

Physicochemical Characterization of Agricultural Run-off and Groundwater Inoculated by *Trichoderma asperellum* and its Effect on Anti-oxidative Enzymes Production by Irrigated *Trifolium alexandrinum* L.

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Water safety and security has become a great problem in recent years, as the shortage of water resources in most countries is becoming an increasingly serious issue. Therefore, searching for new water sources or treating contaminated water has become a major requirement to covering the shortage of fresh water. The current study evaluated the impact of *Trichoderma asperellum* on the physicochemical characterization of agricultural run-off (AR) compared with groundwater (GW). Chemical oxygen demand (COD), biological oxygen demand (BOD₅), ammonia, total nitrogen (TN), and total dissolved salts (TDS) of AR were higher than that of GW with 90, 90.2, 91.9, 78, and 61.6%, respectively. The addition of *T. asperellum* to AR caused the COD, BOD₅, ammonia, TN, and TDS to decrease 47.2, 39.2, 16.2, 12.9, and 4.8%, respectively. Contents of metals in AR or GW were decreased by *T. asperellum*. Microbial analysis of soil irrigated by AR in contrast to irrigated by GW showed the presence of a high number of bacteria (32×10^6 CFU), actinomycetes (18×10^4 CFU), and fungi (13×10^4 CFU). *Trifolium alexandrinum* growth and its antioxidant enzymes activities (catalase, superoxide dismutase, and ascorbate peroxidase) were enhanced by AR inoculated by *T. asperellum* compared with un-inoculated AR or GW.

Keywords: *Trichoderma asperellum*; Agricultural run-off; Oxidative enzymes; Plant growth

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INTRODUCTION

In recent decades, treatment of polluted water either sewage, industrial, or agricultural has attracted attention of researchers and governorates of all countries to compensate for the perceived shortage of fresh water not only for human uses but for agricultural and industrial application utilization.

The surface run-off from farmland outflow, namely agricultural runoff (AR), contains high amounts of pollutants such as phosphorus compounds, nitrates, ammonium, heavy metals, besides persistent organic pollutants (Wang *et al.* 2018). AR and sewage water (SW) have become available in large quantities due to the increase in population and the rational and intensive use of fresh water. Therefore, these are being considered as

alternative sources for irrigation of plants. However, according to numerous literature on the analysis of plant nutrients, AR and other wastewater may contain poisonous organic compounds with highly dangerous impacts on human and animal health. Moreover, municipal wastewater contains high numbers of microorganisms if has not been disinfected or treated by appropriate methods. To avoid the potential toxicity and serious risks of municipal wastewater, crop contamination with pathogens, and intestinal helminths, municipal wastewater must be used for irrigation (Aiello *et al.* 2007; Zavadil 2009). Earlier studies have compared freshwater (FW) or groundwater (GW) with reclaimed water (RW). Higher salts, minerals, such as N, P, and K, and organic nutrients, organic pollutants, heavy metals, and dissolved organic matter (DOM), beside the presence of high content of microbes were detected in RW.

Surprisingly, some studies mentioned the environmental and economic benefits of RW, while others mentioned many defects. For example, of the economic benefits, Singh *et al.* (2012) reported that irrigation with RW minimizes the use of FW or GW, reuses nutrients in RW, and enhances crop productivity. Other scholars' studies (Lado and Ben-Hur 2010; Xue *et al.* 2012) highlight some problems resulting from irrigation by RW. These includes increased salinity of soil, soil greenhouse gas emission, heavy metals, and xenobiotic organic molecules in crops, minimized soil saturated hydraulic conductivity, and contaminated GW. Other studies (Reyes-Solis *et al.* 2003; Bedbabis *et al.* 2010) have shown that salinity, greenhouse gas emission, microbes, and heavy metals were not affected by RW irrigation.

When facing the shortage of clear water resources, municipal wastewater is an essential alternative source to be used for plant irrigation to make up for the shortage of water. Although some contents of municipal wastewater contribute for plant nutrients, the water may contain various potentially toxic elements and organic matters with highly harmful effects on human and animal health. If wastewater is not treated or disinfected, this may promote the growth of microbes, especially pathogens. Therefore, the utilization of un-treated or un-disinfected municipal wastewater for the irrigation of crops is associated with a number of risks. In the current decade, RW has been applied for plant irrigation in many countries but at a secondary treatment stage that was characterized by decreasing contents of salts, xenobiotic organic materials, and heavy metals (Gomez *et al.* 2013). Other benefits of RW application were observed as a result decomposition of organic compounds in municipal wastewater in the agricultural soils; this can increase the population of saprophytic microorganisms as well as fungi such as *Penicillium* spp., *Trichoderma* spp., and *Aspergillus* spp. (Manici *et al.* 2004). Some of these fungi can act as antagonists of soil-borne phyto-pathogens and act as bio-fertilizers as an alternative to chemical fertilizers.

Numerous fungi associated to *Trichoderma*, *Penicillium*, *Aspergillus*, *Spicaria*, *Acremonium*, *Trichothecium*, *Chrysosporium*, and *Hyaloflorae* have been isolated from different sources of polluted water such as wastewater and sewage sludge (Fakhrul-Razi *et al.* 2002). From earlier literature, these fungi and others were applied for treatment of wastewater. According to Behnami *et al.* (2018), fungi were as efficient as bacteria in the elimination of organic pollutants from wastewater. From twenty-two fungi, seven fungi were found to have potential for nitrogen, ammonium and phosphorus treatment of municipal wastewater (Guest and Smith 2007).

Removing of heavy metals from contaminated water was performed recently using *T. brevicompactum* (Zhang *et al.* 2020) and previously by other fungi (Abdel-Razek *et al.* 2009a,b). The presence of multiple functional groups such as amide, hydroxyl, and

carbonyl in fungi play a critical role during bioremediation of heavy metals from water sources (Manna *et al.* 2020). In this sense, the application of AR in irrigation of plants without any treatment is often associated with some health risk. The current study was performed to compare the impact of *Trichoderma asperellum* on physicochemical characteristics of AR and GW, in addition to its impact on oxidative enzyme production by *Trifolium alexandrinum*.

EXPERIMENTAL

Materials

AR and GW Collections

Samples of AR were collected in sterile bottles from an agricultural drainage channel (30° 62'8014" N, 116° 31' 070334" E) (Fig. 1) in Ashmun, Monufia Governorate, Egypt. Over the past 10 years, these channels also have become a place to collect municipal water. The GW was collected from a pump drawing from a depth of 18 m at the same position of AR collection.

Cultures used

Trichoderma asperellum was provided by professor Tarek M. Abdel-Ghany (Abdel-Ghany *et al.* 2018). The species was identified based on DNA's ITS regions, and it was used for co-inoculant in AR and GW for 10 days, followed by physicochemical characterization analysis.

Analytical techniques for physicochemical characterization of AR and GW

All physicochemical analysis, such as pH, dissolved oxygen (DO), chemical oxygen demand (COD), biological oxygen demand (BOD₅), total phosphorous (TP), ammonia nitrogen (NH₄), nitrate (NO₃), total nitrogen (TN), total suspended solids (TSS), and total dissolved salts (TDS) in AR and GW were carried out in accordance with the American Public Health Association (APHA) (Rice *et al.* 2017; Su *et al.* 2019, 2020). Gas chromatography (GC), (Dani, Rome, Italy) using flame ionized detector (FID), column length (L): 60 m, with limit of detection of 1 ug.L⁻¹ was used for analyses of hydrocarbon pollutant determination. An ICE 3000 Series AA spectrometer (Thermo Fisher Scientific China Co., Ltd., Shanghai, China) atomic absorption spectrophotometer (AAS) was used for metal detection. A pH meter (pH-700, EUTECH, Singapore) was used for measuring pH value, with a range of 0.00 to 14.00 and an accuracy of ± 0.02. Millipore (Elix) UV-Milli-Q Advantage A 10 System (Molsheim, France) with double-distilled water (DDW) from Millipore Instrument was used throughout the study for standard preparation.

Seed germination and growth parameters

Twenty five seeds of *Trifolium alexandrinum* (provided from Pure Seeds Company for Trade & Export of Cereals & Agricultural Crops, Egypt) were sterilized by mercuric chloride (0.1% w/v) up to 4 min, followed by washing in sterile distilled water. Germination of seeds was carried on sterile filter paper in plastic dishes (9 cm), the seeds were irrigated by AR and GW inoculated by *T. asperellum*. At 15 days, seed germination %, length, fresh weight, and antioxidant enzymes were recorded. Pure Seeds Co. for Trade & Export of Cereals & Agricultural Crops, Egypt.

Antioxidant enzymes of T. alexandrinum

The enzymes activities were measured in 0.2 g of shoot system (leaves and stems) of *T. alexandrinum* that was ground in 10 mL of 50 mM phosphate buffer pH (7.8) followed by centrifugation at $13000 \times g$ for 15 min. The obtained supernatant was used for the assay of enzymes activity.

Catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD)

According to Havar and McHale (1987), the activity of CAT was assayed using phosphate buffer (100 mmol.L^{-1} , 7.0 pH), H_2O_2 (12.5 mmol.L^{-1}) and 0.1 mL of enzyme extract as a reaction solution (3 mL). At 240 nm absorbance, a reduction in activity of CAT was estimated, which was due to H_2O_2 consumption. The CAT activity (one unit) is defined as a change in absorbance of 0.01 unit/min. According to Nakano and Asada (1981), the activity of APX was assayed using phosphate buffer (100 mmol.L^{-1} , pH 7), 0.5 mmol.L^{-1} ascorbic acid, H_2O_2 (0.1 mmol.L^{-1}), and 0.1 mL enzyme extract as a reaction solution (3 mL) depending on the oxidation of ascorbate. At 290 nm the absorbance was estimated for recording APX activity that was due to the peroxidation of H_2O_2 and guaiacol as an electron donor. The activity was expressed as unit/min. According to Giannopolitis and Ries (1977), the activity of SOD was assayed through measuring the inhibition rate of nitroblue-tetrazolium (NBT) photo-reduction. Where the supernatant of the enzyme was added to the reaction mixture containing of potassium phosphate (50 mmol.L^{-1} , pH 7.8), $75 \mu\text{mol L}^{-1}$ NBT, 14 mmol L^{-1} , methionine, $0.1 \mu\text{mol.L}^{-1}$ ethylene di-amine tetraacetic acid (EDTA), and $2 \mu\text{mol.L}^{-1}$ riboflavin. Then, reaction mixture was exposed to 20-W fluorescent lamp for 6 min, followed by measuring the absorbance at 560 nm using a UV-visible spectrophotometer. The SOD activity (one unit) was defined by 50% photochemical inhibition reduction of NBT.

Microflora of soil irrigated by AR and GW

A plate-counting method was applied for determining fungi, bacteria, and actinomycetes represented by the number of colony forming units (CFU). One gram was taken from 20 cm depth of each fresh soil irrigated by AR and GW was suspended in 9 mL of saline solution (0.9% NaCl). Series dilution of soil suspension ranged from 10^{-1} to 10^7 and was prepared under sterile conditions to avoid the microbial contamination from air or from any other sources. Bacteria, actinomycetes, and fungi were cultivated on nutrient, starch nitrate, Czapek-Dox agar media, and incubated at 2, 3, and 5 days, respectively, at 37°C for bacteria and actinomycetes, at 25°C for fungi. Then, the average CFU was recorded.

RESULTS and DISCUSSION

Physicochemical Characterization of AR and GW

Scarcity of fresh water in numerous countries has increased rapidly in the last decade with increased population and has become one of the main challenges for humanity. Therefore, evaluation and management of sewage water for its re-consumption is an attractive option currently. Water scarcity is one of the main problems for farmers, which tempts them to use wastewater for crop irrigation (Al-Tabbal *et al.* 2016).

Results of the physicochemical parameters of treated and untreated GW and AR by *T. asperellum* are depicted (Table 1). The results of the analysis revealed that *T. asperellum* reduced all detected chemical parameters. There were big differences among chemical contents of GW and AR. The concentration of COD, BOD₅, TP, NH₄, TN, and DO was 18, 10, 0.12, 0.3, 1.9, and 4.15 mg.L⁻¹ in GW (control) while in AR (control) it was 180, 102, 0.72, 3.7, 8.64, and 2.22 mg.L⁻¹, respectively. The higher values of the physicochemical parameters observed in AR indicate higher pollution level.

By treating with *T. asperellum*, a negligible reduction in the GW contents was observed, while an effective reduction was observed in the contents of AR. For example, COD and BOD₅ were reduced from 180 to 95 and from 102 to 62 when reduction reached to 47.22% and 39.22%, respectively. Applications of *T. asperellum* within agriculture was elucidated in many studies as biocontrol agent or as biofertilizer. As previously mentioned by Hultberg and Bodin (2019), treatment by *T. asperellum* achieved a significantly greater COD reduction during real brewery waste streams treatment. Zhang *et al.* (2008) reported a more positive result using the related species *T. viride* to treat winery wastewater, where COD reductions in the range of 86 to 91% after 24 h at a cultivation temperature of 30 °C were achieved. In recent study, Narendran *et al.* (2019) concluded that a *Trichoderma* strain was most effective in the of wastewater treatment, due to its high capability for reducing not only COD, but also other nutrients.

The TP was reduced in GW and AR after *T. asperellum* application (Table 1). These fungi demonstrated an ability to accumulate P in poly-P granules. Ye *et al.* (2015) investigated the potential of *Mucor circinelloides* for phosphorus (P) removal from wastewater, the largest consumption efficiency was 40.1% for P and 7.08% for cellular P content.

Reduction of TSS in GW and AR was observed as a result of *T. asperellum* from 13 mg.L⁻¹ before treatment to 10 mg.L⁻¹ and 115 mg.L⁻¹ before treatment to 75 mg.L⁻¹, respectively. The pH of the GW was 7.79, indicating that it was more alkaline than AR (pH 7.33) but *T. asperellum* lowered alkalinity feature of GW (pH 7.61) unlike sewage water (pH became 7.36) (Table 1). A pH 6.5 to 8 is considered standard for irrigation according to the food and agriculture organization (FAO). From the previous study, *T. harzianum* showed efficient degrading capabilities by degrading the contaminants as they use it for their growth and reproduction (Kashif and Monowar 2017).



Fig. 1. AR collection site. Arrows are pointing to the samples were collected in a row from a line that crossed the channel at distance 2 m.

Table 1. Physicochemical Parameters of GW and AR Inoculated by *T. asperellum*

Physicochemical Parameters	Type of Water			
	GW (Control)	GW (Inoculated)	AR (Control)	AR (Inoculated)
COD (mg.L ⁻¹)	18	16	180	95
BOD ₅ (mg.L ⁻¹)	10	8	102	62
TP (mg.L ⁻¹)	0.12	0.09	0.72	0.53
NH ₄ (mg.L ⁻¹)	0.3	0.1	3.7	3.1
NO ₃ (mg.L ⁻¹)	0.0	0.0	0.0	0.0
TN (mg.L ⁻¹)	1.9	1.3	8.64	7.52
Oils and Greases (mg.L ⁻¹)	0.003	0.004	0.008	0.006
DO (mg.L ⁻¹)	4.15	4.2	2.22	2.20
TSS (mg.L ⁻¹)	13	10	115	75
TDS (mg.L ⁻¹)	480	477	1250	1190
pH	7.79	7.61	7.33	7.36

The current findings indicated that *T. asperellum* was effective in removing or decreasing some detected metal (Table 2). For example, lead was reduced to 57.14% and 37.50% in GW and AR, respectively. Copper completely disappeared as a result of *T. asperellum* treatment in AR. The efficacy of *T. asperellum* on copper and lead predict its efficacy on other non-detected serious metal in either GW or AR, such as chromium, arsenic, and mercury, if present in any contaminated water resources. At application level in the current finding, *T. asperellum* provides good advantages; for example, it is non-pathogenic and a non-toxins producer compared to prior studied mycotoxigenic fungi. A similar result was reported earlier (Mohsenzadeh and Shahrokhi 2014; Hoseinzadeh *et al.* 2017), where *T. asperellum* was used for removing metal from wastewater and heavy metal contaminated soils. Heavy metals eliminating from AR or GW inoculated by *T. asperellum* may be due to the presence of the efficacy groups such as carboxylic, hydroxyl, amine, and amide in fungal cells. These explanations were given in a recent study (Manna *et al.* 2020).

Table 2. Metal Analysis of GW and AR Inoculated by *T. asperellum*

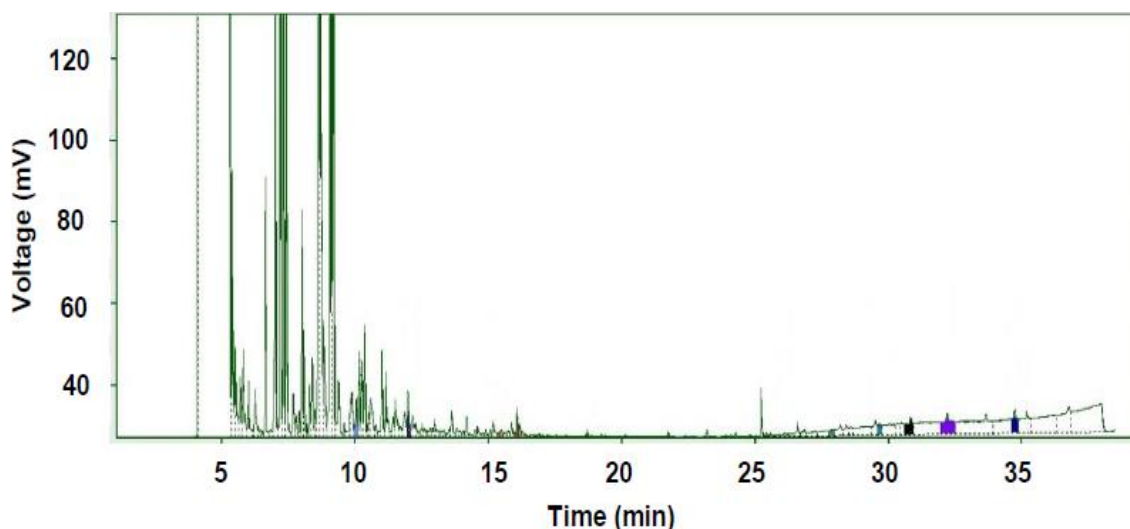
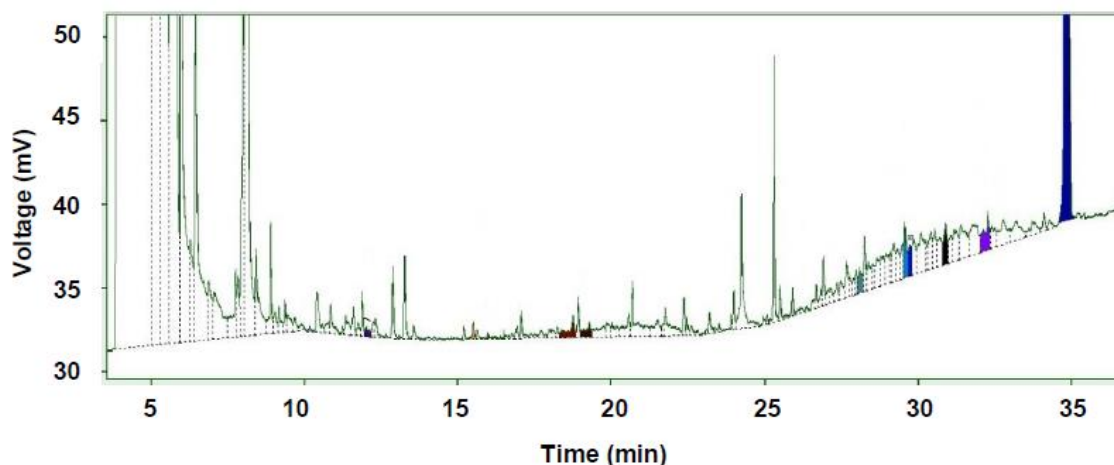
Metal	Metal Concentration (mg.L ⁻¹) of Water			
	GW (Control)	GW (Inoculated)	AR (Control)	AR (Inoculated)
Chromium	0.0	0.0	0.0	0.0
Copper	0.0	0.0	0.002	0.0
Lead	0.007	0.003	0.008	0.005
Cadmium	0.0	0.0	0.0	0.0
Zinc	0.0022	0.0015	0.019	0.013
Arsenic	0.0	0.0	0.0	0.0
Selenium	0.0	0.0	0.0	0.0
Mercury	0.0	0.0	0.0	0.0
Manganese	0.053	0.005	0.003	0.001
Iron	0.065	0.062	0.029	0.022

Analysis of organics of un-inoculated AR (C) and inoculated AR (T) by *T. asperellum* using GC indicated significant removal for the majority of tested compounds after treatment process, as shown in Table 3 and Figs. 2a and 2b.

Table 3. GC Analysis of Hydrocarbons (mg.L⁻¹) in Inoculated and Un-inoculated AR with *T. asperellum*

Compound Name	MF	MW	Inoculated		Un-inoculated		Removal Efficiency (%)
			Concentration	RT	Concentration	RT	
Naphthalene	C ₁₀ H ₈	128.17	28.09	10.06	0.80	10.16	97.15
Acenaphthalene	C ₁₂ H ₈	152.10	35.34	11.99	1.81	12.04	94.88
1-Methyl Naphthalene	C ₁₁ H ₁₀	142.20	3.89	15.37	0.00	--	100.0
2-Methyl Naphthalene	C ₁₁ H ₁₀	142.20	2.83	15.50	1.68	15.53	40.64
Acenaphthene	C ₁₂ H ₁₀	154.21	11.15	16.09	0.51	16.00	95.43
Fluorene	C ₁₃ H ₁₀	166.22	0.28	18.71	0.23	18.78	17.86
Pyrene	C ₁₆ H ₁₀	202.25	0.74	24.80	0.00	--	100.0
Benzo(a)pyrene	C ₂₀ H ₁₂	252.30	1.30	30.84	0.74	30.90	43.08
Di-benzo(a)anthracene	C ₂₂ H ₁₄	278.30	4.87	32.21	2.21	32.28	54.62

MF- Molecular formula; MW- Molecular weight; RT- Retention time

**Fig. 2a.** GC chromatogram of un-inoculated AR by *T. asperellum***Fig. 2b.** GC chromatogram of inoculated AR by *T. asperellum*

The results show 100% removal efficiency for 1-methyl naphthalene and pyrene; 97.2% for naphthalene; 95.4% for acenaphthene; 94.9% for acenaphthalene; 54.6% for di-benzo(a)anthracene; 43.1% for benzo(a)pyrene; 40.6% for 2-methylnaphthalene; and 17.9% for fluorene. The removal efficiency could be due to the biosorption and bioaccumulation capability of *T. asperellum* and the molecular weight of the organic compounds (Mahmoud *et al.* 2019).

Microbial Analysis of Soil Irrigated by GW and AR

Analyzing the microflora of soil irrigated by AR and GW reflected variation in the total number of fungi, bacteria, and actinomycetes (Fig. 3), where the soil irrigated by AR was characterized by the presence of high number of bacteria (32×10^6 CFU//g), actinomycetes (18×10^4 CFU//g), and fungi (13×10^4 CFU/g) compared with the soil irrigated by GW. High TSS of AR may positively affect soil microflora by amending the soil with nutrients from different carbon sources. According to a previous report (Zhang *et al.* 2009; Disciglio *et al.* 2015), the counts of bacteria, actinomycetes, and fungi in soils irrigated by reclaimed-water were highest compared to tap-water irrigated soil, particularly bacteria. Although Yuan *et al.* (2003) found that no difference in the quantity of bacteria, actinomycetes, and fungi isolated from irrigated soils by AR or GW. Research is still being conducted on the effect of wastewater and GW on soil microbial population, where Xu *et al.* (2020) reported that wastewater irrigation changed the bacterial community compared to GW irrigation. Finally, in this section further studies must be carried out to recognize the relationship between microbial species and sewage irrigation soil.

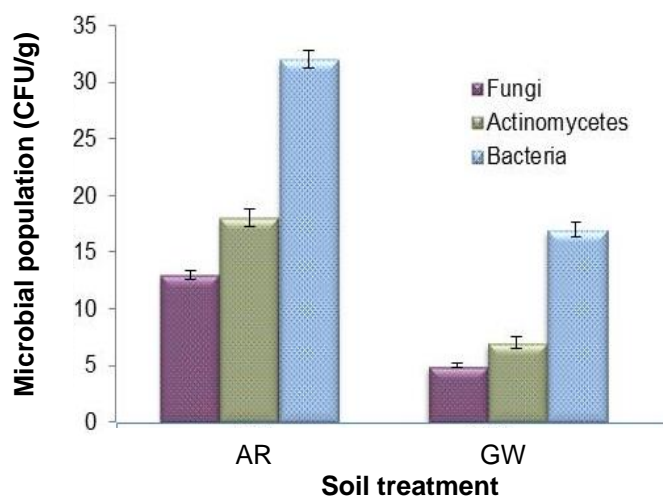


Fig. 3. Microbial population of soil irrigated by AR and GW, fungi (CFU x 10⁴), actinomycetes (CFU x 10⁴), and bacteria (CFU x 10⁶)

Oxidative Enzymes of *Trifolium alexandrinum* Irrigated by GW and AR Inoculated by *T. asperellum*

Decreased plant growth can be induced by disruption of photosynthesis or mineral nutrient imbalance, which causes a decline in yield (Kalaji *et al.* 2016). From Fig. 4, it is clear that there was a difference among plant growth, biomass weight, and plant height irrigated by AR and GW. Inoculated AR by *T. asperellum* induced seed germination with 97.7% followed by GW inoculated by *T. asperellum* (96%), followed by un-inoculated AR (89.3%) and un-inoculated GW (87%).

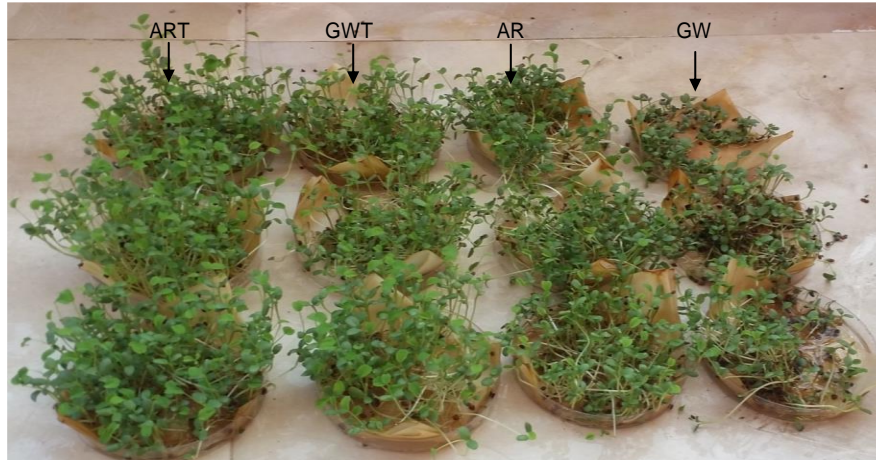


Fig. 4. *T. alexandrinum* irrigated by GW and AR inoculated and un-inoculated by *T. asperellum*

Irrigation with GW without *T. asperellum* treatment attained the lowest means of shoot length (4.5 cm) and biomass (0.95 g) compared with inoculated AR or GW inoculated by *T. asperellum* (Fig. 5). It is clear from the mentioned results that the AR contains at least the necessary nutrients (organic and inorganic substances) for promotion of plant growth compared to GW water. At the same time, *T. asperellum* may excrete substances that induce plant growth. There was agreement with Wafaa *et al.* (2018), who noted that irrigation by AR (50%) improved biomass, plant height, and dry weight of the maize and barley seedlings compared with the control. Wastewater beneficial effects on plant yields were also documented recently (Neamatallah 2018; Manjunatha *et al.* 2020). Khadka and Uphoff (2019) reported the vital role of *Trichoderma* spp. inoculation for plant development, enhanced nutrient uptake, solubilization, sequestration of inorganic nutrients, in addition to the alleviation of biotic and abiotic stresses. The present findings are also in agreement with previous reports (Doni *et al.* 2014) that revealed the role of *T. asperellum* in promoting and enhancing seedling elongation and physiological response to stress condition. Activity of the antioxidant enzymes CAT, SOD, and APX increased (4.25, 1.53, and 8.25 U mg⁻¹, respectively) in plant irrigated with AR with the addition of *T. asperellum* while it reduced to 3.25, 1.12, and 6.56 U mg⁻¹, respectively, when irrigated by AR without inoculation by *T. asperellum* (Fig. 5). No remarkable changes were observed in oxidative enzymes activities in plants irrigated with or without *T. asperellum* (Fig. 5). Indirect effects of *T. asperellum* may act through enhanced production of oxidative enzymes that may enhance the growth in the existence of organic pollutants. The current finding partially agrees with other studies (Joner and Leyval 2001) that reported *Glomus mosseae* induced oxidative enzymes in white clover and ryegrass grown in soil contaminated by chrysene and dibenz(a,h)anthracene. Generally, any pollutants promote oxidative stress through highest productivity of reactive oxygen species (ROS). Oxidative damage of building molecules for living things may be due to ROS. Antioxidant enzymes play an important role against ROS, particularly SOD. Doni *et al.* (2015) observed that irrigation by AR enhanced the germination rate, growth, vigour index, and chlorophyll content of rice. Although the plant growth in the current study is not affected negatively by irrigation with AR, the authors expect that with the use of AR for a long period of time, it may affect the quality of the agricultural soil and hence the plants. In addition, it is possible to induce accumulation of some AR components inside the plant. So, the authors suggest that AR can be applied to irrigate woody trees, not vegetables or crops.

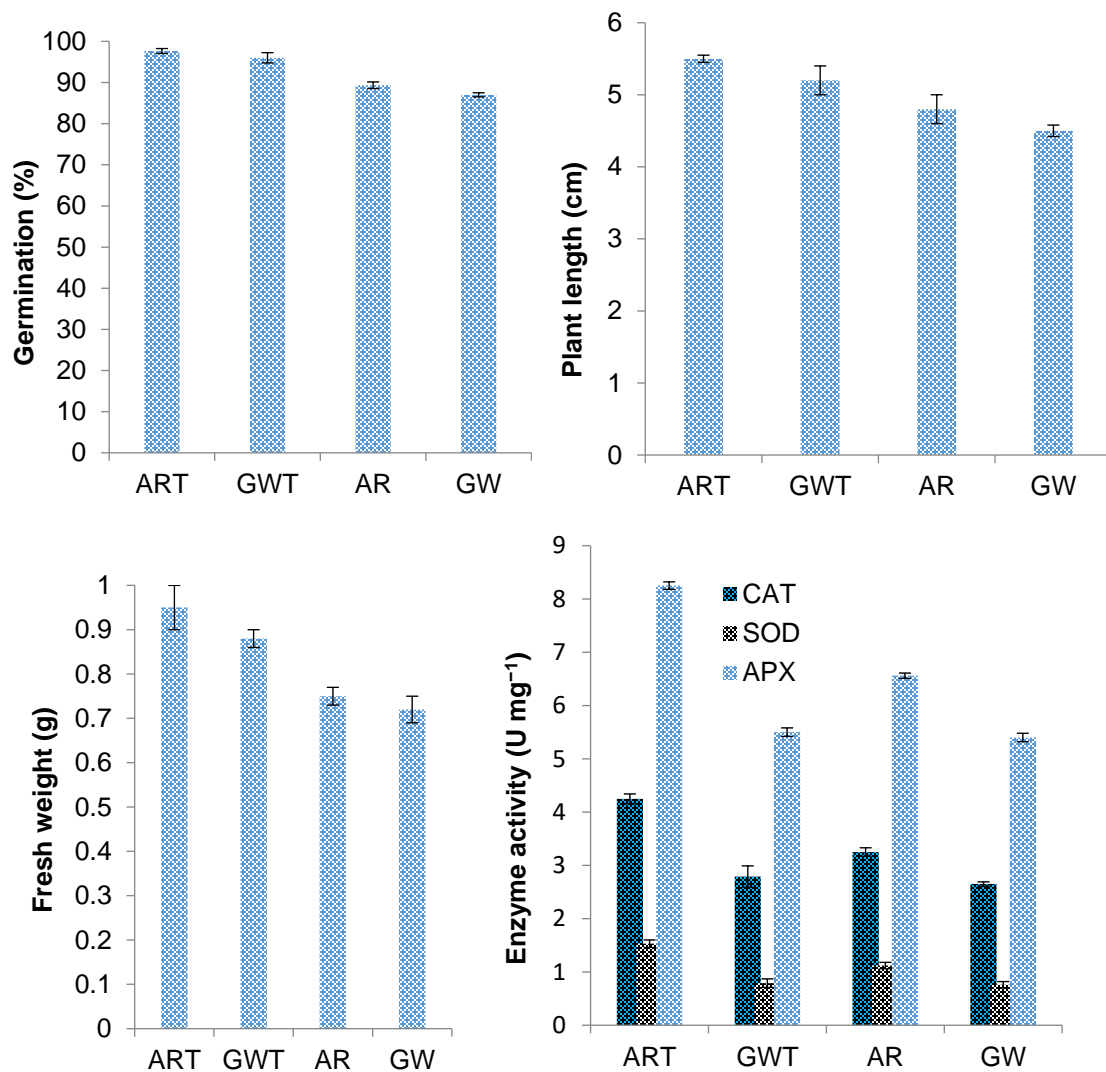


Fig. 5. Growth parameters and oxidative enzymes of *T. alexandrinum* irrigated by GWT and ART inoculated, and by AR and GW un-inoculated by *T. asperellum*

CONCLUSIONS

1. The obtained results suggest that *T. asperellum* may minimize the pollutant contents of agricultural runoff (AR) water.
2. *T. asperellum* enhanced the growth of plants irrigated by AR. Soil irrigated by AR was characterized by the presence of a high content of microorganisms.
3. Irrigation by AR inoculated by *T. asperellum* increased activities of the antioxidant enzymes CAT, SOD, and APX with 23.53%, 26.8%, and 20.5%, respectively, in *T. alexandrinum* compared with the irrigation by un-inoculated AR.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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