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Improvement of Germination, Phosphate Efficiency, Antioxidants, Metabolic Products, and Yield of Wheat Plants by *Aspergillus niger* and *Penicillium chrysogenum*

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INCREASING the efficiency of phosphate fertilizer by biofertilizers is an interesting goal as a way to potentiate the phosphate-fertilizers.

The present study includes the use of culture filtrate (CF) or conidial suspension (CS) of two isolated rhizospheric fungi (RF) in germination solution of wheat plants or pots filled with soils fertilized with superphosphate or rock phosphate.

The RF were morphologically and genetically identified as *Penicillium chrysogenum* (AUMC 14100) and *Aspergillus niger* (AUMC 14260) that were able to produce indole acetic acid (IAA) and solubilize phosphate. Applying CF or CS of the strains separately or in consortium enhanced the germination percentage and vigor index of wheat plants. For pot experiment, CF or CS initiate their positive effect on plant in two ways: a) enhancement of soil properties (increment of organic matter, reduction of pH, enrichment the soil with higher soluble phosphate, calcium, and magnesium); b) stimulation of growth, biochemical status, nutrients content, and the yield of wheat plants. The applied biofertilizers enhanced chlorophyll, primary metabolites, phenolics, ascorbic acid, and tocopherol contents. The application of CF and/or CS exerted no oxidative damage on wheat rather H_2O_2 or lipid peroxidation was reduced due to the activation of various antioxidant enzymes. Furthermore, the root and shoot tissues of treated wheat enriched with high contents of calcium, magnesium, and phosphate improve phosphate-related traits. All up-regulations under biofertilizers application reflected on high yield of wheat plants.

The study recommends application of CF side by side to P-fertilizers for up-scaling their efficiency and enhancing plant development.

Keywords: *Aspergillus niger*, *Penicillium chrysogenum*, Phosphate- related traits, Rock phosphate, Superphosphate, Wheat.

Introduction

The bioavailability of nutrients including phosphorus (P) is one of the most relevant limiting factors for crop development (Iqbal et al., 2019a). Phosphorus has been reported to be involved in various vital processes in plants as new tissues production, cell division, and nucleic acid composition adjusting photosynthesis, protein production, energy convey (Vance et al., 2003; Elhaissoufi et al., 2020). Unfortunately, P-deficiency reached about 40% of cultivated land in the world (Vance et al., 2003; Iqbal et al., 2019b). Up to date, soil fertilization by nitrogen, P and potassium depends on chemical fertilizers improve growth and productiveness of plants.

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Annually, for saving adequate P in the soil, nearly 52.3 billion tons of P-fertilizers were used (FAO, 2017), but a few amount of 0.2% of P is utilized by plants (Islam et al., 2019). Hence, immobilization and leaching reduced the availability of P for suitable crop yield (Niu et al., 2013). On the other hand, the excessive application of P-chemical fertilizers causes an environmental pollution hence elevated the cost of crop yield (Ghaffar et al., 2017). In addition, the natural sources of P-fertilizers as rock phosphate due to high use are depleting, hence the reservoirs become scarcer causing their prices to be elevated (Soumare et al., 2020). Thus, using new techniques for modulation of P-chemical fertilizers are urgently needed for sustainable crop production (Iqbal et al., 2019b).

The application of plant growth–promoting and phosphate solubilizing rhizospheric fungi for increasing yield is getting worldwide acceptance as they efficiently colonize plant roots. The efficiency of plant growth promoting fungi (PGPF) to colonize root is considered to be a tool for preventing phytopathogen infection and also aids in the nutrients uptake for promoting plant growth (Zhang et al., 2018). PGPF play an important role in enhancing plant growth by solubilizing phosphate and biosynthesis of siderophore, indole acetic acid (IAA), chitinase and cellulose (Zhang et al., 2018; Muslim et al., 2019; Naziya et al., 2020). Up to date, only few studies worked on PGPF under P-chemical fertilizer, compared to rhizobacteria for increasing growth and plant productivity (Naziya et al., 2020). Khan et al. (2010) reported that the ability of fungi to increase solubility of phosphate was higher than bacteria in solid and liquid cultures. In addition, soils fungi can move more easily for longer distances compared to bacteria, so P-solubilization is more influential for fungi than bacteria. In addition, the subculture of P-solubilizing bacteria loses the phosphate-solubilizing activity after prolonged cultivation relative to fungi that retain their ability to leach P-containing rocks (Saxena et al., 2013).

Furthermore, mixing chemical fertilizers along with biofertilizers exert more benefits on plant growth and productivity compared to single fertilizer. In this respect, (Chen, 2006) found that the application of beneficial microorganisms mixture in compost along with urea keep the benefits of chemical or organic fertilizers, while reducing the aggregation of P in the soil. (Latha et al., 2014) stated that single application of chemical fertilizers on eggplant had a higher cost-benefit relative to mixing bio- and chemical fertilizers. Other studies reported that mixing P-biofertilizer and chemical fertilizer enhanced growth of various crops as potatoes, soybean, and rice (Munda et al., 2015; Naher et al., 2016). In view of these researches, the insistence to elicit highly efficient fertilizers by mixing chemical, organic materials, and microorganisms is frequently needed and will record high qualifications in the future (Soumare et al., 2020). Thus, the present work is conducted to test the efficacy of P-solubilizing and IAA-producing fungi to upscale the efficiency of superphosphate or rock phosphate in enhancing soil properties by increasing the availability, as well as seed germination, growth, physiology, and yield of wheat plants.

Materials and Methods

Microorganisms and culture conditions

Penicillium chrysogenum and *Aspergillus niger* were isolated from the rhizosphere of wheat and pepper, respectively. The isolates were identified morphologically and confirmed by molecular characterization. They tested for their ability to phosphate solubilization and production of IAA.

Molecular identification of phosphate solubilizing strains and phylogenetic analysis DNA extraction

For DNA extraction, small part of the fungal mycelia of 7-day-old culture grown on Potato dextrose agar (PDA) was gathered and placed individually to Eppendorf tube to be applied according to (Moubasher et al., 2019).

PCR for rDNA and sequencing using ITS1 and ITS4 primers

SolGent EF-Taq was applied for PCR reaction using primer; ITS1 (5′ TCC GTA GGT GAA CCT TGC GG 3′) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) (White et al., 1990). Reaction mixture of DNA template (1µL), Taq polymerase (0.2 unit), dNTP mix (1µL of 2.5mM), 10x complete buffer (5µL), bidistilled water (40µL), and the 10 pmoL of ITS1 and ITS4 were mixed in PCR tubes. The following steps were done for PCR amplification: One amplification round included denaturation for 15min at 95°C, then 30 denaturation cycles at 95°C for 20sec, followed by annealing at 50°C (40 sec), extension at 72°C (1min), and finally extension step was done for 5min at 72°C. Before sequencing, the PCR outputs were purified with the SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea). The

purified PCR outputs were run on agarose gel (1%) using electrophoresis via size marker. The produced bands were eluted and then sequenced in the reverse and forward directions.

Phylogenetic analysis

Contigs were created from the sequence data using CLCBio Main Workbench program. The sequence obtained from each isolate was further analyzed using BLAST from the National Center of Biotechnology Information (NCBI) website. Sequences obtained with those retrieved from GenBank database were subjected to Clustal W analysis using MegAlign (DNAStar) software version 5.05 for the phylogenetic analysis. Sequence data were deposited in GenBank and accession numbers are given for them.

Phosphate solubilization in liquid medium

The isolated fungal strains were inoculated separately in conical flask containing (Pikovskaya) PVK broth medium with 0.5% (v/v) of either superphosphate or rock phosphate. The initial pH of the medium was adjusted to 7.0 before sterilization. Each flask was inoculated with 1 ml spore suspension ($1x10^6$ spore/mL) of the fungal strains, and incubated at 25±2°C for 20 days under agitation at 150rpm. After 5, 10, 15 and 20 day's incubation period, through which, contents of the flasks were filtered and cell-free supernatants were obtained after centrifugation at 8000rpm for 15min. pH of the supernatants were measured and the phosphate concentration was determined using (Jackson, 1973) method.

Auxin production (indole acetic acid, IAA)

The colorimetric method introduced by Salkowski's using Van Urk Salkowski reagent (Ehmann, 1977) was used to determine the concentration of IAA. The two strains, individually or in combination, were grown in PDA supplemented with tryptophan (Smith & Onions, 1994). Flasks were then inoculated, individually with 5% (v/v) spore suspension obtained from 7-day-old cultures of the two strains or their combination (1:1) in a rate of 1×10^8 spore/mL. The inoculated flasks were then incubated at 30ºC for 14 days at 150rpm. Supernatants from cultures of both strains were obtained by centrifugation at 10000 rpm for 20 min (Gordon & Paleg, 1957). A reaction mixture containing 1 mL of the supernatant and 2mL of Salkowski's reagent was kept in the dark for 30min at room temperature. The optical density was recorded at 530nm, and the IAA concentration was calculated in (µg/mL) using the IAA calibration curve.

Preparation of conidial suspension and culture filtrate

Conidial suspension (CS): The phosphate solubilizer fungal strains (*Penicillium chrysogenum* AUMC 14100, and *Aspergillus niger* AUMC 14260) were grown on PDA. 10mL sterilized distilled water was poured on each culture plate and shaken to expel conidia and then placed in sterilized conical flask. The gathered conidial suspension was filtered through some filter papers. Sterilization of the filtrate was done by passing through 0.22µm Millipore membrane. The resulting sterile filtrate was re-suspended in 50mL sterilized distilled water and the concentration of conidia was adjusted to 0.75×10^8 CFUs/mL using haemocytometer. The spore suspension obtained from 7 day-old cultures of the two strains and in combination with 5% spore suspension of both strains (1:1). The spore suspension of the mixed culture was optimized at 0.75×10^8 spore/mL (Niranjana et al., 2009).

Culture filtrate (CF): Five mycelial discs (5 mm diameter) of the phosphate-solubilizing fungal strains (*Penicillium chrysogenum* AUMC 14100 and *Aspergillus niger* AUMC 14260) were grown separately on PDA medium, then were transferred to conical flasks containing potato dextrose broth medium (pH 7). The inoculated flasks were incubated at 25±2°C for 10-12 days. The resulting culture were filtered through three layers of filter papers, and the mixture of the two fungal strains was prepared by mixing 100 mL from each of *Penicillium chrysogenum* and *Aspergillus niger* filtrates (1:1). The sterilized filtrates were collected and stored at 4 °C (Murali & Amruthesh, 2015).

Impact of seed treatment with the inducers of Penicillium chrysogenum (AUMC 14100) and Aspergillus nigerc (AUMC 14260) on the seed germination and seedling vigor

The seeds of wheat (*Triticum aestivium* cv. Gemmiza 9) were surface sterilized with sodium hypochlorite for 2min and rinsed thoroughly in sterile distilled water 2-3 times. The sterilized seeds were treated with the prepared fungal suspensions and culture filtrates of the fungi separately or mixed together by mixing 100 seeds

of each sample with 50mL CS or CF. The control plants were suspended in 50mL distilled water (three replicates were prepared). The treated seeds were kept at 25±2°C in a rotary shaker for 8 h to facilitate the penetration of the inducer. After incubation, the seeds were air-dried aseptically under laboratory conditions and used for further studies. Seeds treated with sterilized distilled water act as control. The treated seeds were plated equidistantly on sterilized petri dishes lined with sterilized filter paper (100 seed per a plate). Three replicates were applied for each treatment and control. After 7 days, percentage germination and vigor index were calculated using the following formula:

Germination percentage= Final number of germinated seeds/ Total number of seeds ×100

Vigor index= Seed germination (%) × [Mean Root Length + Mean Shoot Length]

Impact of phosphate solubilizing fungi (PSF) on growth and nutrient uptake of wheat

Wheat seeds were surface sterilized with 0.1% NaOCl for 10min, then washed thoroughly with distilled water. The sterilized seeds were treated with CS and CF of *Penicillium chrysogenum* (AUMC 14100) and *Aspergillus niger* (AUMC 14260), either separately or in mixture as CS and/ or CF. The treated seeds were kept at 25±2°C in a rotary shaker for 4 h to facilitate the penetration of the inducer. After incubation, the seeds were air dried aseptically under laboratory conditions (25±2°C). Grains soaked in sterilized distilled water were used as control. The inoculated and non-inoculated seeds were sown at 1cm depth in plastic pots filled with 1.5kg sterilized clay soil and classified into three sets of pots (five replicates per a treatment and 10 seeds per a pot) Set I: The seeds were sown at 1cm depth in plastic pots filled with 1.5kg sterilized clay soil without any fertilizer. Set II: The seeds were sown at 1cm depth in plastic pots filled with 1.5kg sterilized clay soil supplemented with superphosphate (0.5g/kg). Set III: The seeds were sown at 1cm depth in 1.5kg plastic pots filled with sterilized clay soil supplemented with rock phosphate (1g/kg). The whole design of the experiment is presented in Fig. 1 and Table 1. Then, the pots were kept under natural conditions of light, moisture as well as temperature for one month after sowing of wheat (from 1st December 2018 to 1st January 2019). After harvest, soil samples were collected to study physical and chemical properties. In addition, root and shoot systems were washed thoroughly for biometric and physiological studies.

Soil analyses

The soil samples were tested for soil moisture content (MC) using the recommended method of Van Reeuwijk (2002), organic matter (Walkley & Black, 1934), electric conductivity (EC) was determined using a conductimeter (YSI Model 35 Yellow Springs, OH, USA), total soluble salts (TSS) (Jackson, 1967) and pH value (pH-meter, Jenway-3540). Sodium and potassium were determined by flame photometry according to Williams & Twine (1960). Calcium and magnesium were determined volumetrically by the Versene titration method described by Johnson & Ulrich (1959). Soluble Phosphates were colorimetrically determined by the method of Murphy & Riley (1962), and total phosphate was estimated based on the technique described by Sommers & Nelson (1972).

Morphological criteria

After harvesting, plant shoot and root of each pot were collected, and then washed well with tap water. The shoots and roots lengths in cm and fresh weight per individual plant were determined in (g), and the samples were oven dried at 70°C for 48h to determine the dry weight in (g).

Physiological analysis

Estimation of pigments and some metabolites

The chlorophyll a, chlorophyll b and carotenoids were evaluated in leaves applying Lichtenthaler (1987). The other metabolites were estimated in shoots; the method published for Homme et al. (1992) and Schlegel (1956) was applied for sugars determination. Proteins were determined using alkaline reagent solution recommended by Lowry et al. (1951). Whilst amino acids were done by the methodology of Sorrequieta et al. (2009) and Kalsoom et al. (2016).

Oxidative criteria markers

Lipid peroxidation was determined by Rao & Sresty (2000) procedures. Hydrogen peroxide (H_2O_2) content of leaf samples was also measured by the protocol of Mukherjee & Choudhuri (1983). Superoxide anion content was estimated by using the detailed methods of Elstner & Heupel (1976).

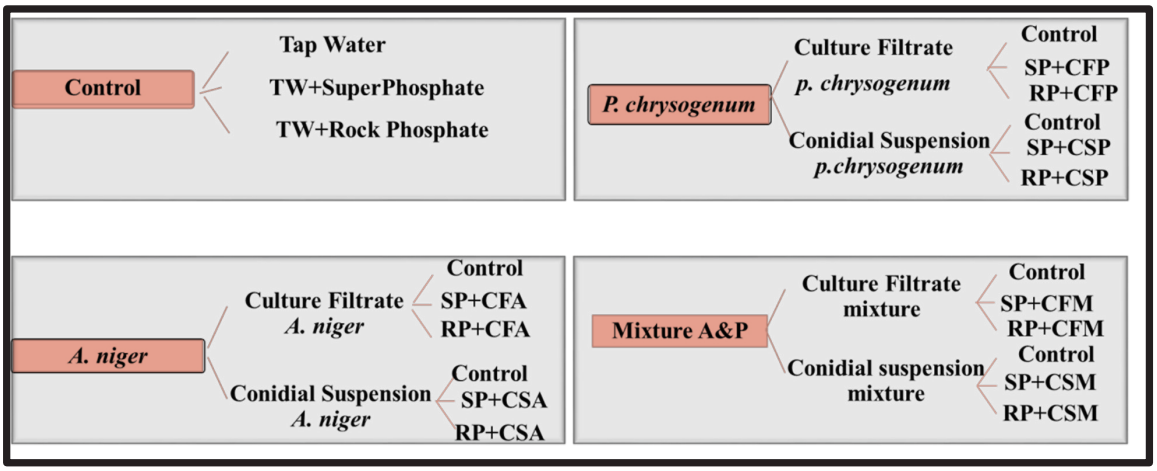

Fig. 1. The design of wheat grains grown under different treatments

TABLE 1. The design of wheat grains grown under different treatments

Treatment 1(T1)	Tap water irrigation only (control)
Treatment 2 (T2)	Superphosphate (0.5g/kg)
Treatment 3 (T3)	Rock phosphate (1g/kg)
Treatment4 (T4)	culture filtrate of *P. chrysogenum* (CFP)
Treatment5 (T5)	Superphosphate (0.5g/kg) + CFP
Treatment6 (T6)	Rock phosphate (1g/kg) + CFP
Treatment 7 (T7)	0.75×10^8 cfu/ ml conidial suspension of *P. chrysogenum* (CSP)
Treatment 8 (T8)	Superphosphate (0.5g/kg) + CSP
Treatment 9 (T9)	Rock phosphate (1g/kg) + CSP
Treatment 10 (T10)	Culture filtrate of *A. niger* (CFA)
Treatment 11 (T11)	Superphosphate (0.5g/kg) + CFA
Treatment 12 (T12)	Rock phosphate (1g/kg) + CFA
Treatment 13 (T13)	Conidial suspension of *A. niger* (CSA)
Treatment 14 (T14)	Superphosphate (0.5g/kg) + CSA
Treatment 15 (T15)	Rock phosphate (1g/kg) + CSA
Treatment 16 (T16)	Culture filtrate of Mixture of *P. chrysogenum* + *A.niger* (CFP+CFA)
Treatment 17 (T17)	Superphosphate (0.5g/kg) + (CFP+CFA)
Treatment 18 (T18)	Rock phosphate (1g/kg) + (CFP+CFA)
Treatment 19 (T19)	Spore suspension of *P. chrysogenum* + spore suspension of *A.niger* (CSP+CSA)
Treatment 20 (T20)	Superphosphate (0.5g/kg) + (CSP+CSA)
Treatment 21 (T21)	Rock phosphate (1g/kg) + (CSP+CSA)

Antioxidant system

Non-enzymatic antioxidants

Free phenolic compounds, ascorbic acid (Vitamins C), and tocopherols were measured in shoot spectrophotometrically using the method described by Kofalvi & Nassuth (1995), Jagota & Dani (1982), and Armstrong (1978), respectively.

Enzymatic antioxidants

The fresh leaves of wheat plants were homogenized in K-phosphate buffer for the determination of catalase (CAT; EC 1.11.1.6, Aebi, 1984), ascorbate peroxidase (APX; EC 1.11.1.11, Nakano & Asada, 1981), guaiacol peroxidase (POD; EC 1.11.1.7, Zaharieva et al., 1999), and superoxide dismutase activity (SOD; EC 1.15.1., Misra & Fridovich, 1972). Polyphenol oxidase PPO activity was detected by protocol of Kumar & Khan (1982). Phenylalanine ammonia lyase, PAL activity was examined by

the protocol of Havir & Hanson (1973). Protein content was estimated in the enzyme extract for calculation of enzymes' specific activities by Lowry et al. (1951).

Nutrients content

Root and shoot samples were collected for determination of Calcium, magnesium and phosphate. Calcium was determined by Johnson & Ulrich (1959) procedures. Soluble Phosphates were colorimetrically determined as phospho-molybdate (Murphy & Riley, 1962), and total phosphate were estimated based on the method used by Sommers & Nelson (1972).

Phosphorus-use efficiency (PUE) indexes

Additionally, PUE definitions of wheat plants grown under various P fertilizers and bio-inoculants were calculated based on the method of Abenavoli et al. (2016) by the following equations:

Total phosphorous accumulation (TPA)= Total phosphorous concentration x total dry weight/ Plant.

Phosphorous utilization efficiency (PUtE)= Total dry matter/ Phosphorous concentration

Phosphorous uptake efficiency (PUpE)= Total phosphorous accumulation/ Dry weight of root.

Physiological P-use efficiency (PPUE)= Shoot P concentration divided by shoot dry matter.

Wheat yield

We allowed the wheat plants to complete their life cycle up to yield production. Plant yield (spike and grain weights) and other related attributes (spike length and the number of spikelet per a spike) were also evaluated.

Statistical analysis

The data were analyzed by one way ANOVA using SPSS 21.0software. Means were calculated for three-biological replicates and analyzed by the Duncan's multiple range where the statistical significance was done $P \leq 0.05$. The heatmap-clustering analysis was done by applying the MetaboAnalyst 4.0 (www.metaboanalyst.ca) as was described by Xia et al. (2009).

Results

Molecular identification of the tested fungal strains

ITS sequences of the strain no. AUMC 14260 showed high similarity (100%) with GenBank database strains; *Aspergillus niger* ATCC 16888 (= AY373852), *A. foetidus* CBS 121.28 (=NR163668), *A. welwitschiae* CBS 139.54 (=NR137513) and *A. awamori* CBC 557.65 (=NR077143). But, morphologically it is closely related to *A. niger*, thus it was deposited in GenBank database giving the accession no. MN70188. While, strain no. AUMC 14100 was found to be identical to three *Penicillium chrysogenum* strains from GenBank database (CBS 306.48 = NR_077145, FRR 807= AY373902, and ATCC 10106= HQ026745) and *P. tardochrysogenum* (CBS 132200= MH865983), resulting 100% similarity with each strain. So, it was deposited in Genbank database as *P. chrysogenum* AUMC 14100 (=MN219732) (Suppl. Tables 1, 2) and Fig. 2.

Phosphate-solubilizing efficiency and IAA production

A. niger AUMC 14260 and *P. chrysogenum* AUMC 14100 exhibited significant reduction in pH compared with the control during the 20 days of incubation on PVK medium amended with superphosphate (SP). The maximum shortage in pH was recorded after 5, 10, and 20 days of incubation. Furthermore, *P. chrysogenum* AUMC 14100 showed its maximum pH decrease from 7.0 to 3.2 after 20 days. Also, when media suspended with rock phosphate, highly significant decline in pH values were observed at the 20th day of incubation. The maximum pH decline was recorded by *P. chrysogenum* AUMC 14100 at the 15th day (from 7.0 to 3.340), at the 20th day (from 7.0 to 3.447) and by *A. niger* AUMC 14260 at the 20th day (from 7.0 to 3.43), but later there was an increase or no change in pH for all culture filtrates (Table 2).

With respect to phosphate solubilization efficiency, the two strains could significantly solubilize the insoluble superphosphate in the broth medium with higher activities, compared to the control. *A. niger* AUMC 14260 had the maximum solubilization potentiality (697.3 µg/mL, 1031.1µg/mL and 1629.3µg/mL) after 5, 10 and 20 days, respectively, while after 15 days' incubation, *P. chrysogenum* AUMC 14100 resulted in the highest concentration of solubilized phosphate compared to *A. niger* AUMC 14260 after 20 days incubation by a

maximum activity of 1478.0µg/mL (Table 2). The obtained results exhibited that, *A. niger* AUMC 14260 was the best rock phosphate solubilizer after 5 days incubation period, recording 511.1µg/mL, followed by *P. chrysogenum* AUMC 14100 (503.3µg/mL). At the 10th, 15th, and 20th days of incubation, *P. chrysogenum* AUMC 14100 was superior in rock phosphate solubilization, recording a potential ability of 659.1µg/mL, 758.0µg/mL, and 982.7 µg/mL, respectively. Then, *A. niger* AUMC 14260 came next by 597.6µg/mL, 706.9µg/mL, and 906.2µg/mL, respectively (Table 2).

The data presented in Table 3 denoted that the mixed culture of *A. niger* AUMC 14260 and *P. chrysogenum* AUMC 14100 showed the highest IAA production (396.4µg/mL), followed by *P. chrysogenum* (355.1µg/mL) while the lowest production was observed by single *A. niger* culture (278.1µg/mL).

Impact of Aspergillus niger AUMC 14260 and Penicillium chrysogenum AUMC 14100 on seed germination and seedling vigor

The data represented in Fig. 3A shows that applying CS or CF of the tested fungal strains improved wheat seed germination and seedling vigor compared to control, with progressing of *P. chrysogenum* CF by 38% (for seed germination) and 65.97% (for seedling vigor). On the other hand, CS of the same fungus improved seed germination and seedling vigor by 29% and 59.2%, respectively relative to control. Generally, co-application of both strains as either CF or CS recorded the highest positive stimulation of seedling vigor of wheat plant.

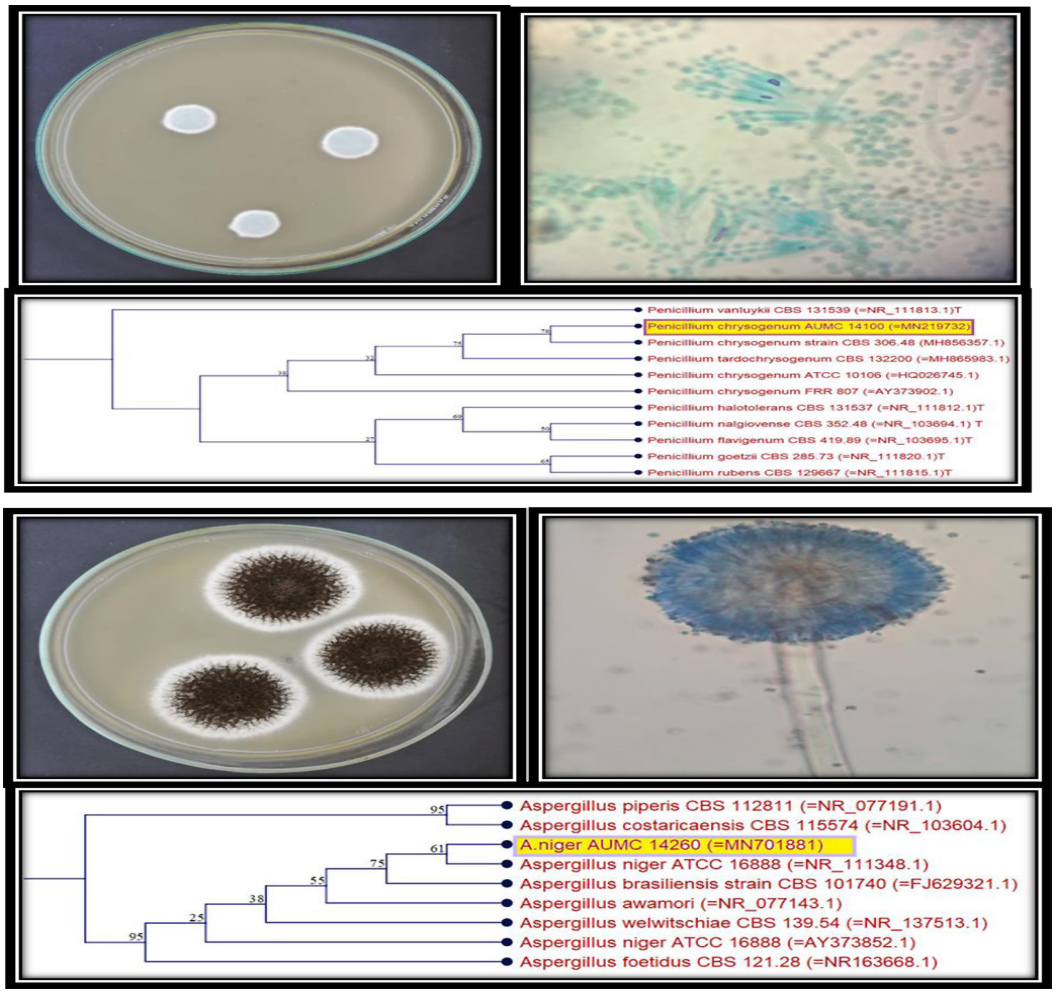

Fig. 2. Phylogenetic tree derived from ITS sequencing of *Aspergillus niger* AUMC 14260 (accession number: MN701881) and *Penicillium chrysogenum* AUMC 14100 (accession number: MN219732) isolated from rhizosphere of wheat with the closest match in the GenBank database and sequence similarity

TABLE 2. Concentrations of solubilized phosphate (mean ± SD µg/mL) by fungal strains *(A. niger* AUMC 14260 and *P. chrysogenum* AUMC 14100) on Pikovskaya's (PVK) medium supplemented with superphosphate or rock phosphate, after 5, 10, 15, and 20 days of incubation at 25±2°C

Incubation time	Traits	Superphosphate			Rock phosphate		
		Control	*A. niger*	*P. chrysogenum*	Control	*A. niger*	*P. chrysogenum*
5 days	PO_4^{3-}	252.7^{e}±1.8	697.3^{a}±8.7	609.8^{c}±16.2	188.7d±2.45	511.1a±7.6	503.3ab±16.0
	pH	7.0^{a}±0.02	4.5^{b}±0.1	4.3b^{c}±0.1	7.0a±0.02	4.0cd±0.24	4.2c±0.12
10 days	PO_4^{3-}	261.3^{f}±2.8	1031.1^{a}±28.5	849.6^{b}±28.7	196.0d±1.42	597.6b±18.3	659.1a±7.8
	pH	7.0^{a}±0.01	4.4^{d}±0.05	4.36^{d}±0.1	7.0a±0.03	3.56d±0.1	3.97c±0.11
15 days	PO_4^{3-}	264.7^{e}±3.6	1238.0^{a}±41.98	1288.7^{a}±26.6	194.7d±1.88	706.9a±32.8	758.0a±50.4
	pH	7.0^{a}±0.03	4.6^{c}±0.06	4.4^{c}±0.1	7.0a±0.02	3.75d±0.1	3.34e±0.12
20 days	PO_4^{3-}	268.7^{f}±3.7	1629.3^{a}±43.3	1478.0^{b}±63.3	200.0e±1.44	906.2b±50.56	982.7a±28.62
	pH	7.0^{a}±0.02	3.98^{d}±0.135	3.2^{f}±0.01	7.0a±0.03	3.43e±0.06	3.45e±0.12

TABLE 3. IAA production by *P. chrysogenum* AUMC 14100 and *A. niger* AUMC 14260

Treatment	*P. chrysogenum* AUMC 14100	*A. niger* AUMC 14260	Mixed culture
IAA (µg/mL)	355.1^{b}±9.19	278.1^{c}±6.55	396.4^{a}±22.88
F-test sig.	**	**	**

Wheat soil properties for different treatments

Results in Suppl. Table 3 reveal that the pH of the blank soil (without cultivation) was neutral (6.96). The incorporation of fungal biofertilizers into the soil either separately or in combination with chemical fertilizers reduced the soil pH significantly especially for mixed cultures. In addition, the joint treatment of fungal biofertilizers with or without chemical fertilizers to the cultivated soils significantly increased the EC and TSS. Furthermore, using *A. niger* or *P. chrysogenum* regardless being CF or CS enhanced the organic matter content significantly, compared to control plants and the lowest values were found for non-fertilized soils. Generally, CF has the highest values of the organic matter relative to CS (Suppl. Table 3). Applying *A. niger*, *P. chrysogenum* or their mixture as CF or CS has a significant role in enhancing the levels of the divalent cations (Mg^{+2} and Ca^{+2}) upmostly recorded for soils received mixed cultures. The data represented in Suppl. Table 4 shows that the peak of total phosphate content was recorded for rock phosphate and superphosphate fertilized soils (with or without plants). Although the content of phosphate was enhanced for plants treated with rhizospheric fungi with or without chemical fertilizers compared to non-fertilized soils, such increment was lower than chemically fertilized soils. Furthermore, the utilization of chemical fertilizers increased the soil content of soluble phosphates. The application of fungal inducers rather CF or CS further increased the content of soluble phosphates compared to control plants or chemically fertilized soils (Suppl. Table 4).

Growth parameters of wheat

According to data illustrated in Fig. 3B, the shoot and root dry weights were significantly increased by the addition of chemical phosphate fertilizers where superphosphate was more efficient than rock phosphate in improving the growth of wheat plant. The biofertilizer application in form of CF or CS positively enhanced the growth of wheat plant with variant magnitude. In general, superphosphate + fungal inducer recorded the highest growth parameters compared to rock phosphate + fungal inducer, and the best results were recorded for the consortium of the two tested strains compared to their single application. Furthermore, for separate treatments, *P. chrysogenum* was more efficient than *Aspergillus niger* in enhancing wheat growth.

Biochemical characteristics

It was detected from Figure 4a, b, c that the foliar chlorophyll a, b , and carotenoids contents of wheat treated with fungal biofertilizers with or without chemical fertilizers increased significantly, compared to the corresponding non-treated controls (with or without chemical P-fertilizers).

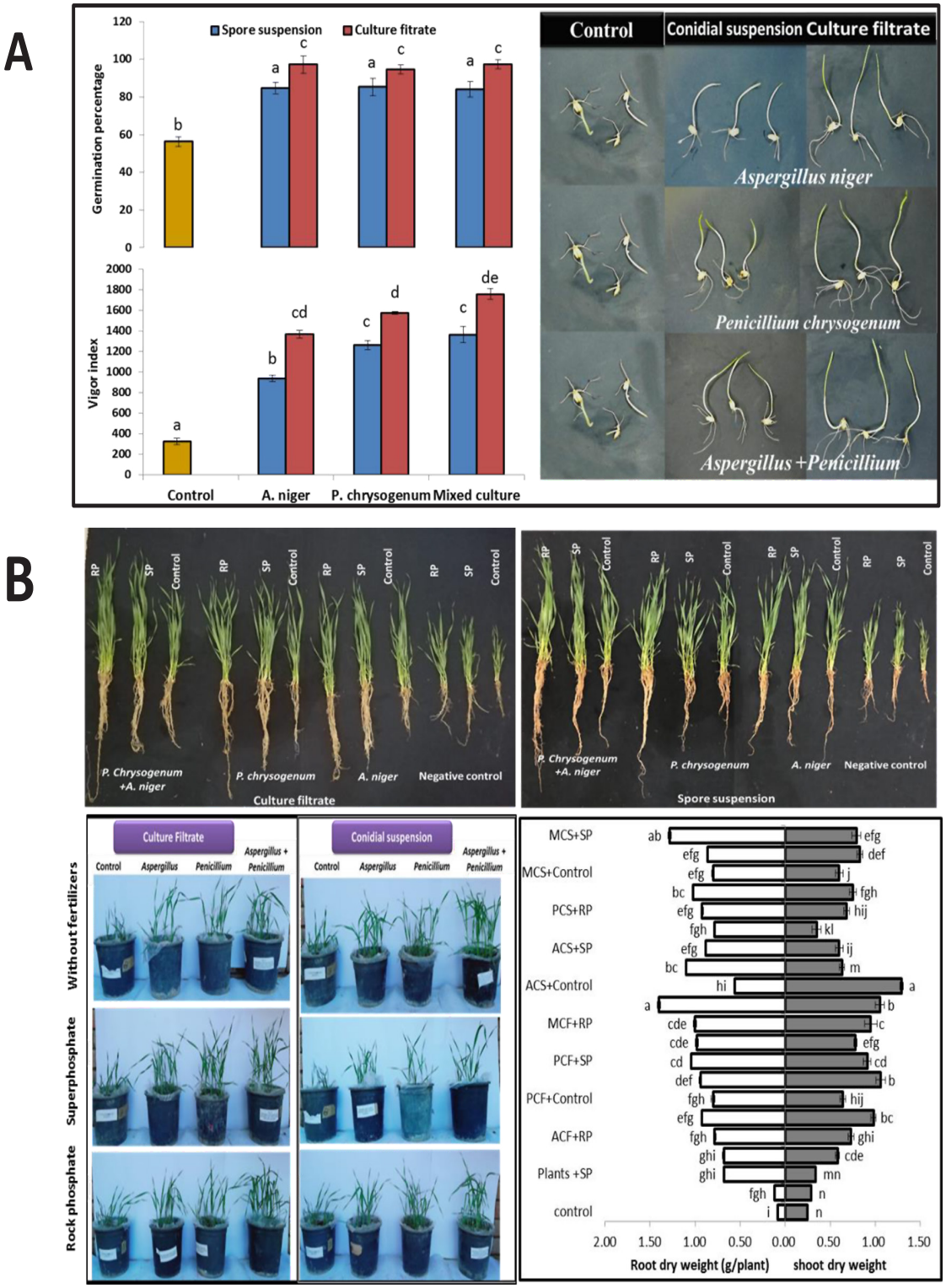

Fig. 3 A. Germination percentage and vigor index of wheat grains treated with *Aspergillus niger* (AUMC 14260), *Penicillium chrysogenum* (AUMC 14100), and their mixture when applied as conidial suspension or culture filtrate. B) Shoot and root dry weight of wheat plants grown in non-fertilized soils, rock phosphate, and superphosphate amended soils, with various fungal treatments: culture filtrates and conidial suspensions of *Aspergillus niger* (ACF, ACS) or *Penicillium* chrysogenum (PCF, PCS), or their mixture (MCF, MCS). [Histograms (*n= 3*) carrying different letters are significantly different at P<0.05]

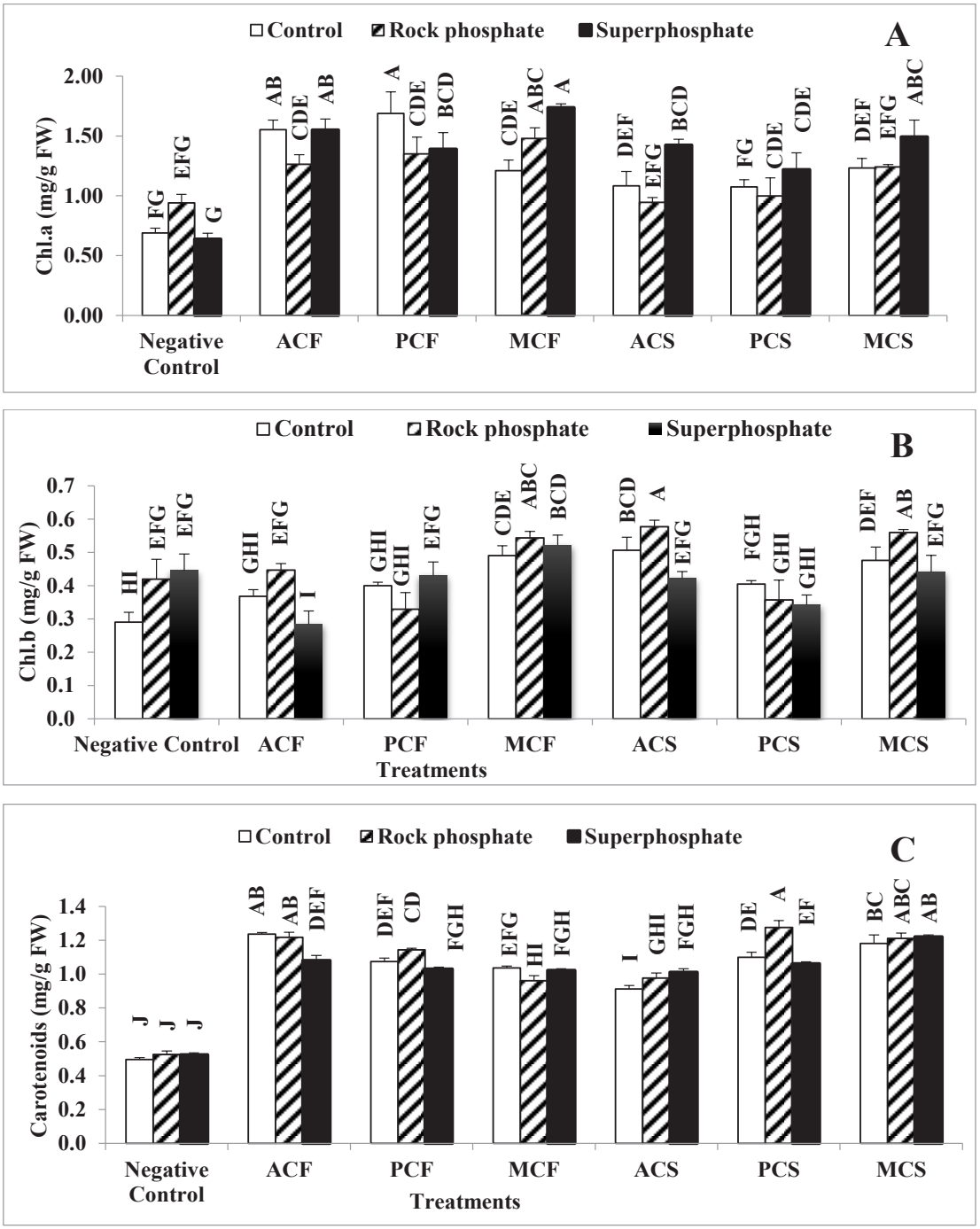

Fig. 4. Chl. a (A), Chl. b (B), and carotenoids (C) of wheat plants grown in non-fertilized soils, rock phosphate, and superphosphate amended soils, with various fungal treatments: culture filtrates and conidial suspensions of *Aspergillus niger* (ACF, ACS) or *Penicillium chrysogenum* (PCF, PCS), or their mixture (MCF, MCS) [Histograms *(n= 3)* carrying different letters are significantly different at P<0.05]

The protein content was significantly increased in wheat plants under different inducers. The data clearly recorded that CF had the highest promotion capacity of protein biosynthesis, compared to conidial suspension, and the consortium of *A. niger* and *P. chrysogenum* had the highest values compared to individual applications (Fig. 5a). The histograms presented in Fig. 5b showed considerable accumulation of amino acids in soil chemically fertilized with superphosphate and rock phosphate,

but much more so for superphosphate. Using *A. niger*, *P. chyrsogenum* or their mixture has a significant further accumulation of amino acids which showed the same magnitude. As illustrated in Fig. 5c, the data showed different magnitudes of carbohydrates accumulation among the different treatments. In this regard, RF treatment increased the carbohydrate content compared to its control while SF was higher than control only in the mixture of CF.

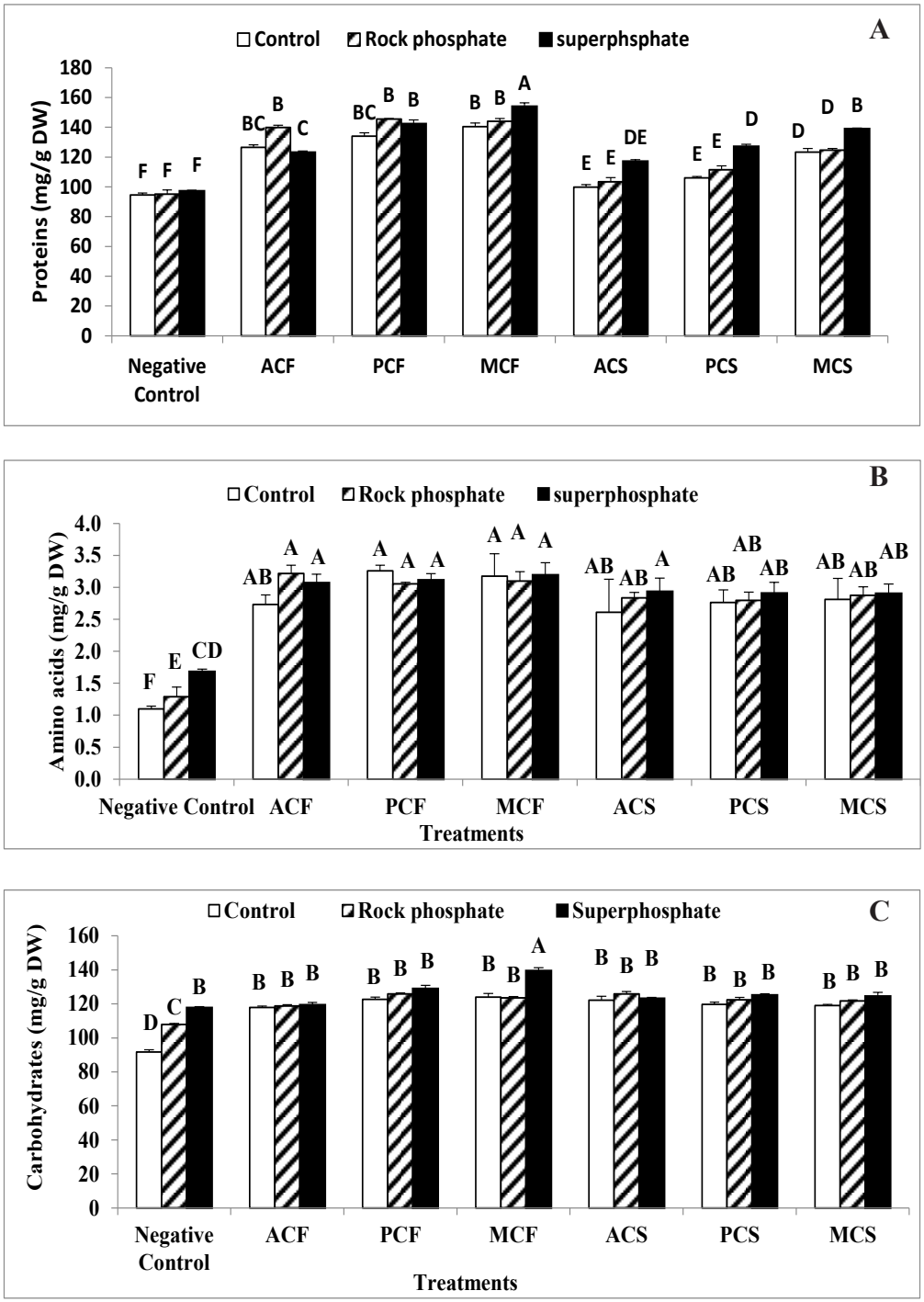

Fig. 5. Proteins, amino acid and carbohydrates content grown in non-fertilized soils, rock phosphate, and superphosphate amended soils non-treated (negative control) or treated with culture filtrates (CF) and conidial suspensions (CS) of *Aspergillus niger* (ACF, ACS), *Penicillium chrysogenum* (PCF, PCS) and their mixture (MCF, MCS) [Histograms *(n= 3)* carrying different letters are significantly different at P<0.05]

Membrane damage and reactive oxygen species

Hydrogen peroxide and superoxide anion were highly significantly reduced under various treatments of fungal biofertilizers and/or chemical fertilizers, compared to non-fertilized control plants. The highest reduction was recorded in plants soaked in mixed CF of *A. niger* and *P. chrysogenum,* followed by CF of *P. chrysogenum* alone, but the lowest reduction was recorded for CS of *A. niger*. Furthermore, CF-treated plants affected highly in reducing H_2O_2 compared to CS with or without chemical fertilizers (Fig. 6a). In the present investigation, superoxide anion recorded its peak values in untreated plants, but rock phosphate and superphosphate had no effect on superoxide anion compared to non-fertilized plants. Interestingly, various treatments of fungal biofertilizers (either single or their consortium as CS or CF) with/without chemical fertilizers reduce the content of superoxide with the priority to culture CF to CS Fig. 6b. Our findings demonstrate in Fig. 6c that MDA content reduced using different applicants with various degrees. The two supplemented phosphate fertilizers slightly reduced the MDA content but significant only for superphosphate fertilizer. All fungal treatments come in agreement to reduce MDA content compared to non-fertilized plants. Interestingly, co-application with culture filtrates of *A. niger* and *P. chrysogenum* with or without phosphate fertilizers had the highest capacity to reduce MDA content, compared to individual fungal treatments.

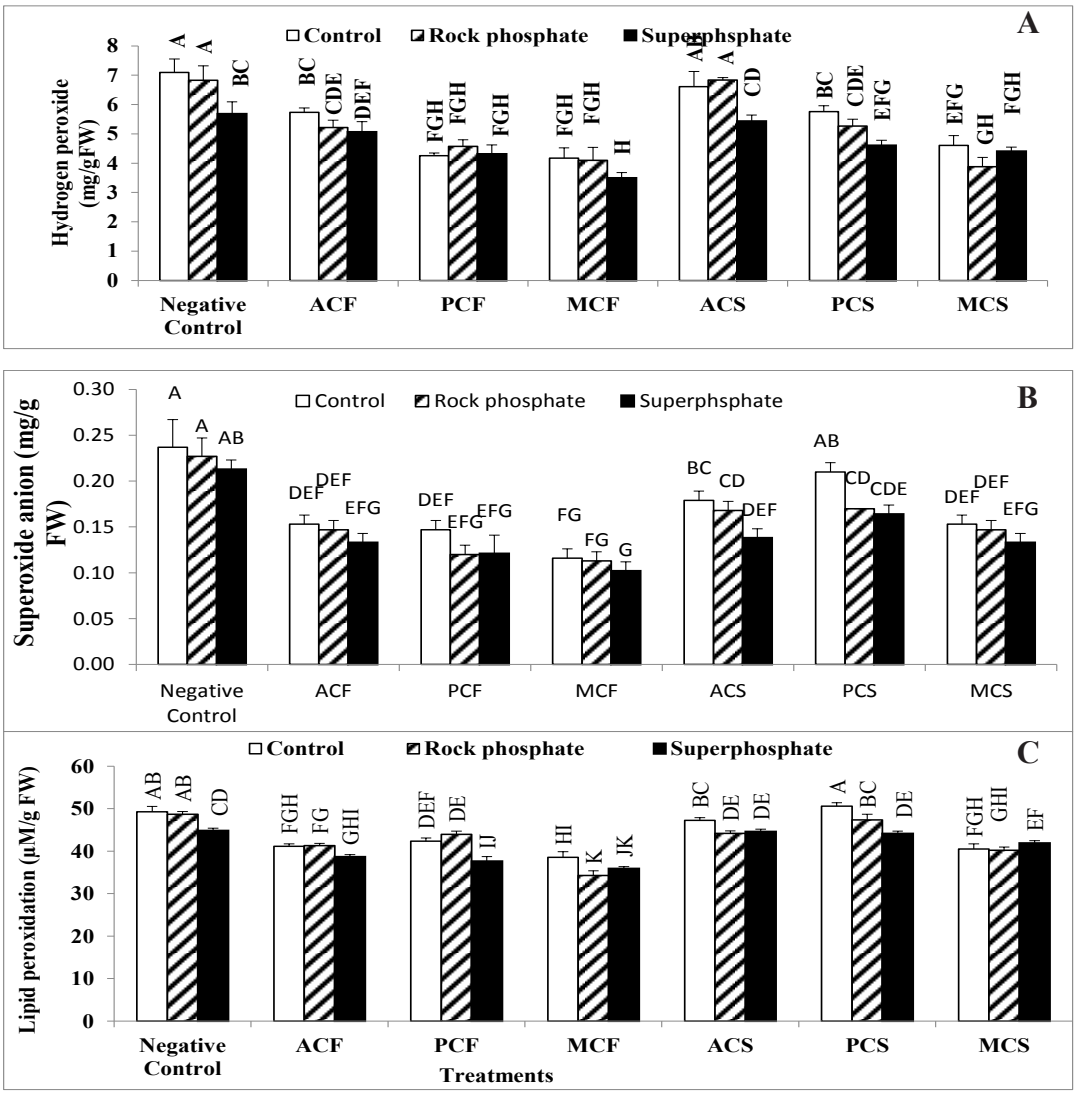

Fig. 6. Hydrogen peroxide, superoxide anion content, and lipid peroxidation grown in non-fertilized soils, rock phosphate, and superphosphate amended soils treated with culture filtrates and conidial suspensions of *Aspergillus niger* (ACF, ACS), *Penicillium chrysogenum* (PCF, PCS), and their mixture (MCF, MCS). [Histograms (*n*= 3) carrying different letters are significantly different at P<0.05]

Non-enzymatic antioxidants

The histograms in Fig. 7a, b, c denote that the chemical phosphate fertilizers had highly significant effect on the accumulation of AsA content compared to non-fertilized plants but had non-significant effect on content of vitamin E and phenolic contents. On the other hand, fungal inducers triggered the biosynthetic pathway of AsA, α-tocopherol, and phenolic compounds under various treatments. The maximum foliar content of AsA was obtained when the plants treated with a mixture of *A. niger* and *P. chrysogenum* compared to the single inoculation. Comparatively, in case of consortium application of both tested fungi, CF is preferred for induction of AsA biosynthesis, compared to CS.

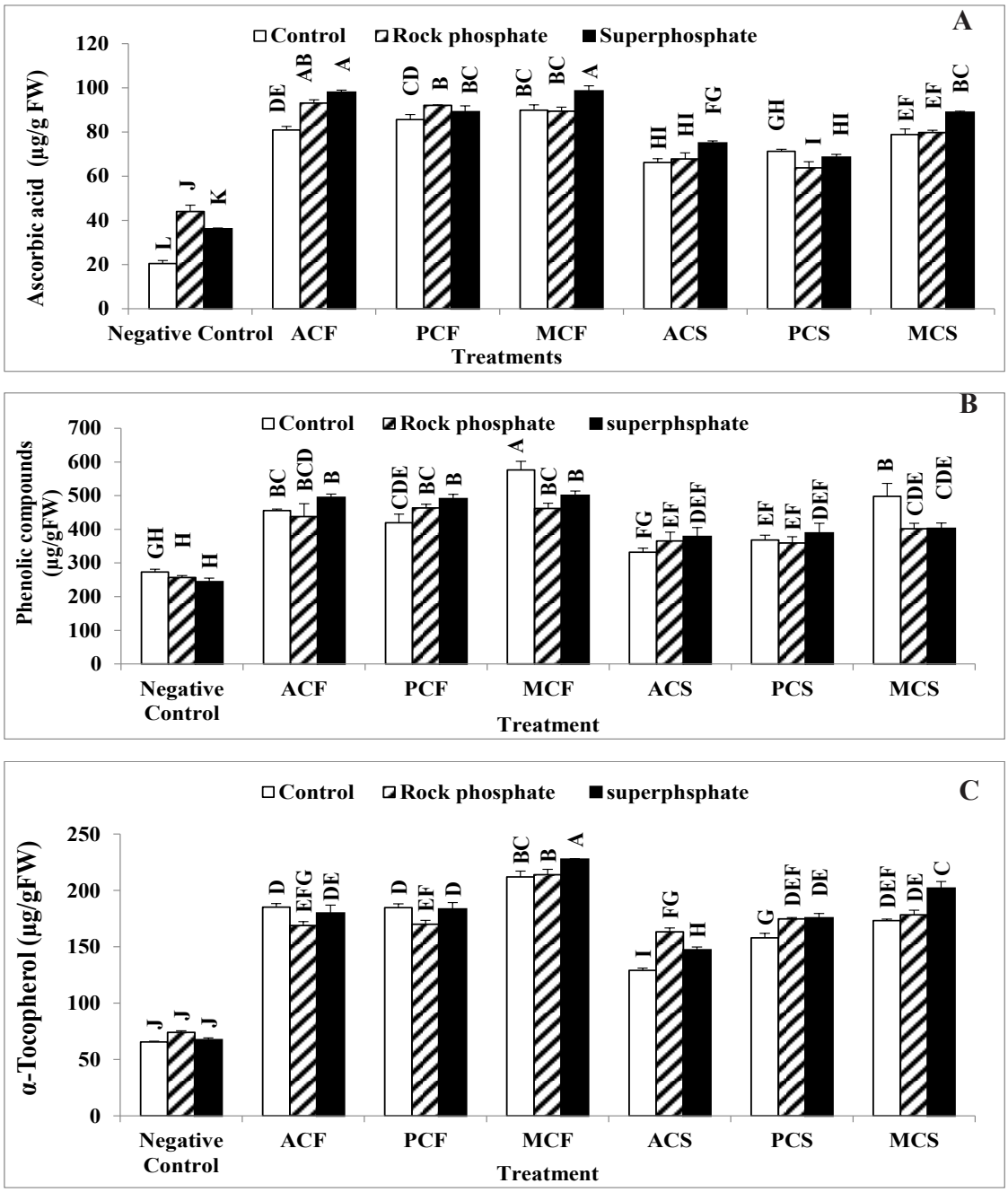

Fig. 7. Ascorbic acid, phenolic compounds and α-Tocopherol content grown in non-fertilized soils, rock phosphate, and superphosphate amended soils treated with culture filtrates and conidial suspensions of *Aspergillus niger* (ACF, ACS), *Penicillium chrysogenum* (PCF, PCS), and their mixture (MCF, MCS) [Histograms (*n*= 3) carrying different letters are significantly different at P<0.05]

Antioxidant enzymes

The presence of chemical fertilizers induced non-significant increase in the activity of SOD (Fig. 8a). Whilst, the different forms of biofertilizers highly significantly raised SOD activity, and the mixture of *A. niger* and *P. chrysogenum* in culture filtrate recorded the maximum activity of SOD. In addition, the data histogrammed in Fig. 8b, c, d, reveals that the lowest CAT, APX and PAL activities were recorded for non-fertilized control plants. The plants treated with biofertilizers (CF or CS) recorded higher catalase activity. Furthermore, the best values were recorded for consortium of *A. niger* and *P. chrysogenum* as culture filtrate. The data represented in Fig. 8e & f denotes that the activity of PAL and PPO were non-significantly changed whatever the inducer used for various soil amendments.

Nutrient contents

Regarding to magnesium (Mg^{+2}) and calcium (Ca^{+2}) ions, a highly significant accumulation in shoots and roots was observed, due to the utilization of fungal biofertilizers as culture filtrate or conidial suspension. Incongruently, there were non-significant differences in their concentrations in response to chemical fertilizers relative to control. A significant difference in Ca^{+2} values was recorded by amendment of *A. niger* and *P. chrysogenum* as CF in soils treated with rock phosphate or without chemical fertilizer (Table 4). The data illustrated in Table 4 shows that all treatments significantly improved the concentration of PO_4^{-3} in wheat tissues. The content of shoot and root phosphate recorded its lowest values for non-fertilized plants. The addition of P-containing chemical fertilizers increased the PO_4^{-3} concentration in the non-inoculated plants. Application of inducers showed high significant promotion of the phosphate content where the highest concentration of PO_4^{-3} in the shoots and roots were obtained (The plants were treated with CF of co-inoculation with both fungal strains.)

The data represented in Table 5 declares the changes of phosphate-related traits for wheat plants under different treatments. With regard to phosphate utilization efficiency (PUE), the applied chemical fertilizers induced significant increment of PUE, but further activation was also induced when fungal inducers were applied. Generally, fungal culture filtrate recorded the highest values of PUE compared to conidial suspension. All treatments with fungal culture filtrates exhibited high significant increase in PUE but much more so for mixed fungi. On the other hand, the total phosphate accumulation, phosphate uptake efficiency, and physiological phosphate use efficiency were represented in Table 5 for wheat under different inducers (conidial suspension or culture filtrate) with/without chemical fertilizers. The used phosphate fertilizers did not affect the phosphate uptake efficiency and physiological phosphate use efficiency. The synergistic application of phosphate fertilizers with fungal inducers recorded highly significant increase of these traits whatever the treatment used, compared to non-fertilized soils. The best results of total phosphate accumulation were coined for culture filtrate treated plants, compared to conidial suspension and the co-inoculation of both *A. niger* and *P. chrysogenum*.

Hierarchical clustering of the tested traits under various treatments using heatmap analysis

The mean values of the biochemical parameters were subjected to perform heatmap with hierarchical clustering which are represented in Fig. 9. Hierarchical clustering illustrated that the applied chemical fertilizers (superphosphate or rock phosphate) showed similar pattern to non-fertilized soils. Compared to non-fertilized soils, using fungal inducers had an intensive positive effect on morphological traits (fresh weights and dry weights of shoots and roots), biochemical traits (Chl. a, Chl. b, Carotenoids, AsA, α-tocopherol, phenolics, PAL, SOD, APX, POD, and CAT), and phosphate related traits (phosphate uptake efficiency, phosphate utilization efficiency, phosphate utilizing efficiency). On the other hand, a decreasing trend has been recorded for reactive oxygen species such as hydrogen peroxide and superoxide anion as well as lipid peroxidation.

Yield traits of wheat

The data represented in Table 6 shows that all yield traits increased with applying phosphate fertilizers to soil but much more so for superphosphate compared to rock phosphate. With soaking wheat in conidial suspension or culture filtrate of *A. niger*, *P. chrysogenum,* or both significantly increased the dry weight of spike/plant, spikes length, and the number of spikelets per spike. In comparison, the CF recorded the highest yield-related traits compared to CS.

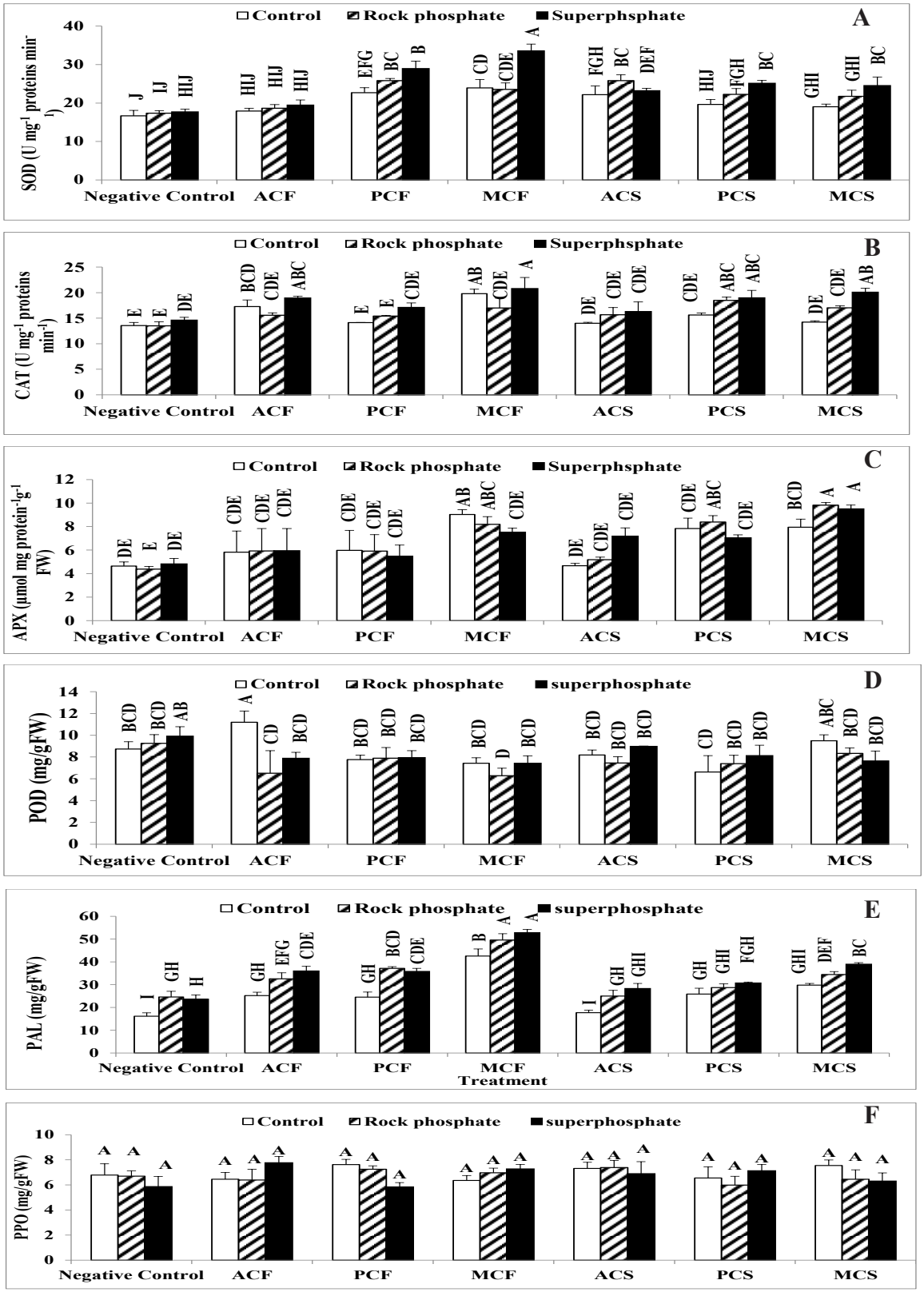

Fig. 8. SOD, catalase, ascorbate peroxidase, guaiacol peroxidase, phenylalanine ammonia-lyase, and polyphenol oxidase of grown in non-fertilized soils, rock phosphate, and superphosphate amended soils treated with culture filtrates and conidial suspensions of *Aspergillus niger* (ACF, ACS), *Penicillium chrysogenum* (PCF, PCS), and their mixture (MCF, MCS) [Histograms (*n= 3*) carrying different letters are significantly different at P<0.05]

TABLE 4. Phosphate, calcium, and magnesium content of wheat roots and shoots under different treatments

Treatments		Roots			Shoots		
		PO_4	Ca^{+2}	Mg^{+2}	PO_4	Ca^{+2}	Mg^{+2}
Negative control	Plants	$0.06^{k}\pm0.007$	$4.88^{g}\pm0.31$	$0.92^{k}\pm0.21$	$0.24^{j}\pm0.021$	$6.91^{b}\pm0.70$	$3.2^{cde}\pm0.01$
	Plants +RP	$0.1^{i}\pm0.006$	$9.15^{e}\pm0.53$	$1.04^{gh}\pm0.21$	$0.23^{j}\pm0.027$	$6.54^{a}\pm0.01$	$2.99^{cde}\pm0.11$
	Plants +SP	$0.08^{j}\pm0.002$	$7.02^{f}\pm0.61$	$1.6^{ij}\pm0.08$	$0.36^{i}\pm0.046$	$6.91^{b}\pm0.70$	$3.31^{cde}\pm0.10$
A. niger	CF+Control	$0.4^{gh}\pm0.012$	$10.68^{d}\pm0.31$	$1.28^{j}\pm0.52$	$0.59^{gh}\pm0.019$	$8.54^{a}\pm0.005$	$3.09^{def}\pm0.21$
	CF+RP	$0.5^{f}\pm0.036$	$12.20^{c}\pm0.31$	$2.48^{def}\pm0.21$	$0.6^{fg}\pm0.012$	$9.53^{a}\pm0.70$	$2.35^{ghi}\pm0.10$
	CF+SP	$0.5^{fgh}\pm0.056$	$13.12^{bc}\pm0.01$	$2.08^{ghi}\pm0.08$	$0.65^{fg}\pm0.017$	$11.79^{a}\pm0.70$	$2.45^{ghi}\pm0.11$
P. chrysogenum	CF+Control	$0.44^{gh}\pm0.027$	$9.15^{e}\pm0.61$	$2.32^{def}\pm0.08$	$0.49^{hi}\pm0.024$	$14.64^{a}\pm1.22$	$2.03^{hi}\pm0.011$
	CF+RP	$0.54^{f}\pm0.045$	$10.68^{d}\pm0.30$	$2^{hi}\pm0.16$	$0.66^{fg}\pm0.015$	$15.45^{a}\pm1.40$	$3.4^{cd}\pm0.010$
	CF+SP	$0.81^{c}\pm0.023$	$13.42^{b}\pm0.31$	$1.28^{j}\pm0.08$	$0.94^{c}\pm0.043$	$16.67^{a}\pm0.70$	$1.8^{i}\pm0.21$
A. niger + *P. chrysogenum*	CF+Control	$0.64^{e}\pm0.023$	$9.76^{de}\pm0.01$	$3.2^{b}\pm0.16$	$0.87^{de}\pm0.034$	$19.52^{a}\pm0.01$	$3.84^{bc}\pm0.32$
	CF+RP	$1.06^{ab}\pm0.056$	$14.64^{a}\pm0.52$	$3.2^{b}\pm0.16$	$1.5^{a}\pm0.066$	$17.49^{a}\pm0.70$	$2.67^{fgh}\pm0.38$
	CF+SP	$1.15^{a}\pm0.027$	$14.64^{a}\pm0.53$	$2.88^{bcd}\pm0.14$	$1.3^{ab}\pm0.057$	$11.79^{a}\pm0.70$	$3.4^{cd}\pm0.11$
A. niger	CS+Control	$0.37^{h}\pm0.01$	$7.32^{f}\pm0.61$	$2.88^{bcd}\pm0.14$	$0.46^{hi}\pm0.011$	$17.41^{a}\pm0.01$	$4.16^{b}\pm0.18$
	CS+RP	$0.61^{e}\pm0.057$	$7.93^{f}\pm0.01$	$2.56^{def}\pm0.35$	$0.66^{fg}\pm0.041$	$14.23^{a}\pm1.40$	$2.77^{efg}\pm0.21$
	CS+SP	$0.5^{f}\pm0.031$	$7.32^{f}\pm0.01$	$2.72^{cde}\pm0.16$	$0.61^{fg}\pm0.013$	$13.01^{a}\pm0.704$	$0.85^{j}\pm0.10$
P. chrysogenum	CS+Control	$0.4^{gh}\pm0.024$	$7.32^{f}\pm0.30$	$2.08^{ghi}\pm0.16$	$0.58^{gh}\pm0.035$	$15.86^{a}\pm0.01$	$2.35^{ghi}\pm0.28$
	CS+RP	$0.57^{f}\pm0.017$	$9.76^{de}\pm0.61$	$1.92^{hi}\pm0.24$	$0.73^{ef}\pm0.043$	$15.45^{a}\pm1.86$	$1.813^{i}\pm0.43$
	CS+SP	$0.56^{f}\pm0.031$	$10.37^{de}\pm0.01$	$2.88^{bcd}\pm0.28$	$0.66^{fg}\pm0.042$	$12.61^{a}\pm0.70$	$2.03^{hi}\pm0.28$
A.niger + *P. chrysogenum*	CS+Control	$0.55^{f}\pm0.028$	$9.15^{e}\pm0.01$	$2^{hi}\pm0.08$	$0.67^{fg}\pm0.013$	$17.49^{a}\pm0.70$	$3.09^{def}\pm0.10$
	CS+RP	$0.75^{ed}\pm0.033$	$10.98^{d}\pm0.30$	$4^{a}\pm0.08$	$0.8^{d}\pm0.007$	$12.61^{a}\pm0.1.40$	$4.8^{a}\pm0.37$
	CS+SP	$0.76^{ed}\pm0.012$	$12.51^{bc}\pm0.34$	$3.04^{bc}\pm0.08$	$0.9^{c}\pm0.025$	$13.01^{a}\pm1.40$	$3.31^{cde}\pm0.10$

CF= Culture filtrate, CS= Conidial suspension, RP= Rock phosphate, SP= Superphosphate

TABLE 5. Total phosphate accumulation, P-utilization efficiency, P-uptake efficiency, and physiological phosphorous use efficiency of wheat under different treatments

Treatments		Total P accumulation	P utilization efficiency	P uptake efficiency	Physiological P-use efficiency
Negative control	Plants	$0.37^{m}\pm0.01$	$0.54^{f}\pm0.03$	$3.85^{g}\pm0.22$	$0.6^{i}\pm0.03$
	Plants +RP	$0.38^{m}\pm0.01$	$0.6^{f}\pm0.04$	$3.77^{g}\pm0.18$	$0.67^{i}\pm0.02$
	Plants +SP	$0.44^{lm}\pm0.01$	$0.6^{e}\pm0.07$	$4.1^{g}\pm0.59$	$0.66^{i}\pm0.02$
A. niger	CF+Control	$1.01^{g}\pm0.04$	$0.99^{a}\pm0.03$	$9.17^{cde}\pm1.07$	$1.22^{cd}\pm0.07$
	CF+RP	$0.97^{g}\pm0.04$	$0.77^{c}\pm0.01$	$8.6^{def}\pm1.01$	$1.21^{cd}\pm0.02$
	CF+SP	$1.31^{ef}\pm0.06$	$0.99^{a}\pm0.04$	$7.5^{ef}\pm0.22$	$1.55^{ab}\pm0.16$
P. chrysogenum	CF+Control	$0.7^{hi}\pm0.02$	$0.84^{b}\pm0.07$	$7.3^{ef}\pm1.09$	$0.78^{hi}\pm0.06$
	CF+RP	$1.46^{de}\pm0.08$	$1.02^{a}\pm0.04$	$7.78^{def}\pm0.85$	$0.84^{ghi}\pm0.02$
	CF+SP	$1.89^{c}\pm0.05$	$0.86^{b}\pm0.01$	$10.05^{bcd}\pm0.234$	$1.03^{fgh}\pm0.06$
A. niger + *P. chrysogenum*	CF+Control	$1.4^{e}\pm0.06$	$0.83^{b}\pm0.01$	$9.3^{cde}\pm0.68$	$1.12^{def}\pm0.03$
	CF+RP	$2.64^{a}\pm0.23$	$0.85^{b}\pm0.03$	$15.6^{a}\pm1.12$	$1.22^{cd}\pm0.09$
	CF+SP	$1.96^{b}\pm0.08$	$0.94^{a}\pm0.03$	$10.8^{bc}\pm0.84$	$1.42^{bc}\pm0.06$
A. niger	CS+Control	$0.3^{kl}\pm0.03$	$0.58^{h}\pm0.04$	$9^{def}\pm0.79$	$1.6^{ab}\pm0.15$
	CS+RP	$1.03^{g}\pm0.04$	$0.74^{c}\pm0.04$	$6.9^{ef}\pm0.05$	$1.06^{fgh}\pm0.09$
	CS+SP	$0.62^{ij}\pm0.04$	$0.74^{c}\pm006$	$7.8^{def}\pm0.63$	$1.55^{ab}\pm0.13$
P. chrysogenum	CS+Control	$0.48^{jk}\pm0.06$	$0.6^{d}\pm0.04$	$7.8^{def}\pm0.47$	$1.72^{a}\pm0.22$
	CS+RP	$1.09^{fg}\pm0.07$	$0.8^{b}\pm0.03$	$8.6^{def}\pm0.72$	$1.07^{def}\pm0.04$
	CS+SP	$1.14^{fg}\pm0.08$	$0.77^{bc}\pm0.02$	$6.7^{f}\pm0.51$	$0.88^{ghi}\pm0.03$
A.niger + *P. chrysogenum*	CS+Control	$0.9^{gh}\pm0.06$	$0.6^{e}\pm0.04$	$9.1^{cde}\pm0.91$	$1.13^{def}\pm0.09$
	CS+RP	$1.53^{de}\pm0.05$	$0.82^{b}\pm0.01$	$11^{b}\pm0.68$	$0.99^{fgh}\pm0.03$
	CS+SP	$1.66^{d}\pm0.09$	$0.8^{b}\pm0.05$	$7.94^{def}\pm0.73$	$1.17^{cde}\pm0.09$

CF= Culture filtrate, CS=Conidial suspension, RP = Rock phosphate, SP = Superphosphate.

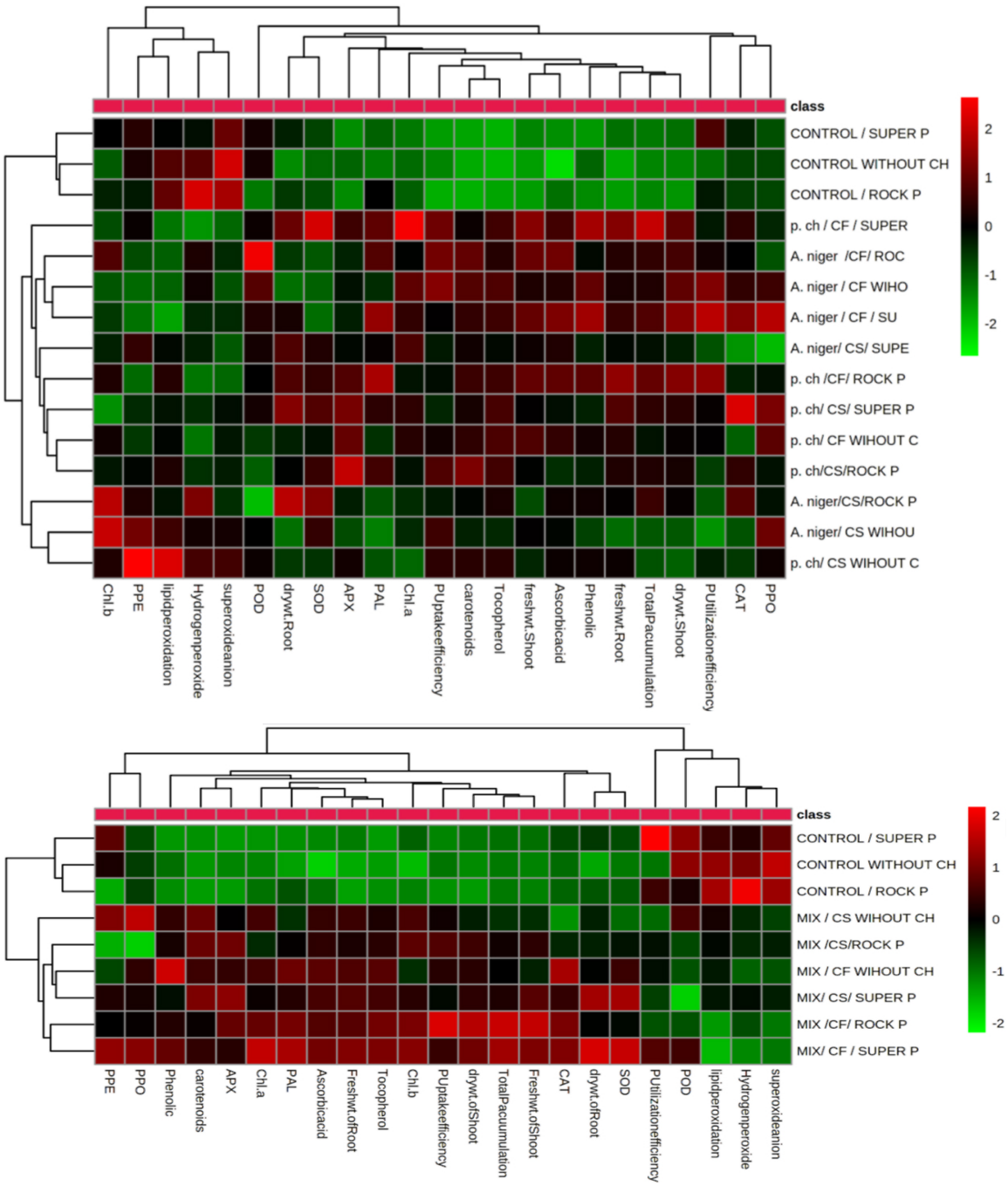

Fig. 9. Hierarchical Clustering of the tested traits under various treatments of wheat using the heatmap. In the heatmap, the color scale shows the intensity of the standardized mean values of different parameters (morphological traits (fresh weights and dry weights of shoots and roots), biochemical traits (Chl. a, Chl. b, carotenoids, AsA, α-tocopherol, phenolics, PAL, SOD, APX, POD, and CAT), phosphate related traits (phosphate uptake efficiency, phosphate utilization efficiency, phosphate utilizing efficiency), reactive oxygen species as hydrogen peroxide and superoxide anion and lipid peroxidation; (a) Non fertilized soil and fertilized soil using chemical fertilizers (rock phosphate and superphosphate) and biofertlizers *(Penicillium chrysogenum or Aspergillus niger)* inducers and (b) Non fertilized soil and fertilized soil using chemical fertilizers (rock phosphate and superphosphate) and biofertlizers (mixed inducers of *Penicillium chrysogenum* and *Aspergillus niger.* In the expression par, the red color shows high, and the green color shows low, trait levels

TABLE 6. Wheat-yield trait, length of spike, number of spikelets/spike, weight of spike (g/plant), and weight of grains (g/spike) under different treatments

Treatments		Number of spikelet/ spike	Length of spike	Weight of spike/ plant	Weight of grains/plant
Negative control	Control	37.33^{f}±1.50	7.5^{f}±0.5	2.52^{g}±0.29	1.93^{f}±0.5
	Control+RP	41^{de}±1.53	8.1^{e}±0.79	3.21^{ef}±0.36	2.62^{e}±0.39
	Control+SP	37.67^{f}±2.30	8.2^{e}±0.64	3.60^{e}±0.42	2.46^{e}±0.64
A. niger	CF+Control	44.67^{d}±1.51	9.5^{cd}±0.87	3.51^{e}±0.23	4.23^{c}±0.47
	CF+RP	48.67^{cd}±1.50	9.7^{cd}±0.53	5.93^{b}±0.38	4^{c}±0.53
	CF+SP	52^{cd}±2.29	10.3^{c}±0.58	5.05^{c}±0.43	4.1^{c}±0.58
P. chrysogenum	CF+Control	38^{f}±3.00	10.1^{c}±12.1	5.36^{c}±0.38	3.23^{d}±0.21
	CF+RP	49.33^{cd}±3.31	12.0^{a}±9.0	5.15^{c}±0.13	4.01^{bc}±0.49
	CF+SP	60.67^{b}±3.055	12.3^{a}±0.58	4.45^{d}±0.12	4.11^{bc}±0.58
A. niger + *P. chrysogenum*	CF+Control	46^{d}±3.11	11.4^{b}±0.63	5.35^{c}±0.19	4.68^{b}±0.63
	CF+RP	59^{b}±3.29	12.3^{a}±0.9	5.98^{b}±0.27	5.34^{a}±0.39
	CF+SP	66.33^{a}±1.52	12^{a}±1.0	6.86^{a}±0.39	4.1^{bc}±0.51
A. niger	CS+Control	39.33^{e}±2.04	10.5^{c}±0.83	5.15^{c}±0.14	3.21^{d}±0.43
	CS+RP	42.33^{de}±3.02	9.4^{cd}±0.68	4.11^{d}±0.26	3.02^{d}±0.38
	CS+SP	42.67^{de}±3.78	9.6^{cd}±0.76	4.32^{d}±0.18	4.2^{bc}±0.46
P. chrysogenum	CS+Control	40.3^{de}±2.13	10.1^{c}±1.0	5.85^{b}±0.17	3.44^{d}±0.51
	CS+RP	46^{d}±2.36	10.5^{c}±0.50	4.29^{d}±0.43	4.30^{bc}±0.5
	CS+SP	49.33^{d}±1.06	11.5^{b}±0.65	5.26^{c}±0.27	3.92^{cd}±0.45
A. niger + *P. chrysogenum*	CS+Control	54^{c}±2.53	11.5^{b}±0.96	4.89^{d}±0.29	4.32^{bc}±0.36
	CS+RP	56^{c}±3.00	10.5^{c}±0.75	5.52^{c}±0.12	4.22^{bc}±0.55
	CS+SP	51.33^{cd}±2.02	12.2^{a}±0.56	4.82^{d}±0.16	4.20^{bc}±0.56

Discussion

The efficacy of phosphate solubilizing fungal strains, *P. chrysogenum* AUMC 14100 and *A. niger* AUMC 14260 to solubilize two different phosphate fertilizers (e.g. rock phosphate and superphosphate) was demonstrated to influence positively wheat life cycle. Such development could be associated with increased P bio-solubilization and growth promoting substances (i.e., IAA). Regarding to germination process, spore suspension and cultural filtrate of the two fungal strains improved seed germination and seedling vigor of treated wheat grains in consistence to the previous studies concerned with treatment of plant seeds with fungal strains (Gizaw et al., 2018; Trizelia, 2020). The positive effect of fungal filtrates (Garuba et al., 2014) and spore suspension (Mahadevamurthy et al., 2016) on germination criteria may be due to the effect of fungal filtrate extract on pre-germinating metabolic activities and mediation of cell division in germinating seeds that make radicle protrusion of the extract treated seed germinated earlier than untreated seeds. In addition, the extrolites of fungal inducers may be associated with the stimulation of reserve mobilization of food material, stimulation and re-production of some enzymes, biosynthesis of DNA and RNA causing improvement of germination and vigor of seed (Kumari& Nanayakkara, 2017).

In the pot experiment, the fungal inducer stimulated their beneficial effect on plants by improving soil properties as well as plant biochemistry. With respect to the improvement of soil characteristics; *A. niger* or/and *P. chrysogenum* is characterized with high ability to solubilize the used phosphate fertilizers. Consequently, the current study confirmed the ability of fungal inducers to increase the efficacy of phosphate fertilizers in the applied soil. This revealed that the plant will take the advantages brought by fungi to the soil where more free forms of phosphate in soil raises its fertility. Similarly, (Singh & Reddy, 2011) illustrated that inoculation with *Penicillium oxalicum* significantly increased the growth and yield of wheat and maize plants compared to the control plants which were concomitant with an increase in plants P content and high level of soil organic carbon levels, compared to the control soil. In conformity, the roots and shoots of these plants recorded high content of soluble phosphate compared to plants non-treated with biofertilizers. Furthermore, the total phosphate accumulation in wheat was enhanced progressively in plants amended with phosphate fertilizers plus fungal inducers where the mixture of *A. niger* and *P. chrysogenum* had the highest values, revealing high efficiency to phosphate use. This is also recommended by the data of phosphorous use efficiency in terms of the capacity of plants to uptake P from the soils (P-uptake efficiency) and to what extent the absorbed phosphate utilized efficiently (P-utilization efficiency) by plants (Bilal et al., 2018; Iqbal et al., 2019b). The mixing of P-fertilizers with phosphate-solubilizing fungi has been found effective in increasing P-use efficiency and crop productivity **(**Abbasi et al., 2015; Giro et al., 2015**)**. Iqbal et al. (2019a) stated that genotypes of cotton with high P-utilization efficiency will produce more dry matter per unit of P-consumption as was reported, in our results, for chemically fertilized or non-fertilized with biofertilizers. In addition to enhanced soil phosphate properties, the fungal treatments, herein, enhanced the organic matter, Ca, and Mg contents compared to their initial values with a noticeable drop in pH values of the soil, compared to the control. The drop in the pH value could be associated with the ability of microorganism to produce organic acids in the growing medium causing solubilization of phosphate (Kang et al., 2012). The pH drop in cultures has been repeatedly reported by several research findings (Janardan et al., 2011; Elias et al., 2016a). (El-Azouni, 2008) reported higher available P, water soluble C, soil total sugars, and lower soil pH under fungal application compared to control soils. This harmony revealed the valuable role of fungal association in changing soil properties, which reflected to improving plant nutrients content and their utilization in plant physiological regulations.

The improvement of shoot and root dry weight values obtained as a result of applying single or combined culture filtrate of *A. niger* or *P. chrysogenum* isolates with rock or superphosphate in soil. IAA is a plant growth regulator induces growth, root elongation, besides augmentation of photosynthesis, sugars metabolism, and the productivity (Li et al., 2019). In this regard, the increased P to be more available, elevated tissue content of P by

the applied inducers may be enhanced the cell elongation, and shoot and root growth of wheat plants. Furthermore, the efficiency of fungal inoculants to solubilize phosphate might be contributed to the enhanced the growth of root, thereby created high area of roots to activate more uptake of nutrients from the soil. (Sharma et al., 2012) suggested that the combined application of phosphate solubilizeing fungi and enrich phosphorus to plants, along with instigate formation of deeper and denser roots. These results are in line with the findings of several works (Elias et al., 2016b; Ye et al., 2020; Ngalimat et al., 2021) which vastly recommended enhanced growth and development of various plants by combining phosphate solubilizing fungi and phosphate fertilizers. These results were further validated by heatmap hierarchical clustering analysis, which signified that plant amendment with various inducers displayed positive relationship with growth parameters and P-related traits in comparison with non-amended plants.

Co-application of both chemical fertilizers and biofertilizers was found to stimulate chlorophylls and carotenoids contents compared non-fertilized plants. This could be associated with the role of rhizospheric fungal treatments in increasing the obtainable nutrients as phosphorous and their related traits as P-physiological use efficiency which could provide energy required for enzymes required for chlorophyll biosynthesis which reflected on high content of sugars, thereby high dry matter acquisition. In addition, the fungal treatments increased nutrients related to chloroplast biosynthesis as magnesium, which is the central component of chlorophyll molecule; hence fungal inducers increased the efficiency of chloroplast and their biosynthesis. Similarly, (Elias et al., 2016b) stated that using P-solubilizers along with rock phosphate increased the number and area of tomato leaves, enhanced the photosynthetic pigments content. This increases the carbohydrate pool of leaves, herein, might be led to the improvement of assimilate transported to the sink organs, thereby increasing the wheat yield. This high carbohydrate content may have reflected positively on carbon storage, osmoprotection, osmotic adjustment, ROS quencher, membrane stability, macromolecules' and DNA structures' saver, and protective of enzymes and proteins (Chehab et al., 2009; Mahmoud, 2017). Thus, the accumulations of sugars using the fungal applicants have a potential role in building the plant body hence giving advent to growth and development.

The accumulation of N-containing compounds had indirect association with photosynthesis activation, revealing metabolic regulation under different fungal applications. Thus, all these changes could favor crop metabolic activity thereby; increase the photosynthetic materials used for growth, hence positively influence late wheat productivity (grains production). These results indicated that single or consortium fungal application acted as elicitor agents for dry matter production and stimulated protoplasm development. Rady et al. (2019) reported that soluble proteins increment by seed soaking may be linked to the formation of osmotin-like or structural protein, which modify cellular walls. It is worth mentioning that, the heatmap hierarchical clustering vastly recommended the positive association of the applied fungi on the main metabolic products (proteins, carbohydrates, and amino acids), that constitute the backbone of plant body. This association was confirmed for plants received synthetic fertilizers or not. Thus, different fungal association with P-fertilized soils or not could instigate osmotic adjustments and may, either directly or indirectly, affect storage functions, metabolic processes and defense responses of plants. Thus, application of such eco-friendly plant growth promoting and phosphate solubilizing fungi can be a viable alternative to synthetic fertilizers for poor quality soils and potent inducers of the efficacy of synthetic phosphate fertilizers.

The most profound reducing effect on superoxide anion was detected for plants received consortium treatment of *A. niger* + *P. chrysogenum* and P-fertilizers, which greatly went in parallelism with high growth, enhanced photosynthetic pigments content, metabolic up-regulation, and the yield of wheat. Further confirmation was denoted from heatmap hierarchical clustering analysis, which revealed that plant amendment with various inducers displayed strong negative correlation with superoxide anion and hydrogen peroxide in comparison with non-amended plants. Mittler (2017) reported there was reduction in

the content of H_2O_2 and $O_2^{\bullet-}$ when the plants provided with P under high $CaCO_3$ stress thereby protecting common bean plants from oxidative stress. The amount of lipid peroxidation increases in damaged membranes, due to the peroxidation of saturated fatty acids in the phospholipid membrane. In the current study, the application of *A. niger* and/or *P. chrysogenum* kept the membranes in a high degree of integrity compared to the non-fertilized plants. Thus, these data recommend a healthiness of biomembranes of the treated plants, which is one of the main strategies of bioaugmentation of plant growth and development under single or combined application of fungal strains tested. These observations successfully confirmed with heatmap hierarchical clustering analysis, which denoted negative association of lipid peroxidation under the applied myco-inculants and the plant growth. Ca^{+2} is an important cation: It maintains cell membrane stability via modulating membrane linkage in terms of restricting ion leakage, it increases the mineral and amino acids uptake, and it keeps the order of binding sites of the cellular enzymes (Fu et al., 2006). We also found that, the used applicants markedly decreased peroxidation of membrane lipids parallel to increment of Ca content which was also linked positively to plant growth and yield. Enhanced Calcium content in plants in response to fungi suggested that these fungi might be achieved membranes stability via enhancing the cellular Ca content which positively associated with stabilization of phospholipids and proteins of cell membranes (Upadhyaya et al., 2011).

ROS production is a key feature of redox reactions in plants which result from the equilibrium between their generation and the functioning of the antioxidant as ROS-eliminator. Therefore, "redox biology" is a term describes the ROS as being signaling products that mediate and uphold the normal physiological processes of plants (Mittler, 2017). The superoxide dismutase activity (SOD) is of the first safeguarding enzymes that catalyzes detoxification of O_2^- to less toxic H_2O_2. The enhancement in SOD activity, herein, with application of biofertilizers (with/without P-fertilizers) is tightly linked with increasing the protection against negative effects of environmental factors, reflecting oxidative homeostasis and redox biology regulation. Further confirmation was attained from the positive association between SOD and the plant growth under various treatments of biofertilizers and chemical fertilizers, as recorded by heatmap hierarchical clustering analysis. In the same trend, Latef et al. (2016) found that mycorrhizal fungi had a protective effect on tomato plants through stimulating SOD content. Catalase (CAT) is H_2O_2-related antioxidant enzyme, by converting it into water and oxygen in peroxisomes to neutralize its noxious damages and ascorbate peroxidase (APX), which eliminates H_2O_2 and modulates its level in chloroplast, cytoplasm, and mitochondria of plants using ascorbate as a substrate (Sadak et al., 2017; Sallam et al., 2019). On the other hand, POD was found to be not affected by the used fungal applicants. In this respect, Bhattacharyya et al. (2020) reported that application of rhizobacterial treatments to rice triggered CAT and APX activities in their shoots and roots, indicating their possible role in the reduction of ROS effect and mitigation stress consequences. Beside antioxidant enzymes, non-enzymatic ones can impulsive supplied the free radicals with electrons, reducing the oxidative burst in plants' tissue. In the present investigation, culture filtrate or conidial suspension priming to wheat increased the ascorbic acid, α─tocopherol content, supplied or not with P-fertilizers. Such accumulation of both molecules suggested that, the applied fungal inducers were not only effective on the ROS-scavenging enzyme activity, but also elevates the antioxidant molecules biosynthesis. The accumulation of AsA in green parts of the plants is associated with photosynthesis up-regulation (Badejo et al., 2012), protects DNA, proteins, and lipids from oxidative damages (Turck et al., 2017). In harmony with our results, Evelin & Kapoor (2014) reported the increment of ascorbic acid and α-tocopherol of fenugreek when supplied with *Glomus intraradices*. Also, Bhattacharyya et al. (2020) recorded stimulation of tocopherol content of rice plants in response to rhizobacterial isolates. Arabidopsis plants with high levels of α-tocopherol instigated higher water content, elevated photosynthesis rate, and lower oxidative stress as evidenced by less MDA content and retarded senescence of leaves (Espinoza et al., 2013). In conformity, Sharma et al. (2014) found described that α-tocopherol implicated in lowering lipid peroxidation by minimizing MDA level and thus

hindering the cell membranes more protected. The data of heatmap hierarchical clustering analysis witnessed on the positive correlation of growth traits and low molecular weight of non-enzymatic antioxidants as α-tocopherol and ascorbic acid.

Another defense technic stimulated in wheat plants by the applied rhizospheric fungi was the secondary metabolites up-regulation in terms of phenolic compounds. Phenolic compounds have been found to be strikingly exacerbated whatever the mode of fungal application compared to their negative control. Phenolics have been assisted in membranes up-regulation via limiting their fluidity, causing them less permeable to free radicals, hence lessening lipid peroxidation (Vogt, 2010). It has been reported that, phenolic compounds have multifunction's associated with antioxidant properties and buffering free oxygen radicals (Sallam et al., 2019). This may be partially accounted for lowering the lipid peroxidation in plants treated by culture filtrate or conidial suspension of rhizospheric fungi hence higher membrane integrity compared to control plants. Consequently, fungal application increased ROS-scavenging enzyme activity, non-enzymatic antioxidants, and phenolic compounds to stabilize ROS molecules at beneficial level, leading to a protection of chlorophyll pigments, which could finally maintain higher development of grain yield in wheat. Similar to our obtained results, *Azotobacter chroococcum* and *Glomus fasciculatum* improved growth and phenolic contents on various plants (Baslam et al., 2011; Teixeira da Silva & Egamberdieva, 2013). Khalid et al. (2017) found that combine application of *Glomus mosseae* and *G. fasciculatum* increased phenolics and flavonoids contents of *Spinacia oleracea* L. Bhattacharyya et al. (2020) recorded stimulation of polyphenolic content of rice plants in response to rhizospheric organism. Furthermore, the progressive accumulation of phenolic compounds could be attributed to enormous increment of PAL activity. This enzyme activates phenylpropanoid pathway which is precursor to a wide range of phenolic compounds, such as flavonoids and anthocyanins (Vogt, 2010). This is also documented by heatmap analysis, which displayed a stronger positive association between growth traits, phenolics and PAL activity in comparison with control plants, recommending the positive contribution of both traits in enhancing dry weight of the wheat plants under rhizospheric fungal application. This was also observed during the application of rhizobacterial treatments that triggered PAL activity of rice plants **(**Bhattacharyya et al., 2020).

By collecting the net advantages of the applied fungal biofertilizers on the studied traits in the presence or absence of P-fertilizers on wheat, it was found that wheat plant yield (spike and grain weights) and other related attributes (spike length and the number of grains per a spike) were significantly enhanced. Similarly, phosphate solubilizing fungi under controlled conditions could increase wheat and common bean grain yields under chemical fertilizers (Steiner et al., 2016). Regarding to the mode of fungal inoculation, culture filtrate of both fungal strains was markedly outperformed on spore suspension in improving growth parameters and plant yield. This could be due to that the culture filtrate had the mixture of fungal inducers and the spores of grown fungi as well as the fungal extracellular metabolites compared to only conidial suspension. These highest values of yield attributes in response to fungal products in the current study could be attributed to improving high content and uptake of nutrients (particularly P) in the soil interpreted the better growth, development, physiological status, and yield of wheat plants. The overall mechanism of culture filtrate or conidial suspension of wheat grains grown under different treatments was summarized in Fig. 10.

Conclusion

Gathering together, the improvement of yield attributes as spike length, number of spikelet/plant, and grains yield/plants were a net result of up-regulation of vegetative, growth, P-related traits, photosynthesis, primary and secondary metabolites, antioxidants, and other nutrients recorded with the application of single or dual inoculation of the tested fungal strains, in the presence or absence of P-fertilizers. Thus, the application of P-solubilizing fungi is highly advocated as a safe, low cost, and sustainable way for improving growth, development, and crop yield, under the studied experimental conditions. Further work should be conducted using the same experimental condition under stress conditions.

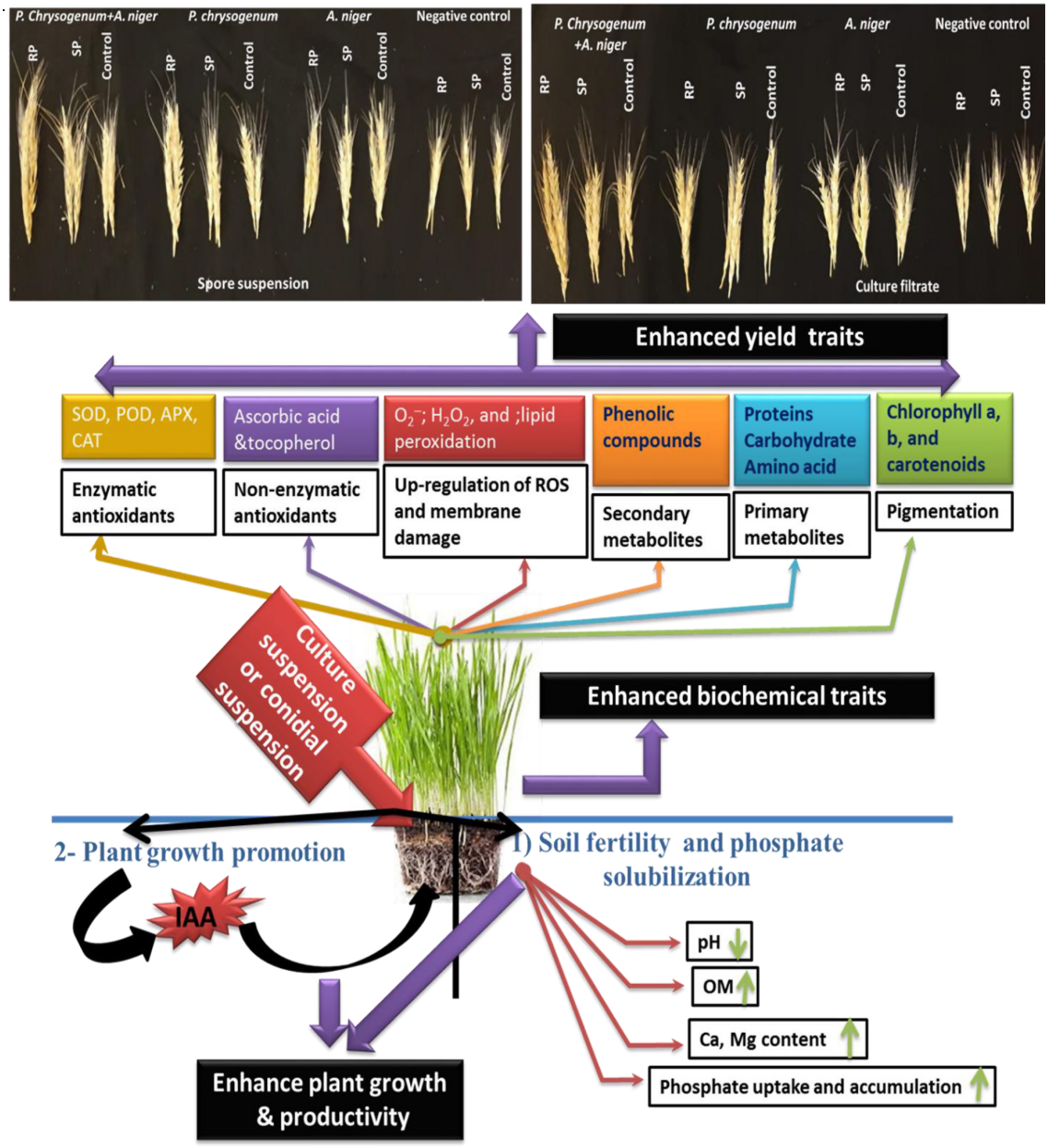

Fig. 10. The overall mechanism of culture filtrate or conidial suspension of wheat grains grown under different treatments. (a) Non fertilized soil and fertilized soil using chemical fertilizers (rock phosphate and superphosphate) and biofertlizers *(Penicillium chrysogenum or Aspergillus niger)* inducers and (b) Non fertilized soil and fertilized soil using chemical fertilizers (rock phosphate and superphosphate) and biofertlizers (mixed inducers of *Penicillium chrysogenum* and *Aspergillus niger).*

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Gawad Hussein, Arwa A.M. El-Khatib and Mona F.A. Dawood; supervision, Mona F.A. Dawood, Nemmat Abdel-Gawad Hussein, Mady A. Ismail and Marwa M. Ragaey. All authors have read and agreed to the published version of the manuscript.

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تحسين الإنبات والكفاءة الفوسفاتية ومضادات الاكسدة والمنتجات الايضية والمحصول لنبات القمح باستخدام *Aspergillus niger* و*Penicillium chrysogenum*

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زيادة كفاءة الأسمدة الفوسفاتية بواسطة الأسمدة الحيوية هدف مثير للاهتمام كطريقة لتقوية الأسمدة الفوسفاتية.

اشتملت الدراسة الحالية على استخدام رشح المستعمرات الفطريه أو معلق الجراثيم الفطريه لفطرين معزولين من التربه **المحيطة بالجذور** في محلول إنبات نباتات القمح أو الأواني المملوءة بالتربة المخصبة بالسوبر فوسفات أو الفوسفات الصخري.

تم تحديد الفطريات التربه المحيطه بالجذور شكليًا ووراثيًا على أنه *Penicillium chrysogenum* (AUMC 14100) و *Aspergillus niger* (AUMC 14260) القادران على إنتاج Indole-acetic-acid وإذابة الفوسفات. عززت اضافه خليط من رشح المستعمرات الفطريه أو معلق الجراثيم الفطريه من السلالات المعزوله او بشكل منفصل على محلول الإنبات نسبة الإنبات ومؤشر النشاط لنباتات القمح. بالنسبة للتجربة الحقليه ، فإن استخدام رشح المستعمرات الفطريه أو معلق الجراثيم الفطريه يبدأ تأثيرهما الإيجابي على النبات بطريقتين: أ) تعزيز خصائص التربة (زيادة المواد العضوية، تقليل الأس الهيدروجيني، إثراء التربة بمعدلات عالية من الفوسفات والكالسيوم والمغنيسيوم القابل للذوبان) ؛ ب) تحفيز النمو والوضع البيوكيميائي ومحتوى المغذيات وإنتاجية نباتات القمح. الأسمدة الحيوية حفزت من الكلوروفيل، مركبات الايض الأولي، الفينولات، حمض الأسكوربيك، ومحتويات توكوفيرول. لم يتسبب استخدام رشح المستعمرات الفطريه و/أو معلق الجراثيم الفطريه في حدوث تلف بالأكسده على القمح و تم تقليل فوق اكسيد الهيدروجين وأكسدة الدهون بسبب تنشيط العديد من الإنزيمات المضادة للأكسدة. علاوة على ذلك، تعمل أنسجة جذر والمجموع الخضري للقمح المعالج بمحتويات عالية من الكالسيوم والمغنيسيوم والفوسفات على تحسين الصفات المرتبطة بالفوسفات. وقد انعكس كل هذا التحسن المنظم في ظل استخدام الأسمدة الحيوية على إنتاجية عالية من نباتات القمح.

أوصت الدراسة باستخدام رشح المستعمرات الفطريه جنبًا إلى جنب مع الأسمدة الكيميائيه لرفع كفاءتها وتعزيز نمو النبات.