004). Vitamin C has numerous metabolic functions that are largely dependent on its reducing properties (Sen et al., 2014). It is required for the maintenance of healthy skin, gums and blood vessels. It is also known to have many biological functions including collagen formation, absorption of inorganic iron, and it is an antioxidant, that reportedly reduces the risk of arteriosclerosis, cardiovascular diseases and some forms of cancers (Amanabo et al., 2011; Nyonje et al., 2014). Iron is a mineral found in every cell of the body. It is considered an essential mineral element because it is needed to make part of blood cells (Abbaspour et al., 2014). Iron deficiency is the main cause of anemia in developing world, and in Tanzania, it affects mostly pregnant women and children below five years of age (Gautam et al., 2008; TDHS-MIS, 2015-2016). Zinc is crucial for normal development and function of cells mediating innate immunity cells and therefore its deficiency can adversely affect the growth and function of immune cells leading to impaired immune system (Roth et al., 2008).

Micronutrient deficiencies can be reduced by educating communities to increase production and consumption of indigenous and traditional dark green leafy vegetables especially in resource poor communities (Aphane et al., 2002; Ismail and Fun, 2003). Green leafy vegetables are harvested from crop fields at different stages of plant growth. For most of green leafy vegetables there is a preferred stage of plant development when flavor and palatability are favorable for human consumption. Moreover, studies have indicated that levels of nutrients and toxic substances in vegetable are influenced by stages of plant development (Khader and Rama, 2003; Modi et al., 2006). Most studies on indigenous vegetables concentrated on nutrient content and authors reported variations in nutrient content of crops depending on cultivar (Singh et al., 2001). However, there is limited information on their nutrient contents when harvested at

various maturity stages. In view of this, the current study aimed to establish the content of vitamin C, iron and zinc at different maturity stages in amaranthus (*A. cruentus*), African nightshade (*S. villosum*) and spider plant (*C. gynandra*) so as to determine best harvesting time for optimal nutritional benefits to the consumer.

## MATERIALS AND METHODS

### Description of the vegetables under study

Five varieties of indigenous green leafy vegetables namely *A. cruentus* (Madiira 1 EX Zim and Madiira II AM 38), *S. villosum* (Nduruma BG 16 and Olevolosi SS49) and *C. gynandra* were used in the study. Seeds of these vegetables were purchased from the Horticulture Training Institute of Tengeru (HORTI - Tengeru), Arusha and from the Horticulture Unit of Sokoine University of Agriculture (SUA), Morogoro, Tanzania. Comparatively, these vegetables were chosen for this research because they are indigenous vegetables that are preferred and consumed by many communities in Tanzania.

### Planting and management

The seeds were sown at the crop museum at SUA. Before sowing the seeds, the land was cleared, ploughed, harrowed and fertilized using poultry manure. Seeds of the five vegetables varieties were sown in three replications. The area was divided into three rows for the three replications and each row was divided into five beds, one for each vegetable variety. The seeds were sown in 30 cm inter and intra spacing. The density of the plants was 45 plants per bed. The vegetables were watered twice daily (mornings and evenings) and weeding was done weekly.

### Harvesting, collection of samples and sample preparation

The three maturity stages were 21, 28 and 35 days from sowing the seeds (named as maturity stages I, II and III, respectively). At each stage, about 500 to 600 g of the edible parts (leaves and young stems) were harvested by uprooting the whole plant and picking the edible leaves and shoots. The picked leaves were placed in dark colored polythene bags and transported to the food laboratory for chemical analyses. The edible portions of the samples were washed with distilled water. Excess water was left to drain off before being homogenized by cutting into small pieces (about 2 mm) using domestic sharp knife and cutting board. About 2 g of each fresh sample was weighed for vitamin C analysis. The remaining portions were then oven dried for 24 h at 60°C. The dried samples were removed from the oven and immediately ground using motor and pestle into a fine powder. The powdered samples were placed in transparent polythene bags, labeled and stored for subsequent chemical analyses.

### Analytical procedure

#### Vitamin C determination

The vitamin C concentration was determined using AOAC procedure (AOAC, 1995) where 2 g of homogenized samples was ground using motor and pestle and vitamin C compounds were extracted using metaphosphoric acid (AOAC, 1995). The pH of the extracts was adjusted to about 1.2. The reducing capacity was then

**Table 1.** Vitamin C contents (mg/100 g) in different vegetables at different stages of maturity.

| Vegetable                                   | Stage of Maturation   |                        |                        |
|---|-----------------------|------------------------|------------------------|
|   | Stage I<br>(21 Days)  | Stage II<br>(28 Days)  | Stage III<br>(35 Days) |
| <i>Solanum villosum</i> (Nduruma BG 16)     | 49.6±0.6 <sup>c</sup> | 49.2±1.6 <sup>c</sup>  | 115.5±1.6 <sup>g</sup> |
| <i>Solanum villosum</i> (Olevolosi SS 49)   | 27.7±3.9 <sup>a</sup> | 41.5±1.3 <sup>b</sup>  | 104.7±3.2 <sup>f</sup> |
| <i>Cleome gynandra</i> (Spider Plant )      | 32.4±2.9 <sup>a</sup> | 63.0±4.5 <sup>de</sup> | 163.4±2.3 <sup>i</sup> |
| <i>Amaranthus cruentus</i> (Madiira Ex Zim) | 51.0±2.3 <sup>c</sup> | 113.5±3.6 <sup>g</sup> | 132.0±3.5 <sup>h</sup> |
| <i>Amaranthus cruentus</i> (Madiira AM 38)  | 69.2±4.6 <sup>e</sup> | 57.1±2.0 <sup>cd</sup> | 102.0±3.4 <sup>f</sup> |

Data presented as arithmetic means ± SD (n = 3). Means within the vegetable variety in row/column with different superscript letters are significantly different at p<0.05.

filtered using No. 1 Whatman filter papers to obtain clear extract and then measured by titrating with standardized 2,6-dichlorophenolindophenol (DCIP). In this oxidation-reduction reaction, ascorbic acid in the extract was oxidized to L-dihydroascorbic acid (DHAA) and the indophenols dye was reduced to a colourless compound. End point of the titration was detected when excess of the unreduced dye gave a rose pink colour in acid solution. The L-dihydroascorbic acid was calculated using the formula below:

$$R = \frac{C \times (V_1 - B) \times V_2}{S_a \times V_3} \times 100$$

Where, R = Concentration of ascorbic acid in mg/100g of the sample; C = Concentration of 2, 6-dichlorophenolindophenol dye; V<sub>1</sub> = Volume of DCIP used for the sample; B = Volume of DCIP used for the blank; V<sub>2</sub> = Total extraction volume; S<sub>a</sub> = Sample weight taken and V<sub>3</sub> = Sample extract analysed.

#### Determination of iron and zinc

Iron and zinc content were determined by following the procedure described by Eslami et al. (2007) where one gram of the sample was incinerated into ash, then the ash obtained was dissolved in 6 M HCl acid. The digest was then filtered using Ashless Whatman filter paper No.1. The clear solution was subjected to atomic absorption spectrophotometer (AAS) (Shmadzu UNICAM 919 - Cambridge, UK) using the hollow cathode lamps set at 248.3 and 213.9 nm, respectively. The AAS was calibrated using standard solutions specific for each mineral element.

#### Statistical analysis

The data obtained was analysed using Statistical Product and Service Solutions (SPSS 16.0) software (Version 20). Analysis of variance (ANOVA) was computed at 5% level of significance to determine any significant difference in the levels of nutrients between the vegetable varieties and the three maturity stages (21, 28 and 35 days).

## RESULTS

### Effect of maturity stage on vitamin C contents

The results of vitamin C content are presented in Table 1. At the first harvest stage, the highest amount of vitamin C

was observed in Madiira AM 38, which was 69.2 ± 4.6 mg/100 g. Madiira EX Zim had highest vitamin C content (113.5 ± 3.6 mg/100 g) at the second stage and Spider plant had highest amount of vitamin C content (163.4 ± 2.3 mg/100 g) at maturity stage III. The lowest vitamin C scores were observed in sample Olevolosi SS 49 (27.7 ± 3.9 mg/100 g and 41.5 ± 1.3 mg/100 g) at maturity stage I and II, respectively and in Madiira AM 38 (102.0 ± 3.4 mg/100 g) and in Olevolosi SS 49 (104.7 ± 3.2 mg/100 g) at maturity stage III. Similar vitamin C content was observed in Nduruma BG 16 at stages I and II and there was a significant decrease observed in Madiira AM 38. In addition, there was a significant difference in vitamin C content among the two cultivars of *S. villosum* and *A. cruentus* at all maturity stages. The highest amount of vitamin C was observed at stage III of harvesting for all the studied vegetables.

### Effect of maturity stage on iron content

Iron content of the five cultivars from three types of vegetables is presented in Table 2. Sample Madiira EX Zim scored the highest iron concentrations (73.3 ± 0.3, 84.6 ± 0.6 and 99.9.0 ± 3.7 mg/100 g), at all three maturity stages I, II and III, respectively while samples Olevolosi SS 49, Madiira AM 38 and Spider plant had the lowest iron concentrations (23.1 ± 1.5 mg/100 g, 31.3 ± 0.8 mg/100 g and 48.6 ± 3.3 mg/100 g) at maturity stages I, II and III, respectively. In all five vegetable, varieties iron content increased continuously from stage I to stage III. Generally, the increase from maturity stage I to stage II was slightly lower compared to that of stage II to stage III. The two cultivars of *A. cruentus*, Madiira EX Zim had higher iron content compared to Madiira AM 38. Likewise, there was a difference in iron concentration in the two cultivars of *S. villosum* (Nduruma BG 16 and Olevolosi SS 49) but they had same concentration at the third stage of maturity.

### Effect of maturity stage on zinc content

The contents of zinc across different stages decreased

**Table 2.** Iron contents (mg/100 g DM) in different stages of maturity of different vegetables.

| Vegetable                                   | Stage of Maturation    |                       |                         |
|---|------------------------|-----------------------|-------------------------|
|   | Stage I<br>(21 Days)   | Stage II<br>(28 Days) | Stage III<br>(35 Days)  |
| <i>Solanum villosum</i> (Nduruma BG 16)     | 27.4±0.8 <sup>b</sup>  | 35.8±0.5 <sup>d</sup> | 85.3±0.8 <sup>i</sup>   |
| <i>Solanum villosum</i> (Olevolosi SS 49)   | 23.1±1.5 <sup>a</sup>  | 42.4±0.4 <sup>e</sup> | 85.1. ±0.6 <sup>i</sup> |
| <i>Cleome gynandra</i> (Spider Plant )      | 36.3±3.3 <sup>d</sup>  | 41.1±3.6 <sup>e</sup> | 48.6±3.3 <sup>f</sup>   |
| <i>Amaranthus cruentus</i> (Madiira Ex Zim) | 73.3±0.3 <sup>h</sup>  | 84.6±0.6 <sup>i</sup> | 99.9±3.7 <sup>j</sup>   |
| <i>Amaranthus cruentus</i> (Madiira AM 38)  | 29.2±0.6 <sup>bc</sup> | 31.3±0.8 <sup>c</sup> | 52.6±1.3 <sup>g</sup>   |

Data presented as arithmetic means ± SD (n = 3). Means within the vegetable variety in row/column with different superscript letters are significantly different at p<0.05.

**Table 3.** Zinc content in different vegetable varieties at different maturity stages.

| Vegetable                                   | Stage of Maturation  |                       |                        |
|---|----------------------|-----------------------|------------------------|
|   | Stage I<br>(21 Days) | Stage II<br>(28 Days) | Stage III<br>(35 Days) |
| <i>Solanum villosum</i> (Nduruma BG 16)     | 7.2±1.8 <sup>f</sup> | 5.4±0.0 <sup>c</sup>  | 4.4±0.9 <sup>b</sup>   |
| <i>Solanum villosum</i> (Olevolosi SS 49)   | 5.8±0.9 <sup>d</sup> | 5.4±0.4 <sup>c</sup>  | 4.5±0.9 <sup>b</sup>   |
| <i>Cleome gynandra</i> (Spider Plant )      | 7.8±0.4 <sup>g</sup> | 5.2±0.9 <sup>c</sup>  | 3.9±0.0 <sup>a</sup>   |
| <i>Amaranthus cruentus</i> (Madiira Ex Zim) | 4.6±0.9 <sup>b</sup> | 4.6±0.8 <sup>b</sup>  | 3.7±0.0 <sup>a</sup>   |
| <i>Amaranthus cruentus</i> (Madiira AM 38)  | 6.2±0.9 <sup>e</sup> | 5.8±0.9 <sup>d</sup>  | 4.0±0.9 <sup>a</sup>   |

Data presented as arithmetic means ± SD (n = 3). Means within the vegetable variety in row/column with different superscript letters are significantly different at p<0.05.

with plant maturity (Table 3). The highest zinc contents were  $7.8 \pm 0.4$  mg/100 g in sample *C. gynandra* at maturity stages I,  $5.8 \pm 0.9$  mg/100 g in Madiira AM 38 at maturity stage II and  $4.5 \pm 0.9$  mg/100 g in Olevolosi SS 49 at maturity stage III. The lowest zinc contents were in sample Madiira EX Zim at all three maturity stages. In all five varieties, zinc content decreased with plant age with the lowest zinc content at stage III of maturity. Significant differences in zinc content were also observed among the two varieties of *S. villosum*, which is Olevolosi SS 49 and Nduruma BG, 16 only in the first maturity stage but no difference was observed in the second and third maturity stages. There were also differences in the two varieties of amaranthus Madiira EX Zim and Madiira AM 38 at the first and second stages of maturity but zinc content was not significantly different at stage III of maturity.

## DISCUSSION

In this study, variation in vitamin C, iron and zinc contents in vegetables harvested at different stages of maturity was investigated. Variation was observed in vitamin C content across the cultivars. Vitamin C content was similar with what was reported by other researchers for vegetables grown in Africa (Ayua et al., 2016). There was

relatively higher vitamin C content at the third stage of maturity in all studied vegetables. *C. gynandra* had the highest vitamin C content at 35 days. These results are in agreement with the work of Amanabo et al. (2011) who found that vitamin C content increased from market maturity ( $94.60 \pm 5.60$  mg/100 g) to reproductive stage ( $160.50 \pm 7.10$  mg/100 g) in *A. cruentus* (Amanabo et al., 2011). Some researchers reported that vitamin C concentration is highest in mature leaves with fully developed chloroplasts (Khan et al., 2011) and regardless of fertilizers used, vitamin C content increased with maturity in spider plant and black nightshade grown in Kenya (Ayua et al., 2016). This suggest that harvesting green leafy vegetables at advanced age potentially provides the greatest concentrations of vitamin C. Apart from maturity stage and variety type, other factors that affect vitamin C concentration in vegetables include postharvest handling conditions (time lapse between harvest to consumption and extent of physical damage), processing methods such as blanching, cooking, drying and canning (Kader and Seung, 2000). This implies that even when vegetables are harvested at the maturity stages when vitamin C content is highest, care should be taken during handling, processing and cooking to ensure maximum intake of this important vitamin.

There was variation in iron content across the varieties

and across the cultivars. *Amaranthus cruentus* (Madiira Ex Zim) had the highest iron content. Iron content in the studied vegetables was higher than other indigenous African vegetables as reported by other researchers (Kamga et al., 2013; Lyimo et al., 2003). In a recent study, variation in nutrient content was reported, specifically zinc and iron contents in vegetables grown from different districts in Tanzania (Amuri et al., 2017). An increase in iron content was observed in all varieties of the studied vegetables across the harvesting stages. The increasing trend of iron suggests that the mineral may be indissociable ion and accumulates as age increase. These findings were similar to those of Flyman and Afolayan who reported increase in iron content at each stage in *Vigna unguiculata* as the plant matured from 21 to 57 days after sowing the seeds (Flyman and Afolayan, 2008). Modi and colleagues found that iron concentration in *A. cruentus* increased significantly ( $p < 0.05$ ) from  $40 \pm 2.5$  mg/100 g at 20 days after sowing to  $58 \pm 2.1$  mg/100 g at 40 days after sowing (Modi et al., 2006). It was reported in other studies that iron concentration decreased in green leafy vegetables as they mature towards fruiting (Khader and Rama, 2003). The possible reason for the increasing trend observed in this study is that the 35 days that were set as maximum for harvesting the leaves was still early for the vegetables to start flowering. Further studies to investigate later harvesting stages on nutrients may be necessary.

Zinc content was significantly higher in *C. gynandra* at the first stage of maturity. The amount was higher than what was reported in other commonly consumed vegetables like sweet potato leaves, lettuce, jute mallow, and kales (Kamga et al., 2013). The observed variations could be attributed by harvesting stage or nature of the soil where the vegetables were grown. The differences in zinc content between them may be attributed to differences in absorption efficiencies between the varieties. Different plant varieties have got different root system and hence different efficiency in exploiting and taking up nutrients from the soil. There was a slight but not significant decrease in zinc concentration at all stages of maturity. The decreasing zinc content may be attributed to diversion of this mineral towards plant development. During fruit initiation and development, some metabolites for cellular synthesis and growth substances are translocated from the leaves, stems, and roots to the developing fruits (Khader and Rama, 2003). Moreover, Lanyasunya et al. (2007) observed that the rapid uptake of mineral by plants during early growth and the gradual dilution that occurs as plant matures would have been responsible for the decrease in some of the mineral content during fruiting. Zinc content decreased with increasing maturity stages in *Amaranthus* and *Spinaces* species (Khader and Rama, 1998).

Flyman and Afolayan (2008) found that zinc content was higher at 21 days ( $389.08 \pm 5.08$  mg/100 kg) and decreased continuously to  $161.53 \pm 3.59$  mg/100 kg at 28

days and decreased further to  $85.83 \pm 1.30$  mg/100 g, at 35 days after sowing. Amanabo et al. (2011) observed that zinc content in *A. cruentus* decreased from  $0.08 \pm 0.01$  mg/100 g dry weight at market maturity (vegetative) stage to  $0.05 \pm 0.01$  mg/100 g dry weight at heading (reproductive) stage. Generally, the variation in nutrient content across the cultivars and across different vegetables can be explained by their genetic variations (Marles, 2017).

### Associations of soil nutrient and concentration in vegetables

Nutrient concentrations in foods depend largely on the quality of soil in which the crops are grown. Soil fertility management practices were reported to affect zinc and iron concentration in vegetables and there was a direct association between soil chemical properties and vegetable mineral concentrations (Amuri et al., 2017). Some studies noted that soil pH may affect zinc content in vegetables and in some study sites zinc in the soil was negatively correlated with zinc content in vegetables (Amuri et al., 2017; Harter, 1983). The authors noted that levels of zinc in vegetables varied due to soil management and not necessarily zinc concentration in the soil (Amuri et al., 2017). A study in Bangladesh found that elemental concentrations vegetables varied in different samples and which reflect the difference in uptake capabilities and their further translocation to the edible portion of the plants (Jolly et al., 2013). They also found variation according to location which could be related to soil properties and soil nutrient management. Other studies found that increased zinc content in the soil increased its concentration in *Amaranthus* leaves (Ondo et al., 2012). Although soil nature and other environmental factors contribute to the mineral deposition in plants, it was not the case in this study since all vegetables were grown in similar soil type.

### Conclusions

There were marked differences in nutrient content among the various varieties and cultivars involved in this investigation. There were variations in vitamin C content in all vegetables investigated where the highest amount was in Madiira AM 38 in stage I, in Madiira Ex Zim in stage II and in *C. gynandra* in stage III. Madiira EX Zim was the best in iron content at all maturity stages. *C. gynandra* contained highest levels of zinc at stage I which decreased to about half at stage III. Iron and vitamin C concentrations increased with plant age implying that the vegetables should be harvested at advanced plant age. On the contrary, levels of zinc decreased with plant maturity implying that vegetables should be harvested at early maturity stages preferably the third week after



sowing. This suggests that consuming different vegetables varieties in combination would be the best approach to get all the essential nutrients in adequate amounts needed to maintain normal body functions. It would be interesting to investigate the consumer acceptance in consuming vegetables harvested at different stages of maturity. While considering nutrient content as an important factor, palatability and other sensory attributes and hence consumer acceptance should not be ignored.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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