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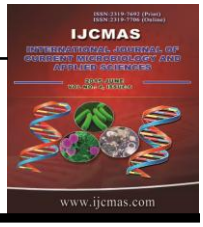

Original Research Article

Potential impact of cyanobacterial exudates on seed germination and antioxidant enzymes of crop plant seedlings

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Keywords

Cyanobacteria, crops, germination, growth, antioxidant enzymes, metabolites.

A B S T R A C T

Cyanobacteria are a diverse group of prokaryotes that are spread worldwide. They improve the growth and development of the plants they share their vicinity via releasing various biologically active substances. The effect of the exudates of the cyanobacterial strains; *Anabaena oryzae, Nostoc ellipsosporum* and *Synechococcus* sp. on the percent of seed germination and the seedling growth criteria of *Sorghum durra* and *Helianthus annuus* was investigated in this study. A marked promotion in the seed germination of *Sorghum durra* was recorded with *Anabaena oryzae* and *Synechococcus* sp. while the three culture filtrates demonstrated a negative consequence with *Helianthus annuus*. At the same time, the seedling growth criteria of the treated plants were highly significantly enhanced especially with *Anabaena oryzae*. Also, A clear augmentation in the activity of the antioxidant enzymes; catalase, peroxidase and polyphenol oxidase was recorded in the treated plants. Analysis of *Anabaena oryzae* exudates by Gas Chromatography Mass Spectrometry (GC-MS) demonstrated the occurrence of gibberellins, n-acetyl-D-glucosamine, linalool, dihydroxyphenyl glycol in addition to niacinamide. The presence of these bioactive compounds in the exudates of *Anabaena oryzae* might be directly or indirectly involved in root initiation, cell division and cell enlargement in addition to their role in the activation of the antioxidative defense enzymes of the treated plants.

Introduction

Cyanobacteria are oxygenic, photosynthetic prokaryotic organisms that are distributed worldwide and can inhabit a wide range of habitats including freshwater, marine and terrestrial environments (Pankratova 2006; Tripathi *et al*., 2007; Nagarajan *et al*., 2012; Whitton 2012). In agricultural soils, they potentially contribute towards biological nitrogen fixation that improve soil fertility and crop productivity. Although, the great majority of cyanobacteria that fix nitrogen are heterocystous (Granhall & Henriksson 1969), non-heterocystous cyanobacteria can fix nitrogen as well (Kallas *et al*., 1983). The fixed nitrogen is then released as available form of ammonia that is required for the growth of higher plants.

The role of cyanobacteria as natural root colonizer in enhancement of rice yield has been well documented in literatures and this effect was mainly attributed to their capacity to fix atmospheric nitrogen (Rodgers *et al*., 1979; De Cano *et al*., 1993). The paddy-field ecosystem is an exclusive habitat that offers a favorable environment for their growth and nitrogen fixation. However, some studies demonstrated the contribution of cyanobacteria in the plant growth of other crop plants such as wheat, maize and cotton which do not require a flooded environment. In this context, Adam (1999) showed the positive effect of cyanobacteria as biofertilizer on seed germination and related processes of wheat, sorghum, maize and lentil plants. Germination and growth parameters of the tested seeds were significantly increased as a result of treating them with the cultural filtrate or cell extract of the nitrogen-fixing cyanobacterium *Nostoc muscorum*.

Another investigation on the morphological and biochemical parameters of *Lupius termis* treated by the extract of *Cylindrospermum muscicola* and *A. oryzae* was carried out by Haroun & Hussein (2003). They recorded an enhancement in the growth parameters such as shoot length, total leaf area and biomass. The culture filtrate also increased the photosynthetic activity, content of nitrogenous compounds and carbohydrates in the shoot of tested plant. Similar observation on morphological and biochemical parameters on pea plant was done by Osman *et al*. (2010). They demonstrated a promotion in the plant growth criteria including root length, shoot length, dry weightsand leaf area in addition to the biochemical constituents of seedling grown in soil that was inoculated with suspensions of *Nostoc entophytum* and *Oscillatoria angustissima*.

Moreover, the cyanobacterial genera *Nostoc, Anabaena, Calothrix, Haplosiphon, Oscillatoria, Lyngbya, Phormidium* were shown to enhance soil microbial biomass, available nitrogen and related soil microbiological parameter, along with the increase in seed germination, root and shoot growth and weight and yield of rice and wheat (Obana *et al*., 2007; Prasanna *et al*., 2013).

Similarly, seed germination, shoot length, tillering number of lateral roots, spike length, grain weight, protein content, micronutrients and endogenous phytohormone pool were significantly enhanced in wheat plants that were inoculated with *Anabaena* sp. (Hussain & Hasnain 2011; Mazhar *et al*., 2013).

It is clear from these studies that the beneficial effect of cyanobacteria on the crop plants may not be restricted to their role in the fixation of atmospheric nitrogen. They can stimulate the growth and development of plants lying in their habitat via different approaches including biofertilization, biological control, soil conditioners, biosorption of heavy metals or by delivering of various biologically active substances (Vaishampayan *et al*., 2001; Ibrahim *et al*., 2010; Essa and Mostafa 2011; Shanab *et al*., 2012).

Sunflower (*Helianthus annuus*, L.) is considered as one of the most important annual crops ifor edible oil (Mohamedin *et al*., 2004) while *Sorghum durra* is a staple food crop of millions of poor in semi-arid tropics of the world. Recently, a great attention has been focused on the possibility of using natural and safety substances in order to improve plant growth. The aim of the present study is to investigate the impact of the culture exudates of *Anabaena oryzae*, *Nostoc ellipsosporum* and *Synechococcus*

sp. on seed germination and the seedling growth criteria of *Sorghum durra* and *Helianthus annuus*.

Materials and Methods

Isolation of axenic cyanobacterial strains

The cyanobacterial strains (*Anabaena oryzae, Nostoc ellipsosporum* and *Synechococcus* sp.) were isolated from soil samples collected from different crop fields in Fayoum Governorate, Egypt. Soil samples were transferred into Petri dishes then wetted by sterilized distilled water and incubated under florescent illumination. After two to three weeks, green mats appeared on the surface of the dishes. The mats were cleaned from mud and soil particles by serial washings with distilled water. The washed mats were inoculated on solid agar BG-11 medium (Rippka *et al*., 1979) and incubated for two weeks in a culture room at 28 ± 1°C under controlled continuous illumination of 40 $\mu Em^{-2}s^{-1}$. The plates were examined and the best colonies were picked up and restreaked to new agar plates. Restreaking and subculturing were repeated several times to obtain unialgal cultures. To get axenic cultures, the tested algae were grown in liquid cultures for 12 days to attain vigorous growth. About 20 mL of each culture were centrifuged at 1500 rpm for 10 minutes. Algal pellets were then streaked on peptone or yeast extract solid media. Those which proved to be axenic were taken into sterilized liquid media to be ready for the desired experimental procedures. The purified cyanobacteria were identified according to Desikachary (1959) and Prescott (1978).

Preparation of the algal filtrate

The selected algal isolates were batch cultured in Erlenmyer flasks (500 mL). Into each flask 200 mL of liquid culture BG-11 medium were added. The initial inoculum used throughout this investigation was approximately 2 x 104 cell/mL of the stock culture at the end of logarithmic phase (7 days old cultures). The culture flasks were kept at 28 ± 1ºC under light intensity of 40 µEm-2s-1 provided by cool white fluorescent lamps for 21 days. Then cultures were centrifuged at 10000 rpm for 10 minutes at 4°C and the supernatant was subjected for dialyzes against running tap water then distilled water in a Spectra/por dialysis tube (Spectra/Por; Spectrum Medical Industries, Los Angeles, CA) for 48 hrs to remove the remaining nutrient from the culture filtrates. The supernatants were lyophilized using Christ L-1, Alpha 2-41 Model at -45oC, re-suspended in 10% of the original volume of sterile water and used to treat plant seeds.

Tested crop plants

Pure identified strains of two economic crop plants were chosen for this study namely *Sorghum durra* var. *aegyptiacum* and *Helianthus annuus* L.var Giza 102. Seeds of the chosen plants were from the Agricultural Research Centre, Giza, Egypt. The two species were Sorghum durra Stapf. var. aegyptiacum.

Seed germination experiment

A homogenous lot of seeds of each tested plant were selected for uniformity of size, shape and viability. Before germination, seeds were surface sterilized by soaking for 30 minutes in 2.5 % sodium hypochlorite solution, rinsed several times with distilled water. Then the sterilized 20 seeds were presoaking in the cynaobacterial filtrates for 18 hrs, and then they were transferred to sterile Petri dish containing two sheets of Whatman No.1 filter paper, moisted with 10 mL of distilled water. The seeds germinating

in the darkness at 25°C. Petri dishes were daily watered with 2 mL of distilled water for 6 days. The percentage of seed germination was calculated as following:

Seed germination (%) = (no. of germinated seeds / no. of seeds in Petri-dish) x 100

At the end of the experimental, the shoot length, root length, fresh weight and dry weight of the seedlings were determined. At the same time, catalase and polyphenol oxidase activities were assayed according to the method of Kar & Mishra (1976) while peroxidase activity was assayed according to the method of Bergmeyer (1974).

Characterization of the cyanobacterial exudates

To determine total and soluble carbohydrates in the culture filtrates, the anthrone- sulfuric acid method (Irigoyen *et al*., 1992) while protein content was assayed using Bradford assay (Bradford, 1976). At the same time, the total phosphorus content in the cyanobacterial filtrates was measured spectrophotometrically at 720 nm according to Pierpoint (1957). Cyanobacterial phytohormones were extracted and fractionated according to the method that was originally described by Horemans *et al.* (1986). A LC/GPC 401 Liquid Chromatography with two Modules 510 pumps, Module 721 Programmer controller, Module U6K injector and Module 441 UV monitors, operating at 254 nm. Radial-Pack A Cartilage C18 (100 x 8 mm) column and Z-module redial compression system were used.

Moreover, GC-MS analysis of the culture filtrates was carried out in Faculty of Agriculture (Cairo University) using a GC-MS system model 7890. An Hp-5MS fused silica capillary column (Hewlett- Packed, 30 m, 0.25 mm i.d., 0.25 µm film Thickness, cross-linked to 5% phenyl methyl siloxane stationary phase) was used. The entire system was controlled by MS ChemStation software (Hewlett- Packed, version A.01. 01). Electron impact mass spectra were recorded at 70 eV. Ultra-high purity helium (99.9%) was used as the carrier gas at flow rate of 1mL/min. The injection volume was 1 µL and all the injections were performed in a splitless mode. Injector temperatures were 250ºC. Temperature program was used: 60 ºc (2 min)–30 ºc /min–170 ºc (5 min)–7 ºc /min–250 ºc (10 min) (Langseth *et al*., 1998).

Statistical analysis

The experimental design was a random complete block, with three replications. The data were analyzed by STATGRAPHICS (Statistical Graphics Corporation, Princeton, USA) statistical package by *t-test* function to assess significant difference.

Results and Discussion

Changes in percent of seed germination

Data in Figure (1) showed the changes in germination percentage of *Sorghum durra* and *Helianthus annuus* that were treated with culture filtrates of *A. oryzae, N. ellipsosporum* and *Synechococcus* sp. It is apparent that the germination percentage increased progressively throughout the germination period. The culture filtrates of *A. oryzae and Synechococcus* sp. caused significant increase ($p \leq 0.05$) in the percentage of germination of *Sorghum durra* with 30% and 26%, respectively *w*hile *N. ellipsosporum* filtrate showed a 20% reduction in the germination. At the same time, a considerable retardation in the seed germination of *Helianthus annuus* was demonstrated with *A. oryzae* (4%), *N. ellipsosporum* (25%) and *Synechococcus sp.* (14%).

Change in seedling growth parameters

The shoot length of two crop plants seedlings was highly significant increased ($p \leq 0.001$) in response to all culture filtrates with amount ranged from 80% to 302% in relation to control (Figure 2). The maximum increase in shoot length of *Sorghum durra* seedlings was induced by the culture filtrate of *A. oryzae*, while the culture filtrate of *A. oryzae* and *N. ellipsosporum* showed the highest shoot length with *Helianthus annuus.* Moreover, the root length of seedlings treated with culture filtrates of the algal isolates were highly significant enhanced. The increment ranged between 120% and 242% as compared to control. The highest increase in root length was induced by *A. oryzae* in the tested crop plants. In the meantime, the changes in the fresh weight of seedlings treated with different culture filtrates. The fresh weight of seedlings was highly significant promoted ($p \leq 0.001$) with all culture filtrates and the maximal increase in seedling fresh weight occurred in case of *A. oryzae*. From data present in Figure (2) it was proved that, the dry weight of two crop plants seedlings increased significantly ($p \leq 0.05$) with all cyanobacterial filtrates where the maximal increase occurred in case of *Synechococcus* sp.

Change in enzymatic activities of seedlings.

It can be seen from Figure (3) that, the enzymatic activities of *Sorghum durra* and *Helianthus annuus* seedlings increased as a result of the application of the cyanobacterial filtrates compared with control. The largest increase in catalase activities was attained with filtrates of *A. oryzae* followed by *Synechococcus* sp. Also, the peroxidase activity of plant seedlings was clearly increased with different culture filtrates. These increments ranged from 54 % to 106% compared to control value. The highest increase in peroxidase activity occurred in case of *N. ellipsosporum*. Regarding polyphenoloxidase activity of seedlings, the maximal increase in enzyme activity was obtained with *Synechococcus sp*.

Characterization of the cyanobacterial filtrates

It is evident from Table (1) that carbohydrate content differs significantly in the three cyanobacterial culture filtrates. The total and soluble carbohydrate content of the filtrate of *Synechococcus* sp. culture filtrate was higher than the other cyanobacteria while the lowest content was present in *N. ellipsosporum*. At the same time, the maximum protein content was recorded in *N. ellipsosporum* and *Synechococcus* sp. while low level of protein was recorded in the filtrate of *A. Oryzae.* Also, data in Table (1) demonstrated that *A. oryzae* culture filtrate contained the highest phosphorus content (42.5 mg/L) while culture filtrate of *N. ellipsosporum* and *Synechococcus* sp. contained the lowest content (5.0 mg/L). The highest level of growth promoting substances were recorded in *Synechococcus* sp. (1.27 µg/100ml) and *N. ellipsosporum* (1.08 µg/100 mL) culture filtrates where the lowest level of total promoting substances was recorded in the filtrate of *A. oryzae* (0.71 µg/100 mL) accompanied with the least amount of auxins and gibberellins.

Furthermore, GC-MS analysis of *A. oryzae* exudates (Fig. 4) showed the presence of niacinamide with molecular formula $C_6H_6N_2O$, (M.wt 122.12 $gmol^{-1}$, A), Linalool with molecular formula $C_{10}H_{18}O$ (M.wt 154.25 $gmol^{-1}$, B), 3,4-dihydroxyphenyl glycol with molecular formula $C_8H_{10}O_4$ (M.wt 170.16 $gmol^{-1}$, C) and N-Acetyl-D-glucosamine with molecular formula $C_8H_{15}NO_6$ (M.wt 221.20

$gmol^{-1}$, D) at retention times 10.37, 14.56, 22.76 and 28.87, respectively.

Effect of culture exudates on seed germination and growth criteria

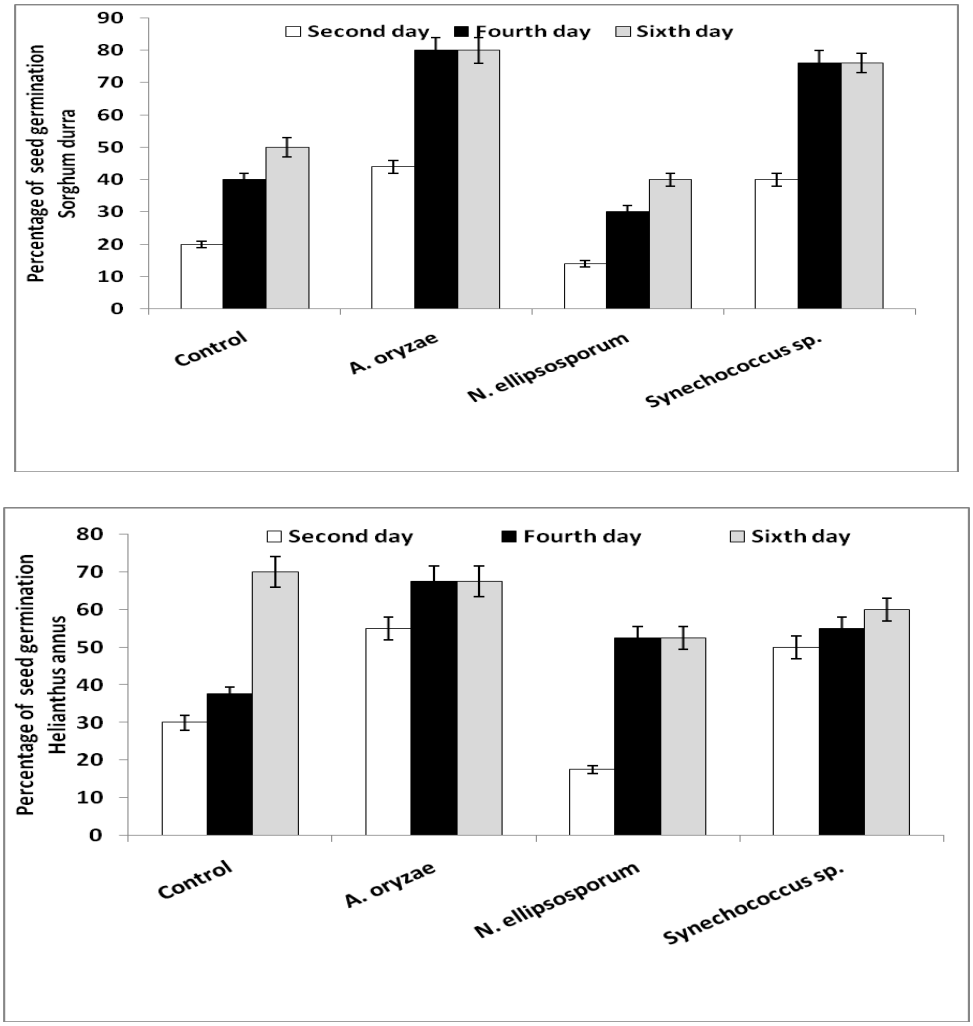
Germination is a vital phase in the life cycle of plants, which aids the embryo to endure the period between seed maturation and seedling establishment. There are many factors affecting on seed germination such as salinity, temperature, moisture and light intensity (Gorai *et al*., 2011). This study clarified an increment in seed germination of *Sorghum durra* treated with the culture filtrates of *A. oryzae* and *Synechococcus* sp. The positive effect of the cyanobacterial filtrate on germination process has been reported by several authors who demonstrated a significant increase in seed germination of some crop plants such as wheat, sorghum, maize, lentil and sugar beet as a result of the treatment with the culture filtrate or cell extract of *Nostoc muscorum* (Adam 1999; Aly *et al*., 2008). Similarly, Hassan & Morcos (2006) showed an enhancement in the germination of rice seeds that were treated with the culture filtrates of *Anabaena oryzae, Nostoe calcicola, Microchaete tenera* and *Cylindrospermum muscicola*. Moreover, Kumar & Kaur (2014) highlighted the positive consequence of the filtrate of *Anabaena variabilis, Nostoc muscorum, Aulosira fertilissima* and *Tolypothrix tenuis* on the germination velocity index and vigor index of wheat seeds. At the same time, the obtained data elucidated a promoting impact of the cyanobacterial filtrates on the seedling growth parameters including shoot length, root length, fresh weight and dry weight of the tested crop plants. In this connection, Hashtroudi *et al*. (2013) and Shariatmadari *et al*. (2013) have recorded a remarkable promotion of morphological and biochemical parameters of some vegetables and herbaceous plants treated with the extracts of *Anabaena vaginicola* and *Nostoc calcicola*. Similarly, Hussain and Hasnain (2011) and Mazhar *et al*. (2013) reported an enhancement in shoot length, number of lateral roots of the wheat plants treated with cyanobacterial strains; *Nostoc* sp. *Phormidium* sp. *Chroococcidiopsis* sp., *Calothrix* sp. and *Anabaena* sp. Apparently, the augmentation impact of the cyanobacterial exudates on seed germination and seedling growth parameters of the tested plants could be attributed to the presence of wide array of bioactive metabolites that are released by cyanobacteria into their environment. These compounds might be directly or indirectly involved in root initiation, cell division and cell enlargement (Prasanna *et al*., 2010).

Surprisingly, this study clarified an inhibitory effect of the exudates of *N. ellipsosporum* on seed germination of *Sorghum durra*. Furthermore, the culture filtrates of the tested cyanobacterial strains showed even neutral or retarding impact on seed germination of *Helianthus annuus*. The negative effect of some cyanobacteria on the germination process has been reported by Pedurand & Reynaud (1989) who reported that about 70% of the 133 cyanobacterial strains showed a negative effect on seed germination of rice while 12% demonstrated an inhibitory impact on the seedling growth. Similarly, Sukkhaeng *et al*. (2014) found that the crude extract of *Nostoc* sp., *Scytonema* sp. and *Lyngbya* sp. had a suppressive effect on the root and shoot growth of *Mimosa pigra* while *Nostoc* sp. extract recorded a marked inhibitory effect on root cell division of onion root tips via reducing the mitotic index. In general, the inhibitory effect of cyanobacteria on seed germination might be ascribed to the presence of secondary metabolites that can interfere and retard the biochemical reactions (Entzeroth *et al*., 1985; Gleason & Case 1986).

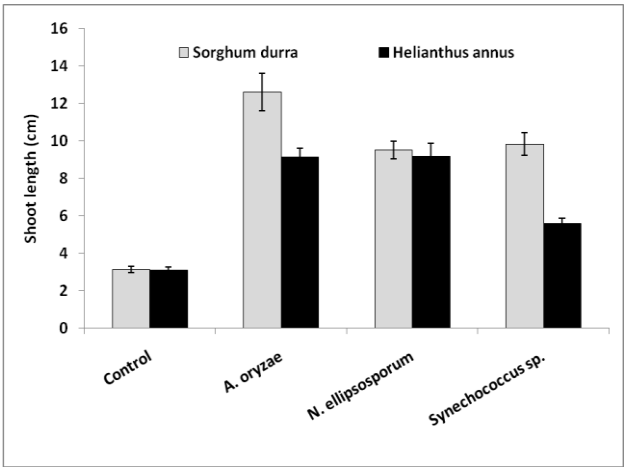
Table.1 Characterization of the cyanobacterial filtrates

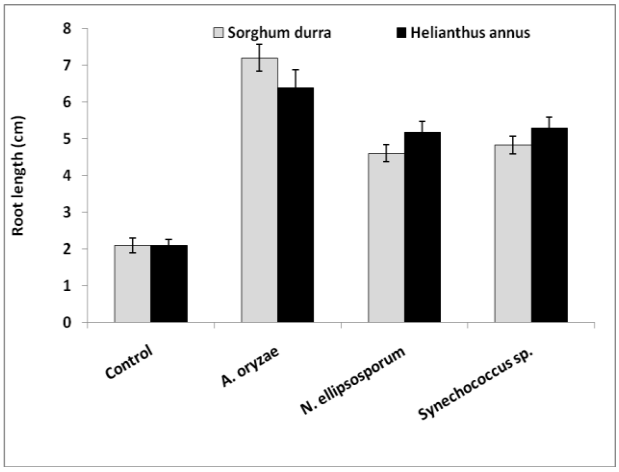
Biochemical analyses	***Anabaena oryzae***	***Nostoc ellipsosporum***	***Synechococcus* Sp.**
Soluble carbohydrate (mg/L)	1.03	1.95	2.03
Total carbohydrate (mg/L)	5.66	2.54	12.18
Total protein content (mg/L)	72.5	90.63	91.88
Total phosphorus content (mg/L)	42.5	5.0	5.0
Auxins (µg/100 mL)	0.0	0.0	0.61
Gibberellins (µg/100 mL)	0.71	1.08	0.66

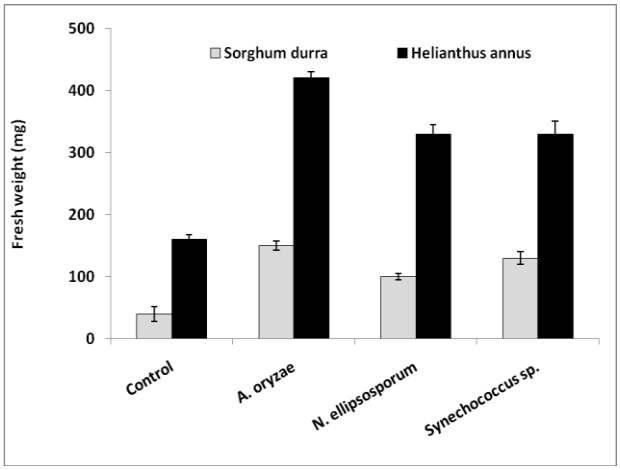
Figure.1 Effect of cyanobacterial filtrates on the seed germination of *Sorgham dura* and *Helianthus annuus.* Data are the means of three replicates and error bars represent the standard errors of the means



Figute 2. Effect of cyanobacterial filtrates on the growth parameters of *Sorgham dura* and *Helianthus annuus.* Data are the means of three replicates and error bars represent the standard errors of the means







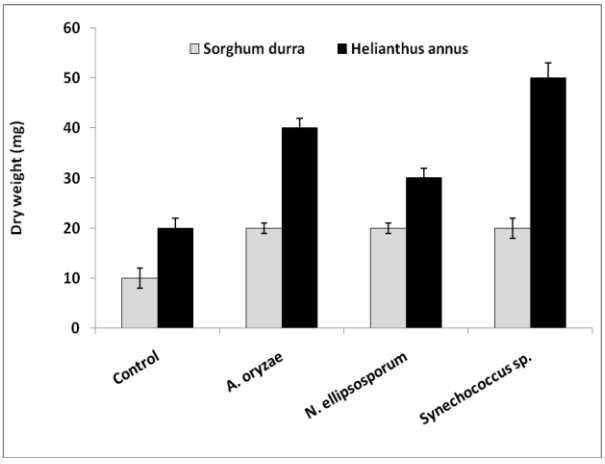


Figure 3. Effect of cyanobacterial filtrates on the activity of the antioxidant enzymes of the treated *Sorgham dura* and *Helianthus annuus* seeds. Data are the means of three replicates and error bars represent the standard errors of the means

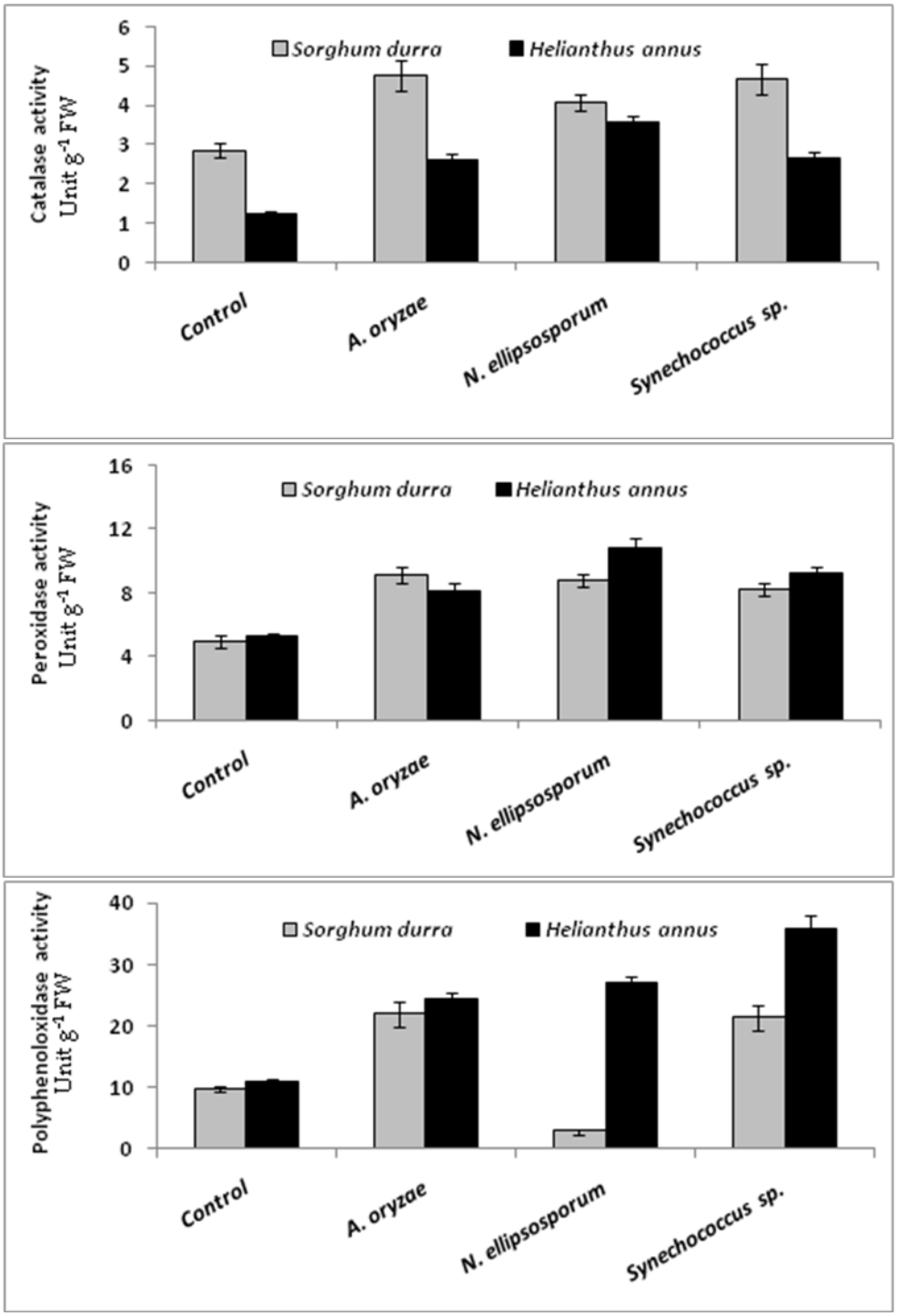
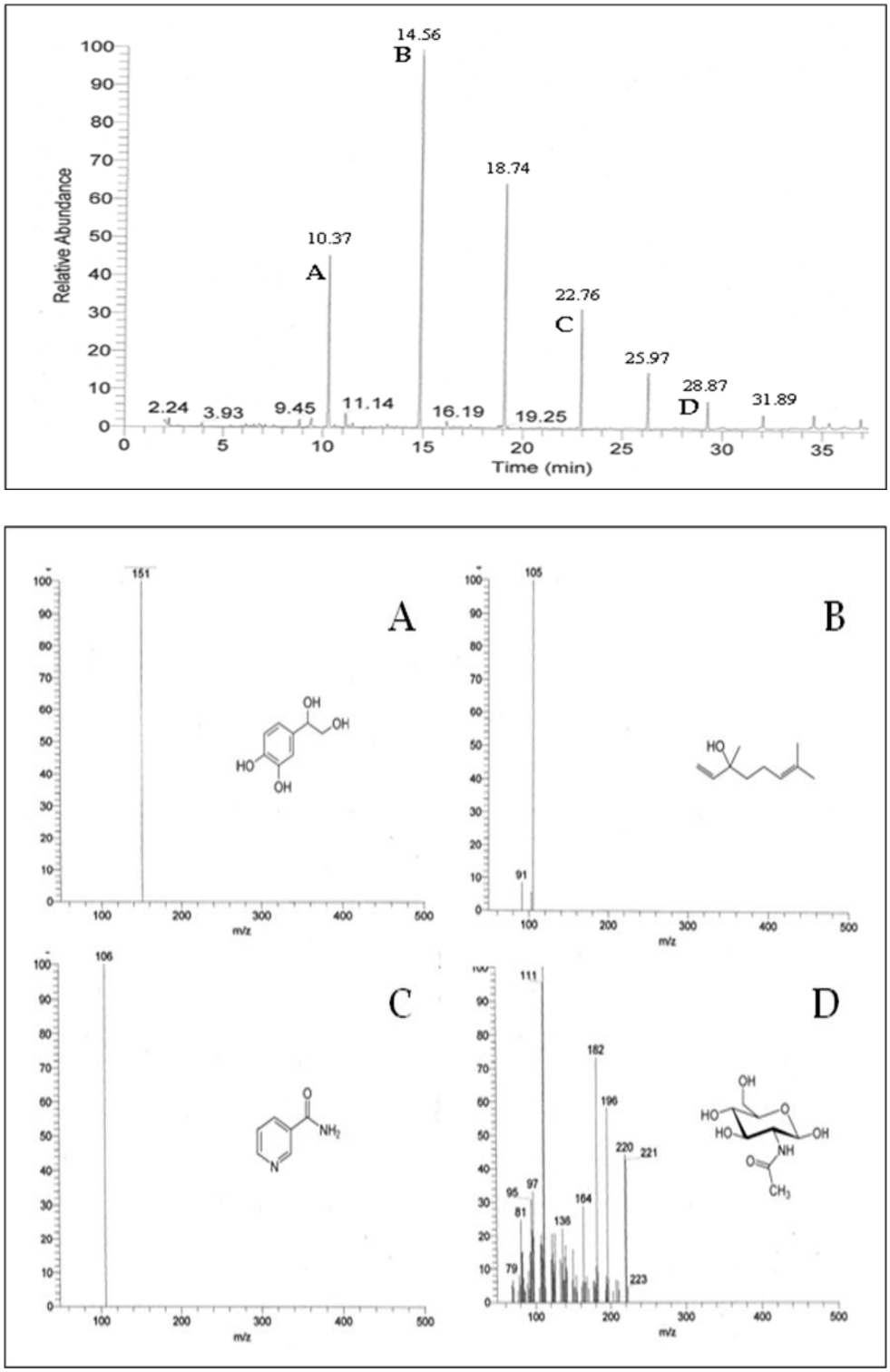


Figure 4. Gas Chromatography mass spectral analysis of the *Anabaena oryzae* filtrate showing the mass spectral analysis of niacinamide (A), linalool (B) and Dihydroxyphenyl glycol (C) and N-acetyl-D-glucosamine (D). Data are representative of at least two independent biological replicates



Effect on the antioxidant enzymes

The present investigation demonstrated a marked increment in the activity of the antioxidant enzymes; catalase, peroxidase and polyphenoloxidase in *Sorghum durra* and *Helianthus annuus* seedlings that were treatment with the cyanobacterial filtrates. In agreement with these findings, Naresh *et al*. (2013) showed an apparent enhancement in the antioxidant system of *Linum usitatissimum* that were treated with *Nostoc muscorum, Nostoc piscinale* and *Anabaena fertilissima* exudates. Similarly, the enzyme activities of peroxidase and phenylalanine ammonialyase in rice leaves were elevated as a result of inoculated with *Oscillatoria acuta* and *Plectonema boryanum* (Singh *et al*., 2011). Actually, cyanobacteria contain various metabolites that might affect positively on plant antioxidative defense system via enhancing the activities of superoxide dismutases, peroxidases, catalases, glutathione S-transferases and glutathione reductases (Chen *et al*., 2004; Pflugmacher *et al*., 2007).

Characterization of the cyanobacterial culture filtrates

It is evident from the obtained results that the cyanobacterial culture filtrates contain variable amount of soluble carbohydrates and proteins in addition to soluble phosphorus. The presence of such these compounds in the culture supernatant could participate in the stimulation of seed germination and growth parameters of plant seedlings (Karthikeyan *et al*., 2009; Xu *et al*., 2013). At the same time, the obtained data showed the presence of auxins and gibberellins in the exudates of the tested cyanobacteria. In harmony with these findings, several studies have recorded the isolation of different growth promoting compounds such as gibberellin-like substances, indole-3-butyric acid, indole-3-propionic acid, indole-3-acetic acid and cytokinins from the cyanobacterial strains of *Anabaena, Oscillatoria, Phormidium, Chroococcidiopsis, Synechocystis, Anabaenopsis, Cylindrospermum* (Osman *et al*., 2010; Singh *et al*., 2011; Hashtroudi *et al*., 2013). The growth promoting substances of the cyanobacterial filtrates can trigger the expression of certain genes responsible for the endogenous phytohormone balance of the treated plants leading to an enhancement of the photosynthetic activity and the regulation of several enzymes (Haroun & Hussein 2003; Singh 2014).

Simultaneously, GC-MS analysis of *A. oryzae* culture filtrate showed the presence of n-cetyl-D-glucosamine, linalool, dihydroxyphenyl glycol and niacinamide. It has been proven that the first three compounds have antioxidant potentiality (Azam *et al*., 2014; Hussain *et al*., 2008; Rodriguez-Gutierrez *et al*., 2012, Essa et al., 2013; Azam *et al*., 2014) while niacinamide is a water-soluble vitamin and is a part of the vitamin B group (Boyle, 2005). In this context, several cyanobacterial strains were reported to contain a wide array of vitamins such as thiamine, riboflavin, folic acid, ascorbic acid, nicotinic acid, cyanocobalamin and pentothene (Aaronson *et al*., 1977; Bonnet *et al*., 2010). The existence of vitamins in the cyanobacterial culture filtrate could act as essential growth factor for plant cells in addition to their capability to stimulate the antioxidative defense enzymes for eliminating reactive oxygen species and free radicals in the treated plants.

In agricultural soil, cyanobacteria as a main constituent of the natural microbiota play a crucial task in improving soil fertility and increasing crop productivity. This study highlighted the beneficial role of the filtrates

of axenic cultures of some cyanobacterial isolates on seed germination and seedling growth parameters of the crop plants *Sorghum durra* and *Helianthus annuus.* These strains demonstrated a great capability for releasing of several bioactive compounds including gibberellins, n-acetyl-D-glucosamine, linalool, dihydroxyphenyl glycol and niacinamide that can trigger and enhance the plant growth and development. Moreover, these compounds positively affected on the treated plants via activating the antioxidative defense enzymes responsible for the elimination oxidative agents and free radicals. The presoaking of plant seeds in the cyanobacterial exudates could be an economical and environment friendly strategy to reduce the utilizing of the expensive chemical fertilizers.

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