

Salicylic Acid Induced Physiological Responses of Microchaete Sp. NCCU-342 Under PQ Stress

Haleema Naaz

Jamia Millia Islamia

Bushra Afzal

Jamia Millia Islamia

Neha Sami

Jamia Millia Islamia

Durdana Yasin

Jamia Millia Islamia

Nida Jamil Khan

Jamia Millia Islamia

Tasneem Fatma (✉ fatma_cbl@yahoo.com)

Jamia Millia Islamia

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Abstract

Weeds are the worst category of agricultural pests as they compete with crops for water, nutrients, and light, grow faster than crops and eventually reduces the crop productivity thereby increasing the production cost. Herbicide has been used as a management tool to control weeds, by modifying their physiological activities like reducing photosynthesis, increasing protease activity and free radicals etc. The application of non-selected herbicides kills not only the weeds but also non-target organisms including the main crop. Herbicides like paraquat have been used in rice fields but its toxic effects on other organisms is at par. Salicylic acid, a plant growth regulator, is known to regulate oxidative stress in plants subjected to unfavourable environmental conditions. Given this, the present study was designed to study the effect of salicylic acid in *Microchaete sp.* NCCU-342 exposed to paraquat. The results obtained demonstrated that the paraquat toxicity elevated MDA and H₂O₂ levels, indicating free radical formation. However, supplementation of salicylic acid in the culture medium reduced the paraquat toxicity. The content of MDA and H₂O₂ in presence of salicylic acid was similar to the control. It enhances phenolic compound (29.07%) and phytohormones content (IAA-2.96%, SA-96.59%) and regulate the redox state of the cells. A significant change (recorded in percentage) was observed in the antioxidant enzyme activities including SOD (121.62 %), CAT (30.38 %), APX (74.38 %), GPX (127 %) and GR (141%) in presence of paraquat and after supplementation of salicylic acid. Antioxidant assay confirmed the change in antioxidants in form of phenolic compounds. Salicylic acid supplementation also enhanced the antibacterial activity of cyanobacteria, highlighting that it induces resistance against biotic stress as well as the abiotic stress. This work provides evidence for the ability of salicylic acid to alleviate the paraquat-induced toxicity, maintain redox state and resist the cell against both biotic and abiotic stress.

Introduction

Herbicide through increase crop yield but their residual toxic impact on both selective and non-selective target, in long run disrupts the ecological balance between soil, crop and microflora¹. Paraquat (PQ) is a very well-known non-selective herbicide that has been widely used in agriculture fields such as Wheat, Rice, Maize, Cotton, Potato and has shown high toxicity against plants, animals and humans². PQ acts by reducing the production of NADPH in a photosynthetic organism. It is an oxidant that interferes with the electron transfer reaction by accepting the electron from photosystem I and transferring it to molecular oxygen and producing reactive oxygen species (ROS) and reducing the formation of NADPH³. Accumulation of ROS leads to lipid peroxidation and induces membrane damage, breakdown of photosynthetic apparatus, ion leakage, eventually leading to cell death and overall reduction in the crop yield⁴. The antioxidant enzymes are known as scavenger of ROS and their expression facilitates plant survival under stress conditions. Superoxide dismutase (SOD) is known to scavenge superoxide radicals, whereas catalase (CAT) and ascorbate peroxidase (APX) function as a scavenger of hydrogen peroxide (H₂O₂). Herbicide toxicity alters activities of these enzymes by activating protease and the plants succumb to the unfavourable conditions⁵.

Cyanobacteria are the pioneer photosynthetic bacteria that are known to play an important role in sustainable agriculture in rice ecosystem. *Nostoc muscorum*, *Anabaena sp.*, *Calothrix*, *Microchaete sp.* are classified as biofertilizers as they fix nitrogen, release plant growth promoter substances such as phenols, phytohormones, and increase the water holding capacity of soil⁶. Application of PQ in rice field is known to effect cyanobacteria that is used as biofertilizer⁷.

Salicylic acid (SA) is an important signalling molecule released by plants under stressful conditions⁸. It is an endogenous plant hormone that modulates metabolic and physiologic response under oxidative stress⁹ as induced by salinity¹⁰, heavy metal¹¹, drought¹², heat¹³, cold¹⁴ and herbicide³. It has been reported that exogenous application of salicylic acid also reduces the toxicity of herbicide by modifying the antioxidant enzyme activity³. SA is known to control the pesticide effect such as H.A.M-45, Termite kill, Anchor in Green gram¹⁵, Napropamide in rapeseed and mustard plants¹⁶ and Clethodium in maize¹⁷. In barley plant under paraquat toxicity, the treatment of salicylic acid-induced the expression of SOD, CAT, APX, and POD³. Exogenous application of SA is known to reduce the quinclorac (herbicide) toxicity by modulating the antioxidant defense enzymes such as SOD, CAT, APX, GPX and GR¹⁸. SA also elevates the content of non-enzymatic antioxidants such as ascorbate and glutathione which are directly involved in the detoxification of ROS and also play an active role in ascorbate-glutathione cycle to maintain the redox state¹⁹. Glutathione performed the redox couple reaction with nicotinamide adenine dinucleotide phosphate and provides stress resistance to the plants²⁰. Supplementation of SA in wheat seedlings showed the increased content of ascorbate, glutathione and other related enzymes²¹.

To validate our hypothesis that SA helps in maintaining the cellular redox state of cyanobacteria under PQ stress. "Cyanobacteria culture treated with PQ was grown in presence and absence of SA for 96 hours. The endogenous phytohormones (IAA and SA), phenolic content, redox biomarker (GSH and GSSH and its ratio), antioxidant enzymes, DPPH (for free radical scavenging potential) and anti-bacterial activity (for biotic potential) were evaluated as these parameters reflect the tolerant potential of the organism.

Materials And Methods

Culture maintenance. *Microchaete sp.* NCCU-342 were procured from IARI, New Delhi and grown in BG-11 medium without sodium nitrate illuminated with the fluorescent light with 2000 ± 200 lux intensity for 12:12 light/dark cycle under the aseptic culture room⁵⁴.

Growth of *Microchaete sp.* NCCU-342 in presence of PQ and SA. *Microchaete sp.* NCCU-342 was grown in presence of PQ (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 μ M) to determine its tolerance limit. After 96 hours, biomass was washed twice with distilled water, harvested by centrifugation (5000 rpm for 10 minutes) and dried at 60 °C. Growth of the organism was check by the chlorophyll content. Total chlorophyll was analyzed according to the modified protocol of Mackinney, (1942)⁵⁵. Dried biomass was dissolved in 95 % methanol (10 ml) and incubated at 65 °C for 30 minutes in a water bath, centrifuged at 5000 rpm for

10 minutes and absorbance of the supernatant was taken at 650 nm and 660 nm against 95 % methanol as blank. Total chlorophyll was calculated by using the following formula.

$$\text{Chlorophyll content (mg/g)} = (2.55 \times OD_{650nm} + 0.4 \times OD_{665nm}) \times 10^{-2}$$

Tolerance limit of PQ was determined through linear regression ⁵⁶. Further, at 50 % inhibitory concentration, 0.3 mM of SA was supplemented to check the physiological parameters i.e., endogenous phytohormones (IAA and SA), phenolic content, redox biomarkers (GSH and GSSH and its ratio), antioxidant enzymes, DPPH (for free radical scavenging potential) and anti-bacterial activity (for biotic potential).

Determination of Malondialdehyde and hydrogen peroxide (supplementary data). Malondialdehyde (MDA) and hydrogen peroxide was estimated as done in our previous study ⁵⁷.

Determination of total Phenol content. The total phenolic contents were estimated by using protocol of Singleton and Rossi (1965) ⁵⁸. Dried biomass (50 mg) was homogenized in 80% methanol (10 ml). Distilled water (5 ml) and Folin Ciocalteu's reagent (0.5 ml) were added to the methanolic extract (40 μ L) and kept for 5 minutes. Then, 20% sodium carbonate (1.2 ml) was added and the volume of the test sample was adjusted to 10 ml with distilled water and allowed to stand in dark at room temperature for 60 minutes. Absorbance was recorded at 750 nm after the development of intense blue colour. This equation was used to calculate the amount of standard gallic acid with the regression coefficient (r^2) being 0.9666.

$$Y = 0.0018 \times X + 0.0132$$

Determination of endogenous indole acetic acid (IAA) and salicylic acid (SA) content. The protocol of Ehmann (1977) ⁵⁹ was used to determine the IAA content produced. A colorimetric technique was adopted by using Salkowski reagent (one ml of 50 mM ferric chloride dissolved in 35 % perchloric acid). Biomass (50 mg) was centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was collected and filtered by Millipore filter, 0.45 μ m and acidified with 1.0 M hydrochloric acid. Extraction was done by partitioning with ethyl acetate (1:3, v/v; thrice) and evaporated using Rotary evaporator (Buchi type). Dried extract was dissolved in 95% methanol (1 ml). Salkowaski reagent (1 ml) was added to this extracted methanolic solution (0.5 ml) and kept in dark. Development of the red-pink colour indicates the presence of IAA and the absorabnce was taken at 530 nm, against Salkowski reagent as blank. Standard IAA (1 mg/ ml) was prepared as stock solution. It was further diluted with distilled water to obtain different working concentrations (0.5 -5 μ g/ml). The calibration curve was described by the equation given below with the regression coefficient (r^2) being 0.9779. This equation was used to calculate the amount of standard IAA.

$$Y = 0.1423 \times X + 0.0035$$

Endogenous salicylic acid production was also checked. Salicylic acid extraction was done by using the protocol of Raskin et al. (1989) ⁶⁰ and quantification was done by following the protocol of Warriar et al.

(2013)⁶¹. For extraction of endogenous salicylic acid, 50 mg biomass was homogenized with 90% methanol (10 ml) and centrifuged at 5000 rpm. Pellet was dissolved in absolute methanol (10 ml) and centrifuged at 8000 rpm for 15 minutes. The supernatants were evaporated at 60°C and the residue was re-suspended in 5 % trichloroacetic acid (5ml) and centrifuged at 5000 rpm. The supernatant was partitioned twice with double the volume of ethyl acetate⁶⁰. The production of salicylic acid was confirmed by simple colorimetric test. To the ethyl extract (1 ml), alcoholic ferric chloride (1 ml) was added, formation of purple-violet colour indicated the presence of salicylic acid that was measured at 540 nm. Salicylic acid stock solution (1mg/ml) was further diluted with distilled water to obtain different working concentrations (0.01–0.5 µg/ml). Absorbance was taken at 540 nm. The calibration curve was described by the equation given below with regression co-efficient (r^2) being 0.9582. The following equation was used to calculate the amount of standard salicylic acid.

$$Y = 1.9821 \times X + 0.0148$$

Where Y is absorbance at 540 nm and X is concentration of salicylic acid in µg/ml.

Determination of reduced and oxidized glutathione (GSH) and redox state. Glutathione was determined by the method of Anderson, (1985)⁶². Fresh biomass (50 mg) was homogenized in 5 % sulfosalicylic acid and centrifuged at 10,000 rpm for 10 minutes under cold condition. After centrifugation, in 500 µl supernatant was added to 600 µl of phosphate buffer (100 mM, pH 7.0) and DTNB (40 µl) and incubate for 2 minutes at room temperature. After incubation absorbance was read at 412 nm. For oxidized glutathione, the GSH was masked by derivatization with 2-vinylpyridine. Reduced glutathione was calculated by subtracting the value of oxidized glutathione from the total glutathione. The redox state was calculated as the ratio of GSH to oxidized glutathione (GSSG).

Determination of Superoxide dismutase (SOD), Catalase (CAT) and Ascorbate peroxidase (APX) activity. SOD, CAT and APX activity was estimated by the method as followed in our previous study⁵⁷

Determination of Glutathione Reductase (GR) activity. GR activity was estimated by the method of Asgher et al., (2014)⁶³. Fresh biomass (50 mg) was homogenized in phosphate buffer (50 mM, pH 7.5) and centrifuged at 15,000 rpm for 15 minutes at 4°C. Reaction mixture (3 ml) was prepared by mixing 0.5 mM oxidized glutathione, 0.2 mM NADPH in phosphate buffer (25 mM, pH 7.8) and 100 µl of supernatant. GR activity was determined by monitoring the glutathione-dependent oxidation of NADPH at 340 nm against blank of the same reaction mixture except for NADPH. 1U of the enzyme is the amount necessary to decompose 1 µl of NADPH per minute at 25°C and GR activity was calculated by taking ϵ_{340} as $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$.

Determination of Glutathione peroxidase (GPX) activity. GPX activity was estimated by the method of Elia et al., (2003)⁶⁴. Fresh biomass (50 mg) was homogenized in 50 mM phosphate buffer (10 ml) having pH 7.5 and centrifuged at 15,000 rpm for 15 minutes at 4°C. Reaction mixture (3 ml) was prepared by the mixing of 0.1 mM EDTA, 0.1 mM NADPH, 0.1 mM FeSO₄, 0.1 mM GSH, 0.1 U glutathione reductase, 0.1

mM hydrogen peroxide in phosphate buffer (50 mM, pH 7.0) and 100 µl of supernatant. GPX Activity was determined by monitoring the glutathione-dependent oxidation of NADPH at 340 nm against blank of the same reaction mixture without NADPH. 1U of the enzyme is the amount necessary to decompose 1 µl of hydrogen peroxide per minute at 25°C and GPX activity was calculated by taking ϵ_{340} as $6.62 \text{ mM}^{-1}\text{cm}^{-1}$.

Determination of anti-oxidant activities. DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay was performed according to the modified protocol of Shimada et al. (1992),⁶⁵ in presence or absence of SA treatments under PQ stress to know the free radical scavenging potential of cells. Dried methanolic extract of each sample (0, 25, 50, 75, 100, 125, 150, µg/ml) was prepared. 3 ml of DPPH solution (0.1 mM DPPH in 80% ethanol) was added in methanolic extract (1 ml). Reaction mixture was shaken vigorously, left to stand in dark for 30 minutes and absorbance was recorded at 517 nm. Percentage DPPH scavenging activity was calculated by using the following formula-

$$DPPH\text{Scavengingactivity} = \frac{(Ac - At)}{Ac} \times 100$$

Determination of anti-bacterial activities. To know whether these treatments are inducing any resistance against bacteria, anti-bacterial activity of aqueous extracts of each culture was tested against *Pseudomonas aeruginosa* by using disc diffusion assay⁶⁶. For which bacteria were streaked on Luria agar media plates and incubated at 37°C for overnight. After that, the pure single bacterial colony was inoculated in 10 ml sterile Luria broth and incubated in a shaker at 37°C for 5 hours at 120 rpm to obtain the log phase bacterial culture. According to 0.5 McFarland ($1-2 \times 10^8$ CFU/ml), the absorbance of culture was adjusted to 0.1 at 600 nm. Then, 50 µL of bacterial culture was spread on Luria agar plates and 5 mm discs were placed at equal distance on each plate. Various concentrations of extracts (20, 40, 60 and 80 µg/ml) from PQ treated biomass, SA treated, PQ and SA treated and control biomass (without any treatment) were taken for testing anti-bacterial activity. Streptomycin and water were used as positive and negative controls respectively. All the plates were overnight incubated at 37°C. Zone of Inhibition (ZOI) was measured to determine their antibacterial potential.

Confocal laser scanning microscopy. Confocal microscopy was done by using Leica true confocal scanner used to check the effect of PQ on *Microchaete* sp. in presence and absence of salicylic acid⁶⁷. In 1ml culture from all sets added in 2.5% (v/v) glutaraldehyde phosphate buffer (0.2 M, pH 7.0) for fixation and kept for 3 hours. Washed thrice with same buffer and stored at 4°C. From this drop of sample was placed on slide and covered with glass slip slowly Slide was adjusted between stage clip and observed with a X63 1.4 numerical aperture and apochromat oil immersion objective lens under an excitation beam with a wavelength of 568 nm by an Ar-Kr laser beam.

Concluding remarks

Paraquat shows toxic effect to the non-target cyanobacterium, *Microchaete* sp. NCCU-342 by changing in their normal biochemical and metabolic activity. PQ increases oxidative damage through free radical

formation. Supplementation of salicylic acid improves the antioxidant machinery, increases phenolic compound and phytohormones that ultimately reduces the free radical level. Salicylic acid application also enhanced the antimicrobial activity of the organism. Supplementation of salicylic acid not only counteracts the toxic effect of herbicide through managing the antioxidant potential but also make it tolerant against biotic stress. Therefore, for the sustainable agriculture, the formulation of salicylic acid with cyanobacterial biofertilizer would be a better option.

Results And Discussion

During last few decades, treatment of herbicide has been adopted for better crop production. The non-selective herbicides like paraquat, glufosinate, basagran, and glyphosate not only affect the weed but also change the physiology of non-target organism like main crop and biofertilizer i.e., cyanobacteria²². PQ (methyl viologen) is a cationic herbicide that disturbs electron transport by accepting the electron from photosystem I. Cyanobacterial rice bio fertilizers like *Microchaete sp.* is also highly sensitive to PQ due to presence of photosystem I²³. *Microchaete sp.* NCCU-342 is dominantly found in the rice field ecosystem as an important component that maintains the nitrogen status of soil to improve crop production.²⁴

Effect of PQ and SA + PQ on the growth of *Microchaete sp.* NCCU-342. To understand the effect of PQ on *Microchaete sp.* NCCU-342, cultures were exposed to 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 μM of PQ. *Microchaete sp.* NCCU-342 showed 86%, 74%, 64%, 50%, 24%, 19% growth reduction in presence of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 μM of PQ, respectively as compared to control (Fig. 1a). The brownish green colour of cultures turned yellow to white and microscopic examination of culture showed cell lysis and depigmentation of cells. The reduction in growth under PQ stress have been also reported in other cyanobacterial strains such as *Nostoc sp.* N1 and *Anabaena sp.* A1²⁵, *Anabaena variabilis* and *Plectonema boryanum*²⁶, *Anabaena oryzae* and *Nostoc ellipsosporum*²⁷ and in microalgae like *Chlorella vulgaris* and *Chlorella pyrenoidosa*²⁸.

For comparative growth analysis, *Microchaete sp.* were grown for 96 hours in presence of only PQ (0.6 μM), and SA supplemented (0.3 mM) PQ exposed cultures. After 96 hours, the growth was found maximum in control. However, growth declined in PQ treated culture (50%) whereas supplementation of SA in the PQ treated culture showed higher growth (35%) (significant at $P \leq 0.05$). Supplementation of exogenous SA in *Microchaete sp.* culture showed good growth than the PQ exposed cultures (Fig. 1b). The growth enhancement by the application of salicylic acid has been also reported in *Chlorella vulgaris*²⁹.

Chlorophylls represent the fundamental part of the whole metabolism of the photosynthetic organisms. Increase in chlorophyll content reflect growth, whereas decrease in chlorophyll reflect the unfavourable condition for growth. Under confocal microscopy, chlorophyll was used as a natural marker. *Microchaete sp.* NCCU-342 grown for 96 hours with 0.6 μM PQ exhibited degradation of chlorophyll. Normal healthy cells of control sample were more fluorescent that confirmed growth. PQ reduced the chlorophyll content

and resulted in poor growth that decreased the fluorescent whereas supplementation of salicylic acid improved chlorophyll and reduced the membrane damage and lysis, and gave higher fluorescent (Fig. 1c-f). *Phormidium corallyticum* under black band disease³⁰ and in *Synechococcus elongatus* PCC 7942 also showed reduced fluorescence under hydrogen peroxide stress.³¹

Effect of PQ and SA + PQ on oxidative stress under PQ stress. PQ induced the production of free radicals MDA and hydrogen peroxide (Fig. 2a-b). MDA level increased by 8.16 % in PQ exposed culture as compared to control and reduced by 15.63 % in culture having both SA and PQ as compared to PQ treated culture (significant at $P \leq 0.05$). As compared to control, the level of hydrogen peroxide in PQ treated set increased by 4.11 % and reduction in hydrogen peroxide level was found as 2.455 % in culture with both SA and PQ (significant at $P \leq 0.05$). PQ (di-cation) disrupts the electron transport system and form the mono-radical cation. This mono-radical cation interacts with molecular oxygen to produce other toxic radical such as $O_2^{\cdot-}$ and hydrogen peroxide³². According to Sood et al. (2011)³³, an increase in the concentration of PQ resulted in the generation of MDA and H_2O_2 in *Azolla-Anabaena* symbiotic system due to disruption of the cell membrane that loosens the integrity of the cell. Production of hydrogen peroxide is mainly due to the reduction of PQ di-cation by PSI that leads to the subsequent formation of various toxic radicals including hydrogen peroxide. Other herbicide like, glyphosate and bentazone increased the MDA and hydrogen peroxide content in *Microcystis aeruginosa*³⁴ and *Anabaena cylindrica*³⁵ SA was found to decrease oxidative damages and worked as a protective compound against PQ induced toxicity. Application of SA on PQ treated culture reduced the lipid peroxidation and generation of hydrogen peroxide. Such observation have been also reported in higher plants e.g. barley under PQ³⁶ maize under clethodium stress and *Arachis hypogaea* under Basagran stress³⁷.

Effect of PQ and SA + PQ on antioxidant enzymes under PQ stress. The toxicity of PQ causes the generation of free radicals. In order to counteract these free radical's antioxidant enzymes like SOD acts as the first-line defense but produces hydrogen peroxide as its end product that gets eliminated by CAT, APX and GPX^{32,38}. As compared to control, a 78.37 % increase in SOD activity was recorded in PQ treated culture set. Supplementation of salicylic acid in PQ-treated culture was found to increase the activity by 121.62 % as compared to control (Fig. 3a). Similarly, CAT (46.38 %), APX (16.73 %), GPX (240 %) and GR (15%) activity also increased in PQ treated culture as compared to control, but their induction was enhanced up to 30.38 % in CAT, 74.38 % in APX, 127 % in GPX and 141% in GR after the supplementation of salicylic acid in PQ culture set as shown in Fig. 3b-e (significant at $P \leq 0.05$).

PQ stimulate the enzyme activity of SOD, CAT, APX GPX and GR in *Azolla-Anabaena* symbiotic system³³ and in *Chlorella vulgaris*². Accumulation of hydrogen peroxide plays an important role as signalling molecule and activates other set of enzymes i.e., CAT GPX and APX. CAT and GPX that catalyse the dismutation of hydrogen peroxide into water and oxygen. APX uses ascorbate as reducing power and potentially catalyze the removal of superoxide and hydrogen peroxide and GR accumulates GSH from GSSH and ascorbate for ascorbate peroxidase in ascorbate-glutathione cycle⁵.

Salicylic acid enhanced the activity of SOD, CAT, APX GPX and GR in the present study. Salicylic acid regulates the antioxidant enzymes under stressful condition and maintain the redox state of cell (Chen et al., 2016)³⁹. It is reported from higher plants that SA + PQ treatment provide the protection against PQ induced toxicity by activation of antioxidant enzymes in to barley³⁶ and in *Vigna radiate* under mancozeb (fungicide), chlorpyrifos (insecticide), under metribuzin (herbicide)¹⁵ and in rice plant¹⁸ under quinclorac toxicity.

Effect of PQ and SA + PQ on GSH Content and Redox State of *Microchaete* sp. under PQ Stress.

Glutathione is a tri-peptide non-enzymatic antioxidant compound that operates in cellular detoxification and maintain the redox homeostasis of the cell. It occurs in two form reduced glutathione (GSH) and oxidized glutathione (GSSH)²⁰. GSSH further gets reduced by glutathione reductase. This cycle regulates the redox homeostasis of the cell⁴⁰. The reduced glutathione (GSH) was decreased in PQ treated culture by 25.29 % as compared to control. The culture supplemented with both SA and PQ showed 67.42 % increased of GSH as compared to control. The GSSH increased by 56.79 % in PQ treated culture as compared to control, whereas 80.69 % reduction was recorded as compared to PQ treated culture. The ratio of reduced glutathione to oxidized glutathione was lowered in PQ treated culture by 20.94 % as compared to control and increased in SA + PQ culture by 28.02 % as compared to PQ treated culture (Fig. 4a-c). The activity of glutathione reductase depends on NADPH, and PQ blocks the regeneration of NADPH from NADP⁺ which may be the reason of reduced GR activity which in turn reduced its activity and destabilizes the redox state. PQ has been also known to reduce the glutathione in cucumber⁴¹ and barley³. Treatment of SA showed protective effect and increased the GSH content in barley under PQ stress³ and watermelons under excess boron⁴².

Effect of PQ and SA + PQ on enhancement of the antioxidant and antibacterial activity of *Microchaete* sp.

DPPH assay was performed to monitor the fluctuation in the antioxidant activity in all cultures. The antioxidant capacity of all sets was evaluated by monitoring DPPH-radical scavenging activity (Fig. 5a). Methanolic extract of all cultures showed improved scavenging effect that indicated the production of the antioxidant compound. DPPH scavenging activity (%) was recorded as 92.11 ± 2.76 in control, 85.97 in PQ treated culture, 73.69 ± 1.90 salicylic acid-treated culture, and 61.80 ± 2.60 in those culture set that contain both SA and PQ (significant at P ≤ 0.05). Moreover, the increased phenols contents in presence of SA + PQ may also be responsible for higher antioxidant activity. The scavenging activity of phenols depends on the losing of a proton from phenolic compound to bounding with DPPH radical⁴³. Studies on abiotic stress have also shown that salicylic acid manages the oxidative stress by enhancing the antioxidant enzyme activity for example in rosemary⁴⁴, in mung bean, alfalfa tomato and lemon balm under salinity^{46, 47, 48}, in *Phaseolus aureus* and flex plant under cadmium stress^{49, 50}.

The antibacterial activity was evaluated by using aqueous extracts of each culture against *Pseudomonas aeruginosa*. The diameters of the zone of inhibition was recorded in millimetres (Fig. 5b). Control sample showed no zone of inhibition but PQ treated samples (60 and 80 µg/ml) exhibited zone of inhibition (9.6 mm and 11 mm diameter). SA supplementation in PQ exposed culture showed zone of

inhibition in all tested concentration of extract (20, 40, 60, 80 µg/ml) that increased in a dose dependent manner (10 mm, 10.4 mm, 16.5 mm and 20 mm). Zone of inhibition by streptomycin was recorded as 10.5 mm, 10.8 mm, 20 mm and 20.2 mm from lower to higher concentration respectively. Thus, the best anti-bacterial activity was found in presence of SA + PQ. Treatment of salicylic acid in rosemary also showed antibacterial activity under salinity. The enhanced phenolic and endogenous IAA and SA content and antioxidant compound by salicylic acid supplementation may be the reason for the enhancement of antioxidant and antibacterial activity. In this study, exogenous application of salicylic acid to PQ exposed cultures alters the antioxidant activities. The antioxidant and antibacterial activity results showed that SA supplementation not only resist abiotic stress but also provide resilience against biotic stress. These scavenging activities showed that SA modulates antioxidant activity. The antioxidant and antibacterial activity showed the tolerance ability of *Microchaete* against PQ after SA supplementation.

Effect of PQ and SA + PQ on phenolic compound and phytohormone of *Microchaete* sp. under PQ stress.

Accumulation of phenolic compounds was found in all cultures. As compared to control, PQ-treated culture showed an 8.85 % increase in total phenolic content (significant at $P \leq 0.05$). Supplementation of salicylic acid showed a 29.07 % increase in total phenolic content as compared to PQ exposed culture (significant at $P \leq 0.05$). SA induced the production of phenolic compound in culture with both salicylic acid and PQ as observed in our study (Fig. 6a). Due to different environmental stress, plant produces secondary metabolites such as phenolic compounds, terpenoids, flavonoids, etc. for protection. These metabolites have high antioxidant potential and play a major role in defense against stress. Radwan. (2012)¹⁷, reported that salicylic acid induced the phenolic compound in clethodium herbicide stressed maize to enable it to survive.

The endogenous phytohormones (IAA & SA) were also induced in all sets (Fig. 6b-c). The endogenous level of IAA increased by 13.43 % in PQ as compared to control, whereas the supplementation of SA in PQ exposed culture resulted in further increase by 2.96 % only as compared to PQ treated cultures. The amount of endogenous salicylic acid also increased in PQ treated culture by 96.59 % as compared to control. The supplementation of salicylic acid with PQ resulted in higher SA accumulation by 4.72 % as compared to PQ treated culture (Fig. 6c). Exogenous supplementation of SA in higher plants induced endogenous SA content e.g. in cucumber under salt stress³¹ and in Baikal skullcap under salt and drought stress⁵¹. Exogenous application of salicylic acid induced both IAA and SA in maize and cucumber under drought stress^{52, 53}.

Declarations

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Declaration of competing interests

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Figures

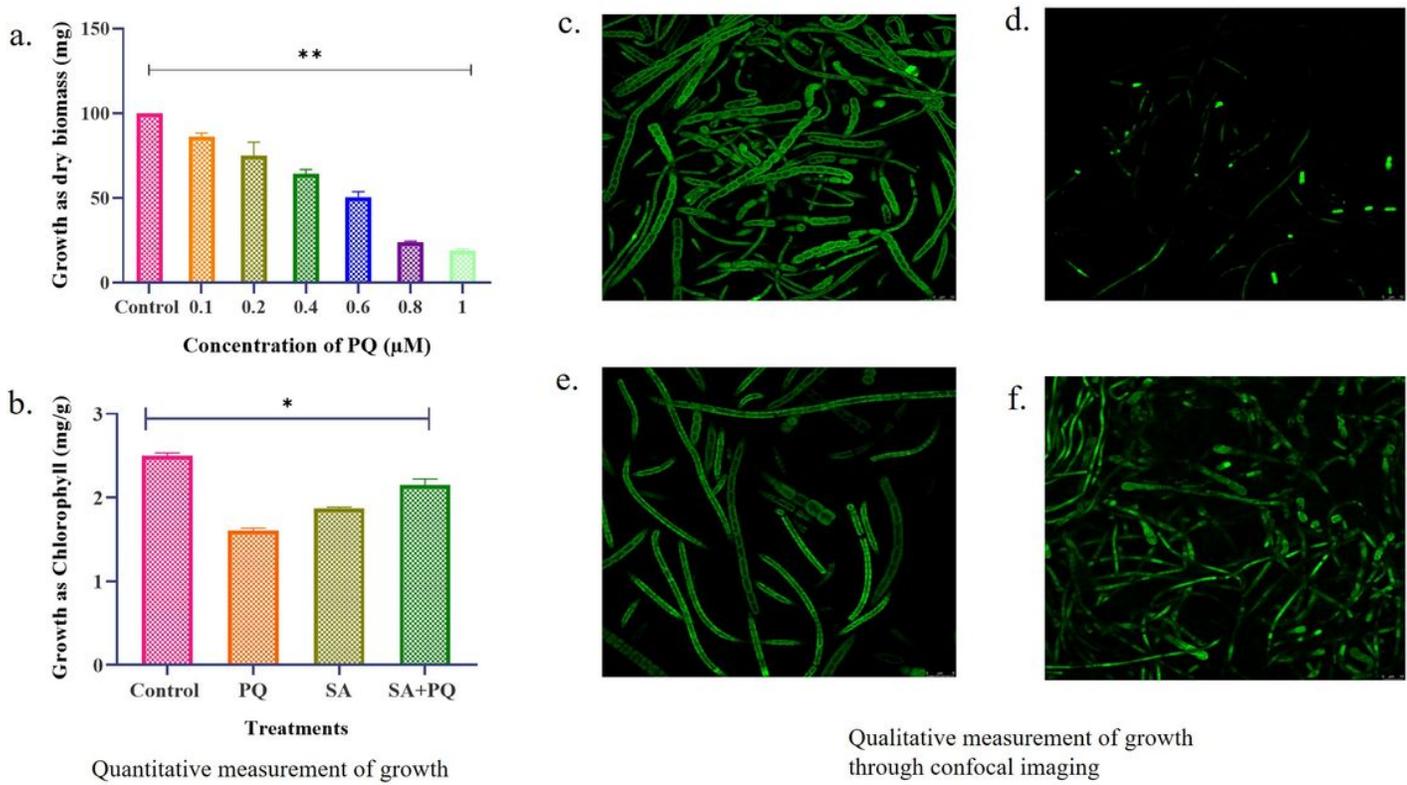


Figure 1

a) Growth of *Microchaete* sp. under different concentration of PQ b) and with salicylic acid, the values are means of three replicates \pm standard deviation (SD). Asterisk showed level of significance at $P < 0.05$. Quantitative measurement of growth through confocal imaging c) control, d) PQ, e) SA, f) SA+PQ.

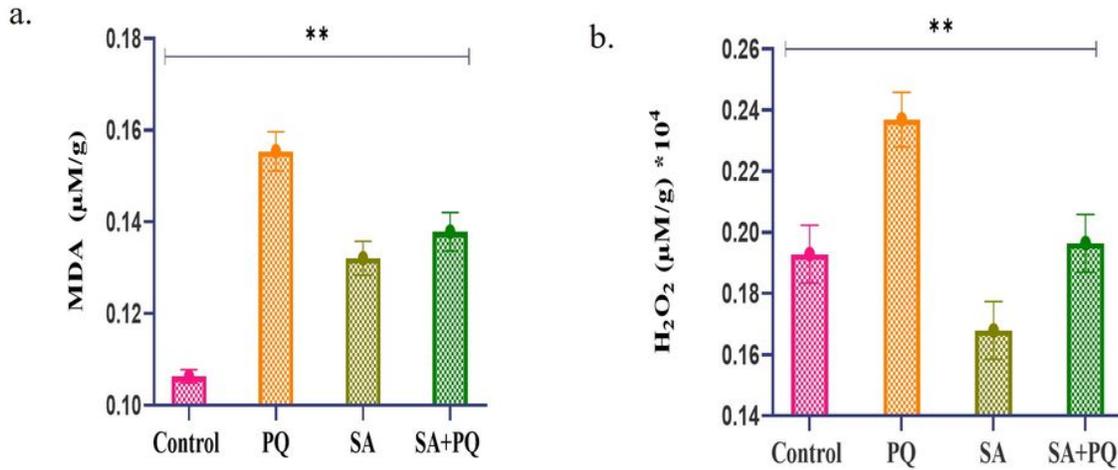


Figure 2

Effect of PQ (0.6 µM), SA (0.3 mM) and SA+PQ on a) MDA (µM/g) and b) H₂O₂ (µM/g) content of *Microchaete* sp. NCCU-342. The values are means of three replicates ± standard deviation (SD). Asterisk showed level of significance at P < 0.05.

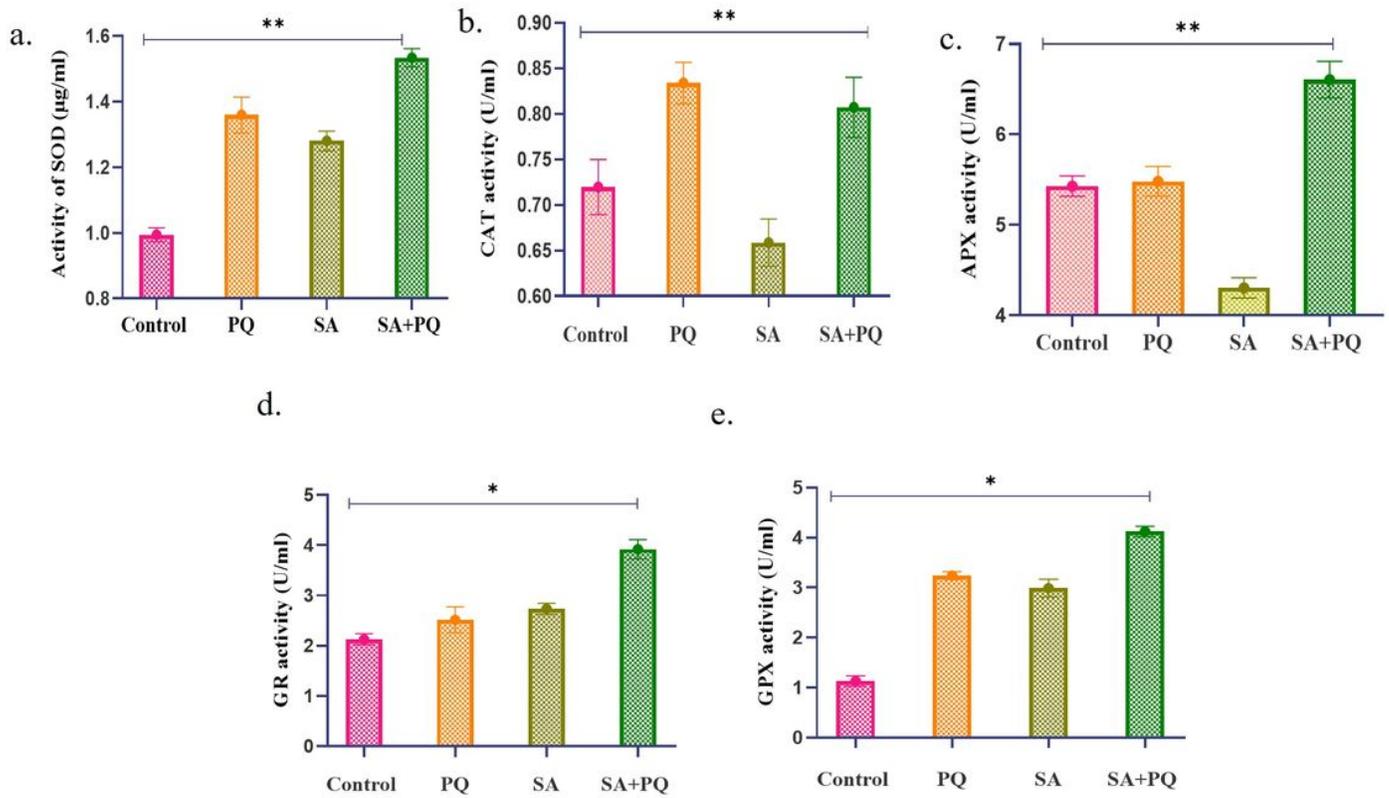


Figure 3

Effect of PQ (0.6 µM), SA (0.3 mM) and SA+PQ on antioxidant enzymes activity of a) SOD, b) CAT and c) APX d) GR, e) GPX of *Microchaete sp. NCCU-342*. The values are means of three replicates ± standard deviation (SD). Asterisk showed level of significance at $P < 0.05$.

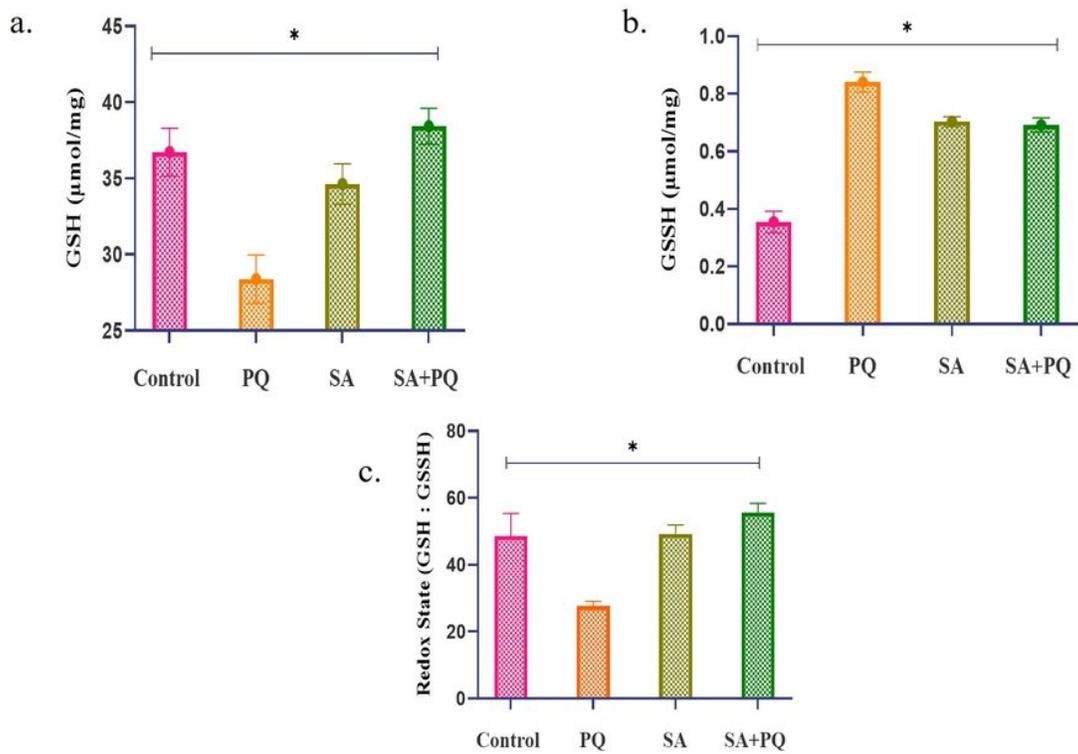
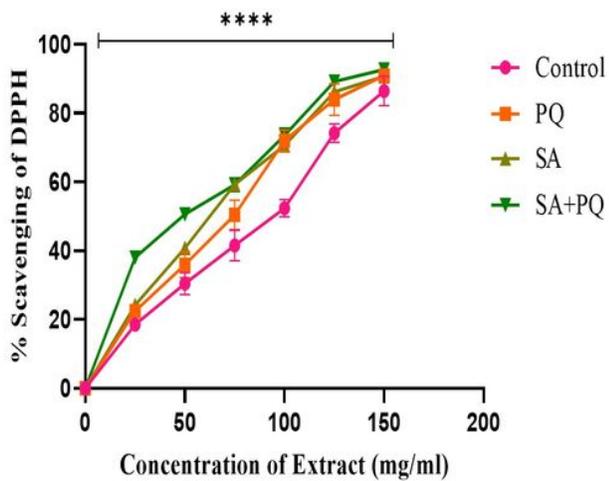


Figure 4

Effect of PQ ($0.6 \mu\text{M}$), SA (0.3 mM) and SA+PQ on glutathione content a) reduced and b) oxidized glutathione and c) redox state of *Microchaete* sp. NCCU-342. The values are means of three replicates \pm standard deviation (SD). Asterisk showed level of significance at $P < 0.05$.

a.



b.

Concentrations	Control	PQ	SA	SA+PQ	Streptomycin
20 µg/ml	No ZOI	No ZOI	No ZOI	10.33±0.054 mm	10.46±0.05 mm
40 µg/ml	No ZOI	No ZOI	7.03±0.03 mm	10.36±0.06 mm	10.86±0.002 mm
60 µg/ml	No ZOI	7.05±0.06 mm	9.03±0.05 mm	16.46±0.057 mm	20.06±0.054 mm
80 µg/ml	No ZOI	10.03±0.061 mm	10.06±0.022 mm	20.01±0.062 mm	20.1±0.033 mm

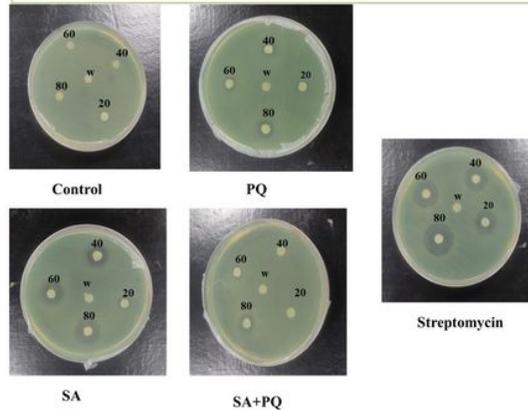


Figure 5

Effect of PQ (0.6 µM), SA (0.3 mM) and SA+PQ on a) antioxidant activity and b) antibacterial activity of *Microchaete* sp. NCCU-342. The values are means of three replicates ± standard deviation (SD). Asterisk showed level of significance at $P < 0.05$.

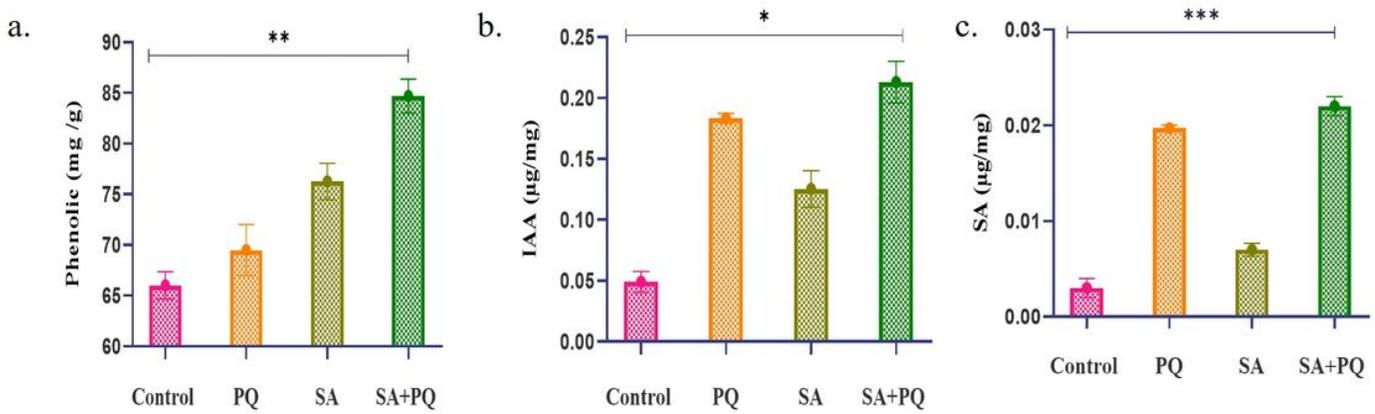


Figure 6

Effect of PQ (0.6 µM), SA (0.3 mM) and SA+PQ on a) Phenolic (mg/g), b) IAA (µg/mg) and c) SA (µg/mg) content of *Microchaete* sp. NCCU-342. The values are means of three replicates ± standard deviation (SD). Asterisk showed level of significance at P < 0.05.