Research Article

Isolation and Characterization of Plant Growth Promoting Fungi from the Rhizosphere and Bulk Soil of *Abelmouschus esculentus* L.

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Abstract |Hormones are chemical substances that exist in trace amounts in definite tissues and control a variety of processes correlated to development, metabolism, and reproduction in the target tissue. The chemical generated by plants are known as phytohormones. During current study we isolated five fungi of which three strains were isolated from the rhizosphere soil of *A. esculentus* L. while the remaining strains were isolated from the bulk soil in the plant vicinity. The result revealed that the rhizosphere and bulk soil fungi have the potential to promote okra growth. It was found that both the rhizosphere and bulk soil had traces of Salicylic acid (SA). The quantities of Salicylic acid (SA) were higher in the bulk soil than the rhizosphere soil. The isolated fungi also released salicylic acid, sugars and proteins as evident from detection in their culture filtrate. The fungal isolates obtained from bulk soil including ZIB1 (Zarab Imad Bulk strain 1) and ZIB8 (Zarab Imad Bulk strain 8) also released proteins in their culture filtrates. Inoculation of fungus strains ZIR1 (Zaryab Imad rhizosphere strain 1), ZIR4 (Zaryab Imad rhizosphere strain 4), ZIR6 (Zaryab Imad rhizosphere strain 6), ZIB1, and ZIB8 enhanced the host growth attribute in term of root and shoot length. Fresh and dry weight of seedlings was also enhanced when associated with fungi. The fungal strains didn't influence each other growth when grown side by side on the agar plate. It concluded that both the fungi can be used as bio-inoculants in combination for plant growth and development. Based on the current study the above mentioned fungal strains are suitable and can be utilized to enhance the growth of okra plant.

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Introduction

The rhizosphere has been characterized as the region encompassing and impacted by the root where plants, roots, soil and microorganism cooperates/interact with each other. These collaborations often benefit plants by enhancing soil fertility and aiding in the breakdown of harmful chemicals (Lynch *et al*., 2001). The rhizosphere microbiome enhances plant growth and stress resistance by influencing nu-

trient uptake, regulating the exchange of chemical signals, and affecting enzyme activity during metabolic processes (Lareen *et al.*, 2016). There are several types of plant microbe's interaction, almost all plant organs interact with microorganisms at some point during their development, and this relation is not always harmful to the plant. In fact, there are numerous interactions where the plant benefits directly or indirectly from the associated microbes. Many plants release substances that attract and nourish associated microorganisms, while also offering a safe habitat for them. The ability of microbes to create phytohormones like auxin (IAA), gibberellin (GAs), cytokinins (CKs), ethylene (ETHY), abscisic acid (ABA), and salicylic acid (SA) leads to microbial-plant interactions (Patel *et al.*, 2015). Various species, such as fungi, bacteria, and plants can be found in soils. Because root exudates contain a high concentration of nutrients, plant roots are extensively inhabited with microorganisms in comparison to soil and other environments (Schlaeppi and Bulgarelli, 2015). Microbe's capacity to synthesize phytohormones in the rhizosphere or root tissue is one of the ways they help plants growth and tolerate stress better (Etesami et al., 2014). Mediating microbial symbiosis, root morphology, and plant immunology, phytohormones are also involved in below-ground interactions between roots, soil, and microbiome (Barker and Tagu, 2000). The ability to produce phytohormones is typically regarded as the most favourable quality when choosing the microorganisms that promote plant growth (Pérez-Montaño *et al.*, 2014). These phytohormones regulate root activity, microbial activity, or interactions between microbial species or with soil in various ways (Egamberdieva *et al.*, 2017). The plant hormones are involved in multiple function in plants like cell elongation, cell division, apical dominance, seed germination etc. The activity of several phytohormones, including IAA, GA, ETHY, CKs, BRs, and SA which regulate numerous corporal and biochemical processes, regulates growth and improvement in the plants in a interrelated manner (Iqbal *et al.*, 2014).

While extensive research has been conducted on the role of rhizosphere fungi in promoting plant growth, particularly in various crops, there is a significant gap in understanding the specific contributions of fungi isolated from both the rhizosphere and bulk soil of *A. esculentus* L. Furthermore, the possible synergistic or antagonistic interactions between diverse fungal strains when utilized together as bio-inoculants is little unknown. This work addresses these gaps with the objectives of isolating and characterizing fungal strains from both the rhizosphere and bulk soil, assessing their individual and combined impacts on *A. esculentus* development and investigating their phytohormone production in culture filterate.

Materials and Methods

Plant materials and treatment condition

Okra plants were collected along with roots from village toru district mardan Khyber Pakhtunkhwa (KPK) Pakistan and brought to the lab for Salicylic acid determination in their different parts. For the isolation of Rhizospheric and bulk soil fungi, the serial dilution method was used. The procedure of serial dilution was to fill 9 ml of distilled water for each in six test tubes which were then sterilized by autoclaving at 15 lb pressure and 121 ˚C for 15 minutes. Weighing 1 gram of soil sample was then put into a test tube containing sterilized 10ml distilled water and homogenized for a few minutes using vortex. 1 ml of homogeneous soil sample was added into the test tube using a micropipette to obtained a 10^{-1} dilution. From this dilution, 1 ml suspension was shifted to another test tube containing 9 mL autoclaved distilled water to obtained a 10^{-2} dilution. The process was repeated till 10^{-6} dilution was made (Reynolds, *et al.*, 2005). The test tubes were then covered with aluminium foil, typically for Potato dextrose agar (PDA) medium preparation, 250g of potatoes was boiled in water for 20 minutes and the extract of potato was taken to prepare 1000ml of media, started by dissolving 15g of dextrose agar in 350ml of extract under continuous stirring and gentle heat. Once dissolved, added 650ml of distilled water and 20g of glucose to the mixture. Autoclaved the mixture for 20 minutes at 122 ˚C to sterilized it. After autoclaving, let the temperature of the PDA medium cool down to 40°C. Then antibiotic added and 25ml media was allowed to solidify before they were used for inoculation. The prepared PDA medium was poured into sterilized petri dishes (approximately 20 ml in each), in such manner that molten medium covered the bottom of petri dishes completely, then the plates were covered with their lids and allowed to cool and solidify the medium in laminar flow. A drop (100 µL) of soil suspension from each dilution was put on the centre of agar plates containing PDA and labeled accordingly. The drop was spread on the surface of agar until the drop dried completely in order to evenly distribute the

fungal spores/hyphae. The plates were then incubated at 30 °C for 3-7 days and observed daily for fungal growth. For comparison and contamination check, PDA plates were inoculated with autoclaved distilled water and put under described conditions. Distinctly separated fungal colonies were selected and shifted to fresh PDA plates.

The soil was collected in clean plastic pots from the field. Then the collected soil was sieved to removed any extra material like stone, garbage etc. then the soil were autoclaved for 30 minutes at 121^{0}C. The okra seeds was bought from the market. The seeds were surface sterilized for 30 seconds with 70% ethanol and then washed 5 times with autoclaved distilled water. The sterilized seeds were sown randomly per plastic pots containing 300g soil and 1g fungal biomass. Control pots did not received fungal biomass. Each treatment was replicated three times. The seedlings were allowed to grow for 30 days.

Interaction of rhizospheric and bulk soil fungus with each other

To check the interaction and effects of two fungal strains on each other both the fungal strains were inoculated on PDA medium in such a manner that one fungus was inoculated in one half side of the plate and other fungus was inoculated in the other half side of the plate. The cultures were incubated at 28°C and regularly observed for 14 days.

Table 1: *Ingredients and their quantities for Czapek broth.*

S.No	Chemical's Name	Concentration (g/mL)
1	Peptone	10
2	Glucose	10
3	$MgSo_4$	0.5
4	KCl	0.5
5	$FeSo_4$	0.02

The purified fungal strains were inoculated to czapek medium and placed in shaking incubator at 30 °C for 7 days at 120 rpm. Filter paper was used to separate filtrate and biomass.

Preparation of Czapek medium

In 1000ml volume of conical flask, 1000ml of distilled water was taken. Other ingredients including 10 gm of peptone, 10 gm of glucose, 0.5g KCl, 0.5g $MgSO_4$, and 0.02g $FeSO_4$ were sequentially added (Table 1). The media was sterilized and autoclaved at 121 °C and 16 lb pressure for 30 minutes. After cooling the media was down to room temperature and was used for shaking fungal cultures in a shaking incubator set at 30 °C for 7 days at 120 rpm.

Determination of Salicylic acid (SA), Sugar and Protein in okra plant

To determin salicylic acid (SA), 1g of fresh leaf sample was ground in 70% ethanol. The homogenate was cleared of debris by centrifugation at 10000 rpm for 10 minutes. The supernatant (0.1 mL) was taken in a test tube and $FeCl_3$ (0.01%) was then added in it until the total volume of the sample reached to 3 ml. Optical density (OD) of the reaction was then taken in a UV/Vis spectrophotometer at 540 nm. $FeCl_3$ was used as blank.

The soluble sugar content of fresh plant leaves and exudates was calculated using the following methods. The totally expanded fresh plant leaves (1 g) were mixed with 10 mL of distilled water in a pestle and mortar and then rotated for at least five (5) minutes at 3000 rpm. At room temperature, 1 mL of 80% (w/v) phenol was added to 100 µl of supernatant and incubated. After incubation, 5mL of concentrated sulfuric acid was added. The resulting mixture was allowed to sit for 60 minutes before the absorbance and O.D of each concentration were calculated at 485 nm. A glucose standard curve was used to calculate the amount of sugar in sample.

To determine protein, 0.1 g of fresh leaf of okra plant was ground in 1 ml of phosphate buffer, afterward it was centrifuge at 3000 rpm for 10 minutes. Supernatant of 0.1 mL was poured in test tube, the supernatant then diluted by adding 1 mL distal water, then added 1 mL of reagent C then stir for 10 minutes, then added 0.1 mL reagent D and incubate the solution for 30 minutes, and then the OD was taken at 650 nm and the foline reagent was used as a blank.

Results and Discussion

Isolation of fungi from Rhizospheric and bulk soil of A. esculentus L.

During current study we isolated fungal strains ZIR1, ZIR4, ZIR6 from the Rhizospheric and ZIB1 and ZIB8 were isolated from bulk soil of *A. esculentus* L. (Figure 1). Colonies of the isolated strains were distinctly different from each other when cultured on Potato dextrose agar (PDA) medium. The ZIR1 strain had several small colonies with black center

and white margins. The ZIR4 had a large white colony with irregular margins. The strain ZIR6 had black colony with entire margin. ZIB1 Brown and ZIB8 black colonies were formed by the fungi isolated from bulk soil (Figure 1).

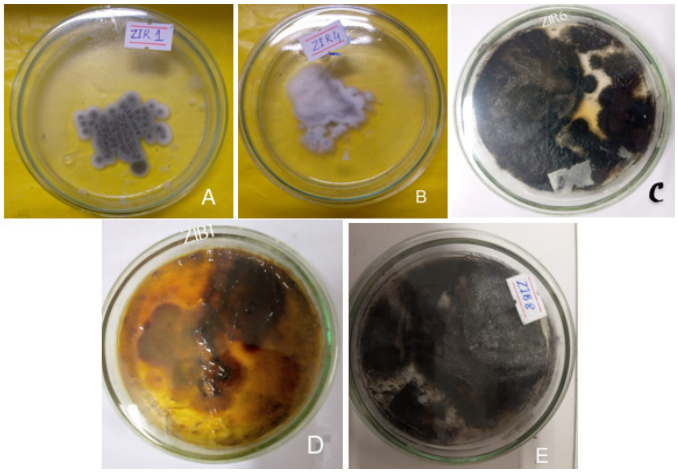

Figure 1: *Fungal strains a) ZIR1, b) ZIR4 and c) ZIR6 isolated from the rhizosphere, and strains d) ZIB1 and e) ZIB8 isolated from the bulk soil of the okra plant.*

Effect of isolated fungi on root length, shoot length, fresh weight and dry weight

The root and shoot lengths of both control and treated plants were measured 30 days after seed germination. With the exception of ZIR6 and ZIB8, all isolated fungal strains promoted the growth of *A. esculentus* L. seedlings. The ZIR4-associated seedlings exhibited the greatest root length, which was 62% higher than the control, while Strain ZIB1 increased shoot length by 39% compared to the control. Seedlings associated with ZIR1 showed a 26% greater root length than the control. Fungi isolated from bulk soil (ZIB1 and ZIB8) of *A. esculentus* L. also significantly enhanced seedling root length by 39% and 26%, respectively, compared to the control, whereas ZIR6 reduced root length by 5.79%. Rhizospheric fungal strain ZIR1 and ZIR4 increased shoot length by 20% and 10%, respectively while the fungal strains ZIB8 and ZIR6 significantly decreased shoot length by 15% and 2.4%, respectively as compared to the control (Figure 2 a and b).

All the fungal strains show various effect on fresh and dry weight of okra plant. The isolated fungal strains ZIR1 and ZIB1 increased fresh weight in fungus associated okra plant by 7.5% and 15%, respectively as compared to the control. Fungus ZIR4, ZIR6, ZIB8 have decreased fresh weight by 7.5%, 15% and 11.3%, respectively (Figure 2c). The strains ZIR1, ZIB1 and ZIB8 have increased dry weight in fungus associated okra plant by 21%, 57% and 5%, respectively while the fungal strains ZIR4 and ZIR6 decreased the dry weight in fungus associated okra plant by 15% and 15.7%, respectively as compared to the controls (Figure 2d)

Determination of Salicylic acid, Sugar and Protein in cultured medium

The selected five strains of fungi produced salicylic acid which was then released in culture broth. Among the selected strains, ZIB8 released the highest quantity (550.67±6.66 µg/mL) of SA followed by ZIR6 (516.22±5.09 µg/mL). Amount of SA released by the remaining strains ZIB1, ZIR4 and ZIR1, were, 467.33±8.81 µg/mL, 292.88±18.95 µg/mL and 135.11±18.35µg/mL, respectively (Figure 3a).

The rhizosphere fungus ZIR1, ZIR4, ZIR6 and the Bulk fungus ZIB1 and ZIB8 showed variable production of sugar in their culture medium. The rhizosphere fungus ZIR4, ZIR1 and ZIR6 have sugar concentration of 51.6±0.54 µg/mL, 33.5±.167 µg/mL, and 27.1±0.167 µg/mL respectively, in their culture medium. While the bulk isolated fungus ZIB1 and ZIB8 have sugar concentration of 57.61±0.27 µg/mL and 45.86±0.38 µg/mL, respectively. Among all the fungal strains, Z1R6 has produced low concentration, while ZIB8 has produced a high concentration of sugar in cultured medium (Figure 3b).

The selected five fungal strains showed a dissimilar production of protein concentration in their cultured medium, in which the isolated rhizosphere fungal strains ZIR1 produced 157.60±1.03 µg/mL, ZIR4 142.90±0.53µg/mL, and ZIR6 108.88±4.81µg/mL, while the fungus isolated from bulk soil ZIB1 produced 183.84±0.51 µg/mL and ZIB8 203.76±0.90 µg/mL. The strain ZIR6 produced the lowest while the strain ZIB8 produced the highest concentration of protein in their cultured medium among all the fungal strains (Figure 3c).

Determination of salicylic acid, sugar and protein in A. esculentus L.

All the selected strain showed significant enhancement of endogenous level of salicylic acid in the host plant, the rhizospheric fungal strains, ZIR4, ZIR6 and ZIR1 have increased the salicylic acid level by, 36%, 31% and 22% respectively as compared to control.

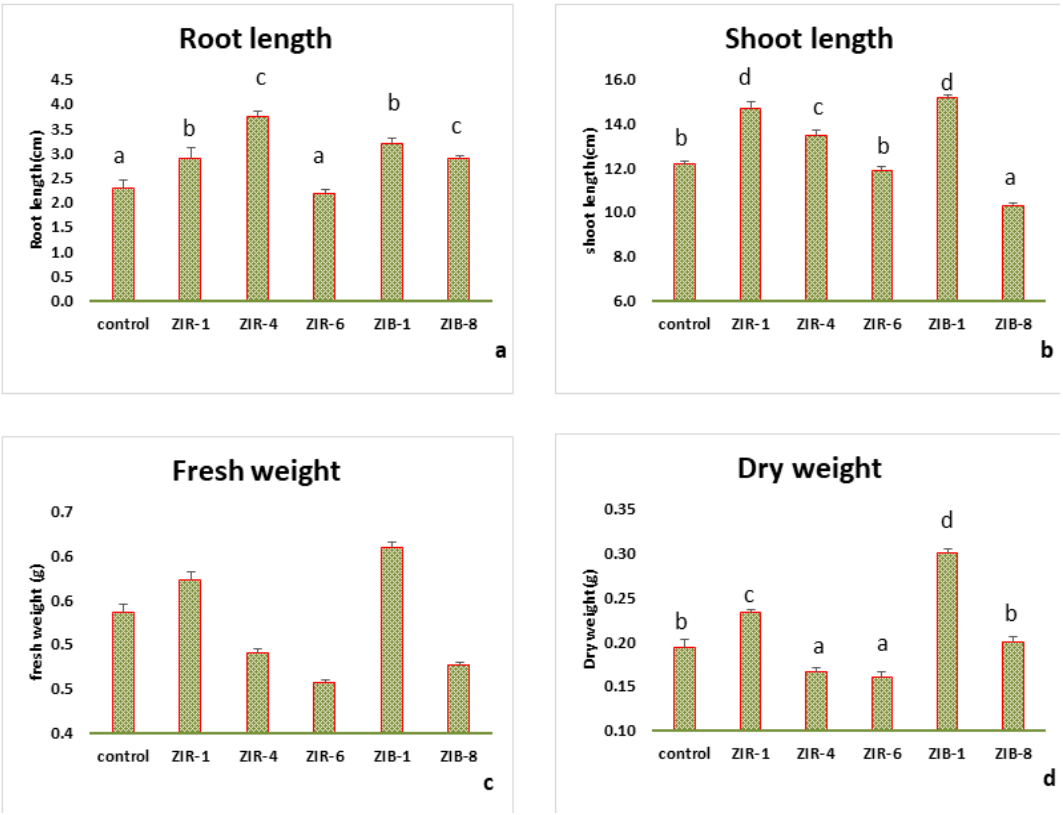

Figure 2: *Effects of isolated fungi on a) root length, b) shoot length, c) number of roots, d) fresh weight and e) dry weight of okra seedlings. Mean values (± standard deviation) are shown, with significant differences between treatments indicated by different letters ($p < 0.05$).*

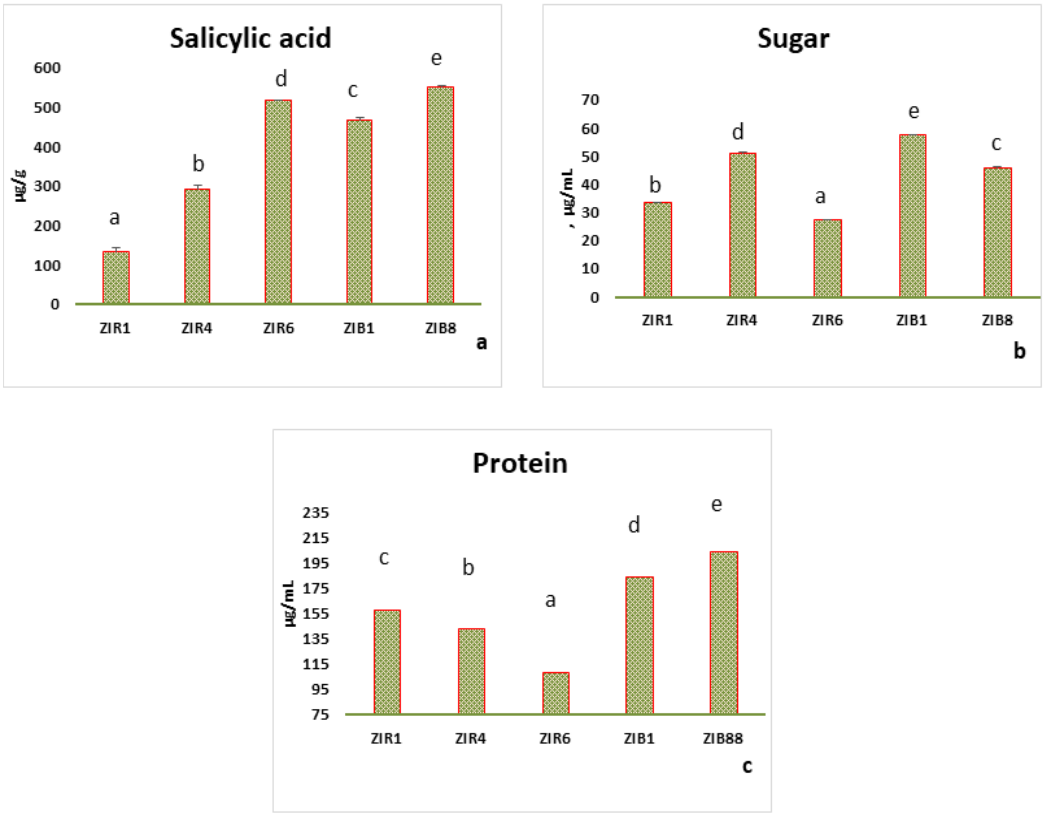

Figure 3: *Ability of fungal strains isolated from the rhizosphere and bulk soil of okra to release a) salicylic acid, b) sugars and c) proteins in the culture broth. The strains were cultivated for 10 days in Czapek liquid broth, and the culture filtrate was analyzed for the selected metabolites. Mean values (± standard deviation) are shown, with significant differences between strains indicated by different letters ($p < 0.05$).*

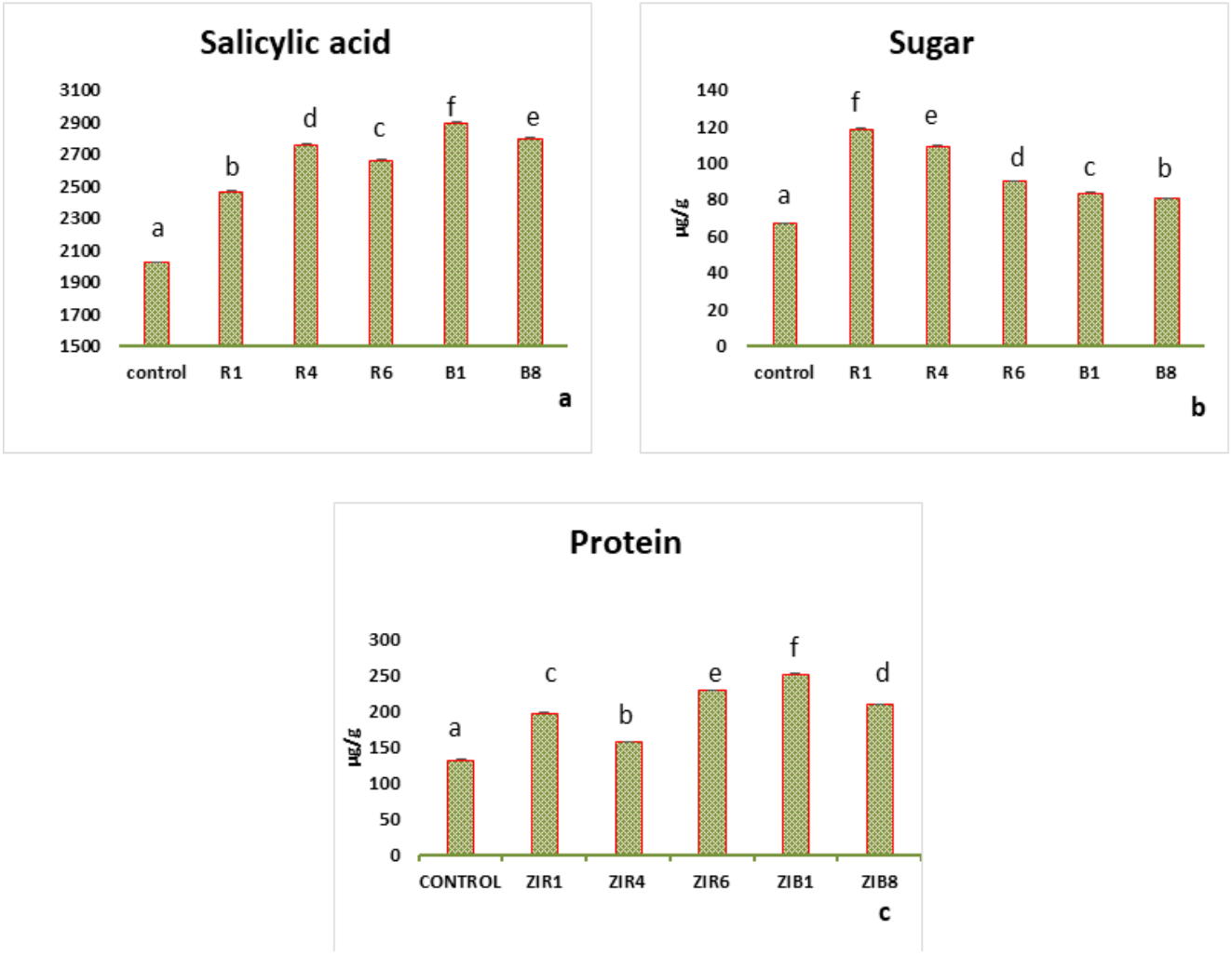

Figure 4: *Effect of fungal strains isolated from the rhizosphere and bulk soil of okra on the endogenous levels of a) salicylic acid, b) sugars and c) proteins in okra seedlings grown for 30 days in fungal-inoculated soil. Mean values (± standard deviation) are presented. Significant differences between strains are indicated by different letters ($p < 0.05$).*

The fungus isolated from bulk soil such as ZIB1 and ZIB8 also enhanced the salicylic acid by 43% and 38 %, respectively (Figure 4a).

Concentration of sugar was determined in the leaves of fungi associated okra seedlings. The fungal strains had a positive effect on okra plant in term of increase in sugar concentration (Figure 4 b). The Rhizospheric fungal strains ZIR1, ZIR4, and ZIR6 increased the sugar level by 24%, 53% and 29%, respectively, as compared to the control. The fungus isolated from bulk soil such as ZIB1 and ZIB8 also enhanced the sugar by 93% and 61%, respectively (Figure 4b).

All the selected strain showed significant enhancement of endogenous level of protein in fungus associated plants. The Rhizospheric fungal strains ZIR6, ZIR1 and ZIR4 have increased the protein levels by 85, 55 and 22 %, respectively, as compared to control. The fungus isolated from bulk soil such as ZIB1 and ZIB8 also enhanced the protein level by 90% and 57 %, respectively (Figure 4 c).

Determination of salicylic acid in different parts of A. esculentus L.

Salicylic acid concentration was determined in different parts of *A. esculentus* L, the highest concentration was found in leaf (1772±10.18 µg/g) while the lower concentration was measured in Rhizospheric soil (200±13.33 µg/g). It concluded that the salicylic acid concentration decreased towards base as the rest of the parts such as stem and root have 1452±15.03 µg/g and 1330±3.33 µg/g of SA, respectively. The salicylic concentration determined in bulk soil was 717±18.55 µg/g (Figure 5).

Interaction between two fungi

Interaction between rhizosphere ZIR6 and fungal strain isolated from bulk soil ZIB1 was studied on agar plate. Both the isolates grow normally without

inhibiting each other growth. It's concluded that both the fungus can be used as bio-inoculant in combination for plant growth and development (Figure 6).

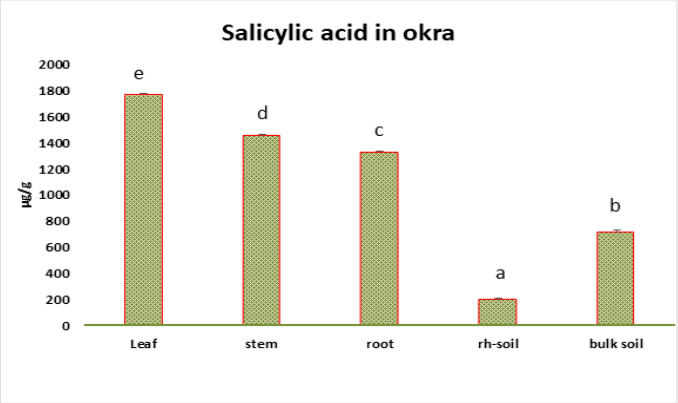

Figure 5: *Figure shows mean salicylic acid concentrations (± standard deviation) in different parts of the okra plant. Significant differences among concentrations in various plant parts are indicated by different letters ($p < 0.05$).*

The concentration of salicylic acid was determined in field grown okra plants. Its concentration was also tested in the rhizosphere soil of these plants. Concentration of Salicylic acid (SA) was lower in the rhizosphere in comparison to the leaves of the plants. Rhizosphere is the region where SA is released not only by plant roots but also by microbes including bacteria, fungi etc (Curl and Truelove, 2012). Five various fungi were isolated from the rhizosphere and bulk soil of *A. esculentus (*okra) plant, their colony morphology and other microscopic characters showed that these isolated fungi belong to diverse background. In isolated fungi most of them were efficient plant growth promoters. Micro-organism are more active in rhizosphere soil but relatively passive in bulk soil which serves as soil hots spot (Schloter *et al.*, 2018), particularly durinsg the phase of plant development (Houlden *et al.*, 2008). The range of micro-organism in bulk soil may be of greater importance as rhizosphere resource library (Schloter *et al.*, 2018). Rhizospheric fungi promotes the growth of plants by releasing the secondary metabolites such as sugar, protein and plant hormones especially salicylic acid (Fouda *et al.*, 2015). The isolated fungal strains enhanced the dry and fresh weight of okra plant as compared to control by fungus associated with okra seedlings (Mastouri *et al.*, 2012). The fungal strains ZIR1, ZIR4, ZIB1 and ZIB8 enhanced the endogenous level of salicylic acid in plants. According to our results, the endogenous

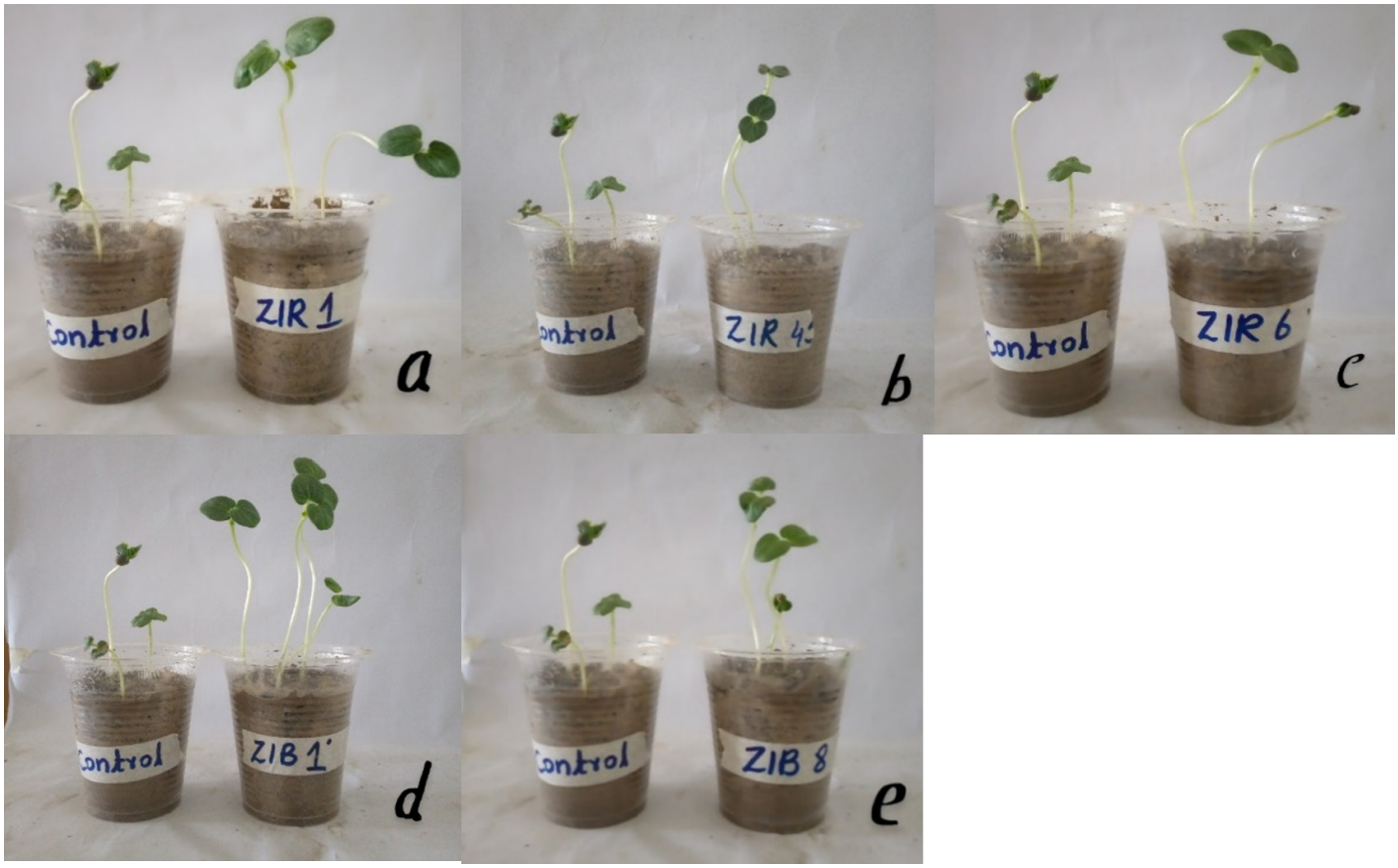

Figure 6: *Pots containing okra seedlings in soil inoculated with fungi compared to those in non-inaculated soil. a) ZIR1 treated vs control seedling b) ZIR4 treated vs control seedling c) ZIR6 treated vs control seedling d) ZIB1*

treated vs control seedling and e) ZIB8 treated vs control seedling of okra are shown for comparison.

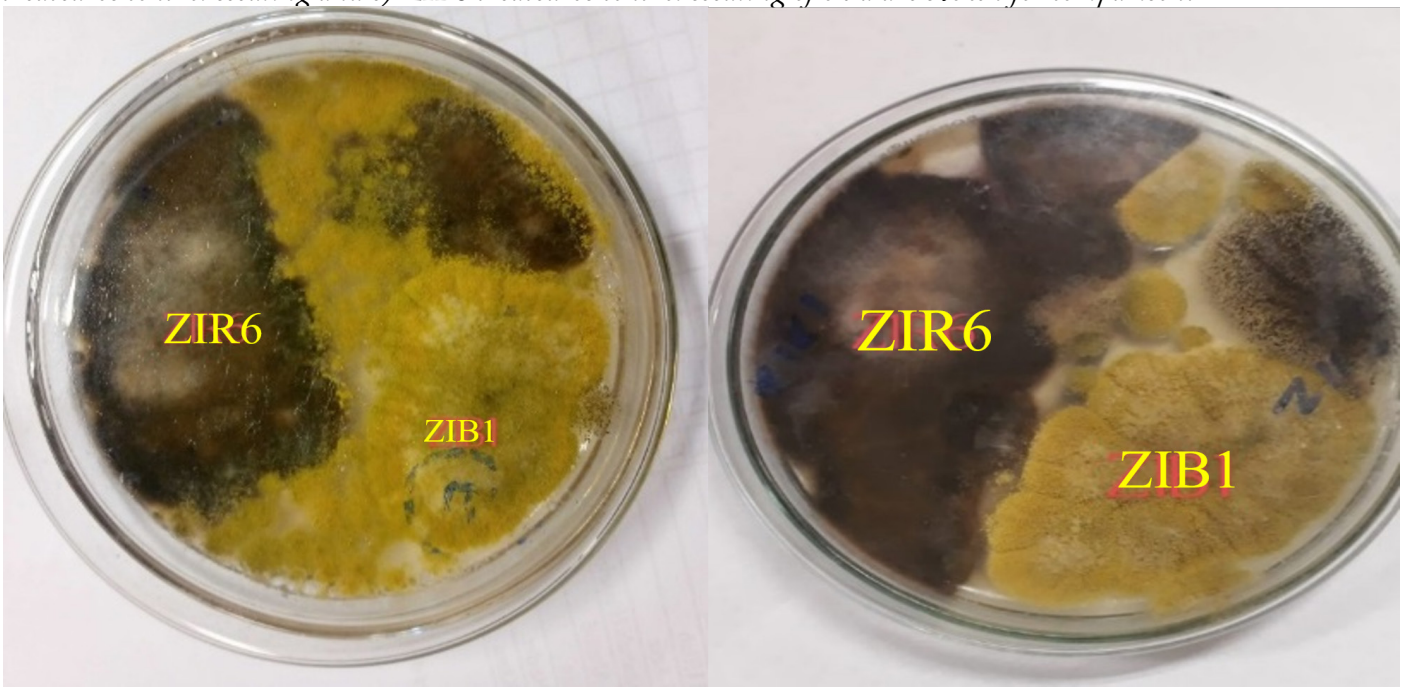

Figure 7: *Interaction between ZIR6 and ZIB1 grown on PDA plate. Both the strains grow without any inhibition.*

concentration of salicylic acid was significantly enhanced by 22-43 % as compared to control. The three mentioned secondary metabolites (sugar, protein and salicylic acid) were also found in the isolated fungus culture filtrate. Microbes as well as plants developed salicylic acid, which regulates plant growth and development. Salicylic acid is essential plant hormone that regulates plant growth and development and involved in defense, thermogenesis, photosynthesis, stomatal conductance, disease resistance, and seed germination (Ding and Wang, 2003). Sugar concentration was significantly enhanced in fungus-associated okra plant as compared to control. Sugar play an important role in the life of plants: they are structural and storage substances, respiratory substrates, and intermediate metabolites of many biochemical processes (Ciereszko, 2018). The fungal strains ZIR1, ZIR6 ZIB1 and ZIB8 enhanced the endogenous concentration of protein in okra seedlings by 48-90 % as compared to control. Proteins play various enzymatic, structural and functional roles (photosynthesis, biosynthesis, transport, immunity, etc.). They also act as storage mediums to meet the growth and nutritional demands of developing seedlings (Hemsley and Grierson, 2008). The Interaction between rhizosphere ZIR6 and fungal strain isolated from bulk soil ZIB1 was studied on agar plate. Both the isolates grow normally without inhibiting each other's growth. It's concluded that both the fungus can be used as bio-inoculant in combination for plant growth and development (Zeilinger-Migsich and Mukherjee, 2014).

Conclusions and Recommendations

Based on the data, it is concluded that both the rhizosphere and bulk soil contain fungi that interact with okra plants, promoting their growth by releasing salicylic acid and other metabolites. The fungi isolated during current study modulated host plant metabolites and SA. Concentration of SA was determined in different parts of okra plant and it was noted that leaves of okra had higher levels of SA than the roots. SA was also present in the soil which is released by roots as well as microbes inhabiting the rhizosphere. The fungi inhabiting rhizosphere and bulk soil interact and grow together without any apparent competition.

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Novelty Statement

This study uniquely demonstrates that rhizosphere and bulk soil fungi actively enhance okra growth by

releasing and modulating salicylic acid (SA) and other growth-promoting metabolites, with significantly higher SA concentrations observed in okra leaves compared to roots. Additionally, it reveals a novel, cooperative relationship among soil fungi, which thrive without apparent competition, collectively supporting plant health and resilience.

Author's Contribution

Imad Ahmad Noor and Abdullah: Designed the study and wrote the manuscript.
Imad Ahmad Noor, Abdullah and Imtiaz Ahmed: conducted the experiment.
Imad Ahmad Noor and Kashif ur Rahman: Analyzed the data.
Imad Ahmad Noor and Habib Ur Rahman: eviewed the manuscript.

Conflict of interest
The authors have declared no conflict of interest.

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