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Un nuovo ceppo di PGPR (*Kocuria rhizophila* Y1) migliora la tolleranza allo stress salino in mais

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Indice

Abstract

L'elevata salinità è dannosa per la fisiologia e la resa delle colture, ma prove crescenti indicano che i rizobatteri che promuovono la crescita delle piante (PGPR) possano migliorare la crescita delle colture e ridurre gli impatti negativi dello stress salino attraverso la regolazione di alcune caratteristiche molecolari, biochimiche e fisiologiche. Nello studio preso in analisi in questo elaborato un nuovo ceppo batterico alo-tollerante che ha mostrato due tratti di promozione della crescita è stato isolato dal suolo della rizosfera del mais ed è stato denominato Y1. Esso è stato poi attribuito alla specie *Kocuria rhizophila*, e sono stati ulteriormente esaminati i suoi effetti sulla crescita e lo sviluppo del mais (*Zea mays*) sotto trattamento con NaCl. I risultati hanno mostrato che in condizioni di salinità l'inoculazione con il ceppo Y1 di *K. rhizophila* potrebbe proteggere il mais dallo stress salino migliorando l'acquisizione di nutrienti, le prestazioni di crescita, la produzione di biomassa e la capacità fotosintetica, contribuendo all'omeostasi ionica, regolando i livelli di fitormoni e aumentando la risposta di detossificazione dalle ROS e in generale la risposta allo stress. Ciò rende il ceppo Y1 un ottimo candidato per possibili applicazioni nel settore agricolo in quanto PGPR.

1. Introduzione

1.1 Stress salino nelle piante

La salinità del suolo rappresenta uno dei principali problemi per l'agricoltura e la produzione di cibo globale: si stima che fino al 20% delle aree coltivate in tutto il mondo siano colpite da ipersalinità, e nonostante il processo di salinizzazione sia causato da fattori naturali come la presenza di climi secchi, scarse precipitazioni, un alto tasso di evaporazione e scarso ristagno idrico, ci sono una serie di fattori antropici che lo velocizzano e lo alimentano come pratiche di irrigazione inappropriate, sistemi di drenaggio scadenti, uso eccessivo di fertilizzanti e la crescente estremizzazione del clima derivata dall'impatto delle attività umane. Trovare dei modi per rendere le piante meno vulnerabili è dunque essenziale per diminuire le perdite economiche e soprattutto per tutelare e migliorare la produzione alimentare, in modo da contrastare gli effetti dei cambiamenti climatici e stare al passo con la costante crescita demografica globale (Tarolli et al., 2024).

Gli effetti negativi della salinità sulle piante sono molteplici ed interconnessi come si può notare anche dallo schema nella *figura 1*. I più importanti sono identificabili in stress osmotico, stress ionico e stress ossidativo. Il processo inizia dal suolo, dove in primo luogo i sali solubili in eccesso quali sodio e cloro diminuiscono il potenziale idrico del terreno, riducendo la capacità di assorbimento di acqua per la pianta, che dipende proprio dalla differenza di potenziale idrico tra le radici e il suolo. La carenza di acqua (o stress osmotico) altera il bilancio idrico, influisce sull'efficienza dell'utilizzo dell'acqua e riduce il turgore cellulare; quest'ultimo processo induce la chiusura degli stomi, portando ad un apporto più basso di CO_2 e dunque ad un minor tasso di fotosintesi.
Dopo essere entrati nella pianta attraverso una serie di vie di trasporto, gli ioni presenti in eccesso nel suolo possono accumularsi negli organi della pianta, e in particolare è l'accumulo intracellulare di sodio (Na^+) e cloro (Cl^-) che induce stress ionico. Il sodio possiede una somiglianza elevata con il potassio dal punto di vista molecolare, infatti i due ioni condividono sistemi di trasporto, e un assorbimento elevato del sodio può inibire l'assimilazione del potassio causandone una carenza; inoltre un eccesso di sodio nel citoplasma può provocare una sostituzione del potassio in enzimi potassio-dipendenti che di fatto non possono essere attivati dal sodio, portando ad uno squilibrio che compromette processi metabolici essenziali come la fotosintesi e la sintesi proteica. Il sodio, infine, contribuisce

assieme alla carenza idrica alla riduzione della conduttanza stomatica perturbando la regolazione dell'apertura degli stomi. Per quanto riguarda il cloro invece esso esplica la sua tossicità interferendo soprattutto con la produzione di clorofilla (causando un'ulteriore riduzione del tasso di fotosintesi) e con l'assorbimento di nitriti; in generale l'assimilazione di nutrienti e ioni come potassio, calcio, fosforo e azoto viene ridotto in presenza elevata di NaCl.
Il terzo effetto fondamentale sulle piante della salinità del terreno è lo stress ossidativo che si genera con l'aumentata produzione di specie reattive dell'ossigeno (*Reactive Oxygen Species* o ROS), che è dovuta alla maggiore attività ossigenasica della RuBisCO (causata dal ridotto tasso di assimilazione del carbonio a seguito della chiusura stomatica), alla dispersione di elettroni da parte della catena respiratoria dei mitocondri, e alla maggiore attività dei perossisomi a causa di un incrementato tasso di fotorespirazione. La presenza di ROS porta a danno cellulare attraverso l'ossidazione di lipidi, proteine, acidi nucleici e altre macromolecole, il che è negativo per la pianta.
Complessivamente la salinità del terreno porta ad una crescita ridotta (sia a causa della riduzione del tasso di fotosintesi sia a causa dei danni cellulari) e ad una condizione di stress per le piante, e in ambito agricolo ciò determina una produzione quantitativamente e qualitativamente più scarsa (Zhou et al., 2024, Parihar et al., 2015).

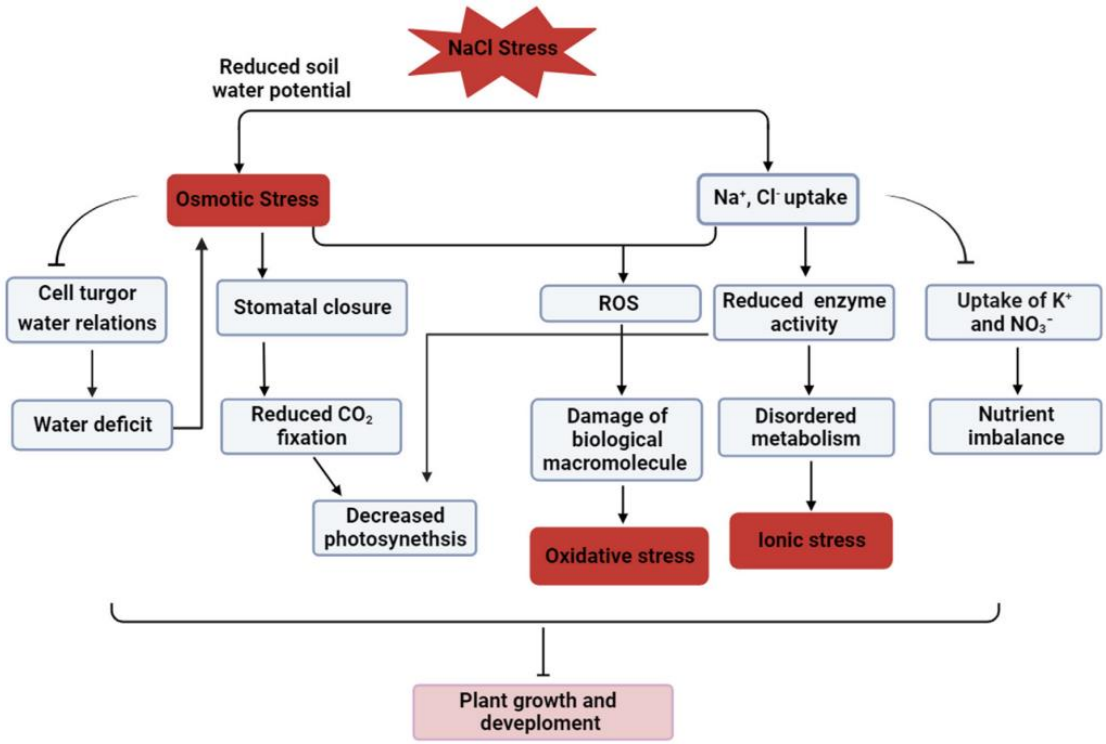

Figura 1. Schema riassuntivo degli effetti negativi sulle piante della salinità (Zhou et al., 2024).

1.2 Meccanismi di tolleranza al sale

Come visto prima, la salinità è uno dei principali fattori ambientali che limita la crescita e la produttività delle piante, infatti esse possiedono una serie di meccanismi fisiologici, molecolari e biochimici per rispondere allo stress salino.
Innanzitutto, per mantenere l'omeostasi ionica e prevenire l'accumulo di sodio nel citoplasma vengono impiegati trasportatori ionici specializzati che aiutano a escludere il sodio dal citoplasma o a sequestrarlo nei vacuoli, mantenendo l'equilibrio con il potassio che è vitale per i processi cellulari. Per contrastare la perdita di acqua vengono prodotti osmoliti come prolina, glicina, betaina e zuccheri, che non interferiscono con il metabolismo cellulare e aiutano a trattenere l'acqua all'interno delle cellule, contribuendo a mantenere il turgore e stabilizzando proteine e membrane. Per quanto riguarda le ROS queste fungono da importanti molecole di segnale soprattutto nelle fasi iniziali di stress salino, ma la loro concentrazione deve poi essere drasticamente ridotta attraverso enzimi antiossidanti per limitare i danni cellulari. Un altro essenziale iniziatore di cascate di segnale in situazioni di stress salino è il calcio, il cui influsso porta a regolazione dell'espressione genica per mediare la tolleranza al sale, ad esempio portando proprio all'espressione di enzimi che processano le ROS. Un ruolo essenziale è infine svolto dagli ormoni, in particolare l'acido abscissico (ABA) e l'auxina, che influenzano la risposta della pianta attraverso adattamenti cellulari e modulazione della crescita: l'ABA, un ormone dello stress, influenza l'espressione genica e induce la chiusura degli stomi riducendo la perdita d'acqua, mentre l'auxina aiuta il rimodellamento dell'architettura delle radici, promuovendo la crescita di radici laterali e la formazione di peli radicali per accedere all'acqua e ai nutrienti in aree del suolo meno saline (Van Zelm et al., 2020, Zhou et al., 2024).

Dal punto di vista della ricerca scientifica è importante continuare a studiare i meccanismi intrinseci di tolleranza al sale presenti nelle piante, ma soprattutto per le specie di interesse produttivo ed economico è utile andare ad indagare dei metodi per migliorare ulteriormente la loro resistenza e produttività in condizioni di stress. Tra gli approcci bio-ingegneristici c'è l'utilizzo di batteri che promuovono la crescita delle piante (*Plant Growth-Promoting Rhizobacteria*, o PGPR), che sono già ampiamente utilizzati per ridurre l'uso di pesticidi e di fertilizzanti chimici (Zhou et al., 2024).

1.3 PGPR (*Plant Growth-Promoting Rhizobacteria*)

I rizobatteri che promuovono la crescita delle piante (PGPR) sono un gruppo di batteri che si trovano nella rizosfera, ovvero l'ambiente del suolo in prossimità della radice della pianta. Essa presenta una maggiore concentrazione di nutrienti, una maggiore densità microbica e anche una composizione microbica diversa rispetto a quella del resto del terreno. È stato ipotizzato che i microrganismi che occupano la rizosfera vengano selezionati da quelli del microbiota generale del suolo prima attraverso la presenza degli essudati radicali, che cambiano la composizione chimica del terreno e aumentano la disponibilità di sostanze organiche, e in seguito attraverso processi basati sulla compatibilità con le risposte immunitarie e l'ambiente biochimico specifici della pianta. I PGPR in particolare formano poi una relazione mutualistica con le piante, ricevendo nutrienti dai rizodepositi e portando in cambio ad esse dei benefici: essi infatti promuovono la crescita delle piante attraverso diversi meccanismi, sia diretti che indiretti, che sono analizzati nei seguenti paragrafi (Bulgarelli et al., 2013).

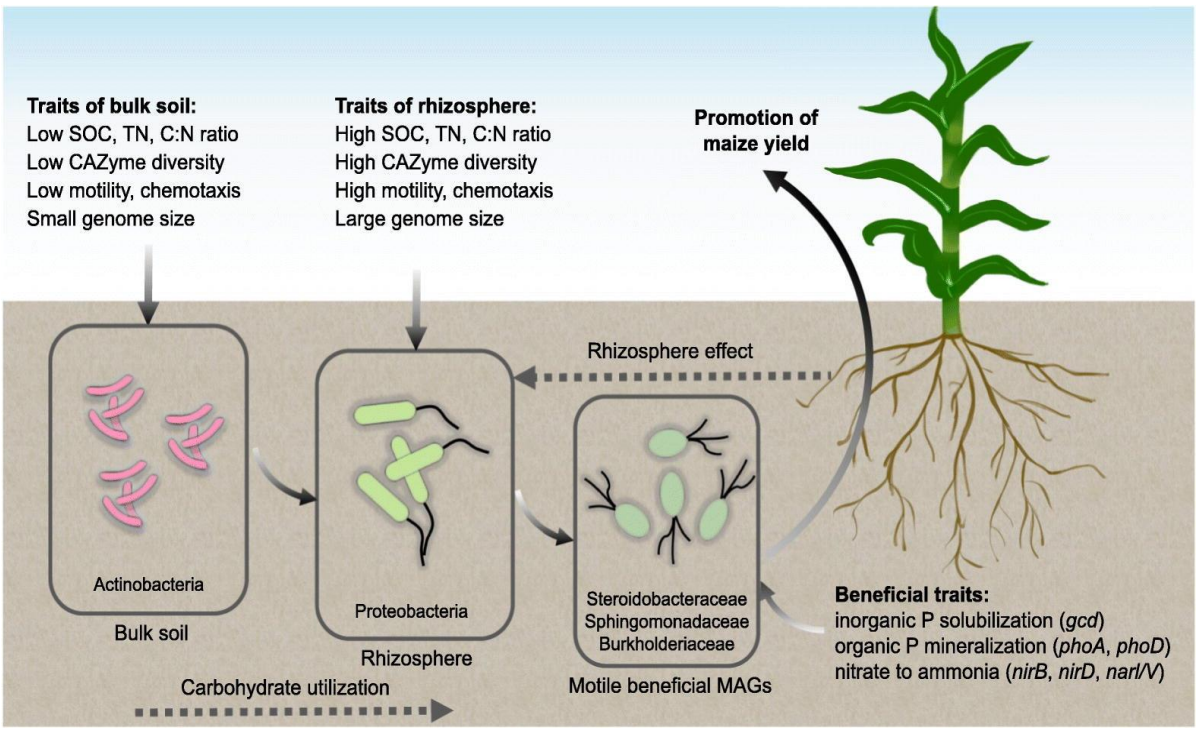

Figura 2. L'immagine illustra le differenze tra i batteri della rizosfera e quelli della massa del suolo lontano dalle radici (Wu, X. et al., 2023)

Tra quelli diretti troviamo l'aumento della disponibilità di nutrienti, tra cui l'azoto, il fosforo ed il ferro. L'azoto è un nutriente essenziale per le piante e spesso un fattore limitante per la loro crescita, e il suo metabolismo può essere influenzato dai PGPR: alcune specie forniscono forme di assorbimento più facilmente utilizzabili quali ammoniaca e nitriti tramite la fissazione del carbonio, l'ammonificazione e la nitrificazione, mentre altre incrementano l'attività degli enzimi coinvolti nel metabolismo dell'azoto, come la nitrato reduttasi e la glutammina sintetasi, o in generale migliorano l'efficienza di utilizzo dell' azoto. Anche il fosforo è un elemento particolarmente importante per le piante, e il suo assorbimento è critico in quanto meno del 5% del contenuto di fosforo del terreno è biodisponibile per le piante. I PGPR intervengono proprio sulla sua disponibilità andando a mineralizzarlo, ovvero rendere inorganico il fosforo organico, e solubilizzarlo. Similmente al fosforo, il ferro è generalmente presente in quantità considerevoli nel suolo, ma è poco disponibile alle piante a causa di numerosi fattori, tra cui la bassa solubilità dello stato di ossidazione più comune, Fe^{3+}. I PGPR possono mitigare questa scarsità attraverso la produzione di siderofori, ovvero piccole molecole con alta affinità per il ferro, che in realtà vengono prodotte in minore quantità anche dalle piante stesse. La presenza di siderofori batterici, che hanno affinità maggiore dei fitosiderofori, aumenta la biodisponibilità del ferro nella rizosfera e quindi migliora l'assimilazione di esso e la crescita vegetale, ed inoltre influisce sulla competizione contro patogeni batterici e fungini.
L'altra tipologia di meccanismo diretto per promuovere la crescita è la produzione di fitormoni e l'interferenza con essi. Il più importante fitormone prodotto dai PGPR è l'acido acido indol-3-acetico (*indole-3-acetic acid* o IAA), che è anche la principale auxina naturale; come accennato in precedenza, l'auxina stimola l'allungamento delle radici, la formazione delle radici laterali e lo sviluppo dei peli radicali, il che aumenta la capacità di assorbimento di nutrienti e acqua della pianta e dunque promuove la crescita in condizioni di stress, come quello salino. L'interferenza con gli ormoni della pianta riguarda invece l'etilene, uno dei maggiori fitormoni prodotti durante condizioni di stress, che va in queste situazioni ad inibire la crescita delle radici e in generale della pianta. L'enzima batterico ACC-deaminasi è coinvolto nella degradazione del precursore dell'etilene, l'ACC (acido 1-amminociclopropano-1-carbossilico), a livello della rizosfera; ciò porta ad una ridotta biosintesi dell'etilene nella pianta, dunque ad una diminuzione delle risposte inibitorie mediate da questo ormone, e di conseguenza ad un aumento della crescita in condizioni di stress (Bulgarelli et al., 2013, Hasan et al., 2024).

I meccanismi indiretti di promozione della crescita si basano principalmente sulla soppressione di microrganismi patogeni e sull'induzione della resistenza sistemica indotta (*induced systemic resistance* o ISR). Per prima cosa i PGPR competono con i potenziali patogeni per ossigeno, spazio e nutrienti attuando delle strategie per prevalere, come la produzione di siderofori ad alta affinità (che possono ad esempio prevalere su quelli fungini) o la produzione di sostanze con attività antimicrobica, quali antibiotici o enzimi idrolitici. In secondo luogo la presenza di batteri non patogeni come i PGPR porta all'attivazione della ISR, un tipo di risposta immunitaria vegetale che rende le piante più resistenti e meno vulnerabili alle infezioni, non solo a livello locale delle radici ma sistemico (Bulgarelli et al., 2013, Hasan et al., 2024).

In conclusione, per quanto riguarda il problema della salinità del suolo l'utilizzo di PGPR che tollerano la presenza di sale e che promuovono la crescita delle piante in queste condizioni potrebbe risultare una valida alternativa o un'implementazione all'utilizzo di fertilizzanti chimici, ai metodi tradizionali di incrocio per ottenere varietà alo-tolleranti e alla manipolazione genetica delle piante. L'articolo analizzato si concentra sull'isolamento di un particolare ceppo di PGPR e sugli effetti benefici che esso ha sulla tolleranza al sale nella pianta di mais, una delle specie vegetali più importanti a livello globale in agricoltura e per la produzione alimentare.

2. Materiali e metodi

2.1 Identificazione dell'isolato batterico e analisi delle caratteristiche di promozione della crescita

Il ceppo batterico Y1 è stato ottenuto a partire da campioni di suolo della rizosfera di piante di mais, che sono stati lavati e filtrati e poi utilizzati per allestire delle colture su terreno LB agar addizionato con NaCl a diverse concentrazioni. A partire da 18 colonie singole che tolleravano la presenza di sale e che sono state isolate in coltura pura è stata selezionata quella con la maggiore abilità di produzione di IAA e di solubilizzazione del fosfato, che è stata denominata ceppo Y1. Questo è stato ulteriormente caratterizzato, e quindi utilizzato nello studio dell'articolo in analisi.
Per identificare il ceppo è stata utilizzata l'analisi dell'RNA ribosomale 16S, un metodo molto comune per l'identificazione batterica che prevede l'estrazione del DNA, l'amplificazione del gene 16s rRNA tramite PCR, il suo sequenziamento e quindi un allineamento con le sequenze simili presenti nel database GenBank del NCBI (National Center for Biotechnology Information).
Per testare le principali caratteristiche di promozione della crescita del ceppo, ovvero la solubilizzazione del fosfato, la produzione di IAA e l'attività di ACC deaminasi, sono stati svolti dei saggi specifici:

- la capacità di solubilizzazione del fosfato è stata inizialmente determinata utilizzando il terreno di crescita Pikovskaya agar, dove la presenza di aloni circolari intorno alla colonia indica proprio la capacità dei batteri di solubilizzare il fosfato, e poi per avere una misura quantitativa della solubilizzazione è stato usato il metodo di spettrofotometria molibdeno-antimonio.
- la quantità di IAA prodotta è stata determinata su coltura liquida con il metodo colorimetrico, basato sull'aggiunta del reagente di Salkowski al surnatante della coltura batterica e poi sulla lettura della densità ottica con spettrofotometro UV a 530 nm.
- l'attività di ACC deaminasi è stata misurata in modo indiretto usando la quantità di α-chetobutirrato prodotto dall'enzima, il quale catalizza la reazione di scissione dell'ACC in α-chetobutirrato e ammoniaca. Anche in questo caso è stata effettuata una misurazione di densità ottica con spettrofotometro UV a 540 nm.

2.2 Preparazione dell'inoculo batterico e pretrattamento dei semi di mais

Una volta isolato e caratterizzato il ceppo batterico, questo è stato utilizzato per preparare un inoculo aggiungendo un piccolo volume di sospensione batterica a del nuovo terreno LB liquido e facendolo crescere per 24 ore in agitazione a 28°C. Dopo aver sterilizzato i semi di mais con etanolo (75%) e ipoclorito di sodio (10g/L) e averli lasciati reidratare in acqua sterile per 12 ore, questi sono stati immersi nell'inoculo batterico per 2 ore e quindi successivamente usati per l'esperimento.

2.3 Tasso di germinazione

Il tasso di germinazione è stato calcolato con semi sterilizzati e lasciati crescere a 25°C per 7 giorni in quattro diverse condizioni, ognuna con tre repliche biologiche: i primi due gruppi, di cui uno era costituito da semi inoculati con il ceppo Y1 e l'altro da semi non inoculati, sono stati trattati con una soluzione priva di NaCl; gli altri due gruppi, di cui uno era sempre costituito da semi inoculati con il ceppo Y1 e l'altro da semi non inoculati, sono stati invece trattati con NaCl (30 mmol/L).

2.4 Condizioni di crescita e trattamenti

Sono state prese in considerazione sei diverse condizioni di crescita: i primi tre gruppi, denominati I+0, I+100 e I+200, sono stati inoculati con il ceppo Y1 e poi sottoposti a trattamento con NaCl in concentrazione 0 mmol/L, 100 mmol/L e 200 mmol/L rispettivamente. Gli altri tre gruppi, denominati N+0, N+100 e N+200, non sono stati inoculati con il ceppo Y1 e sono stati sottoposti a trattamento con NaCl in concentrazione 0 mmol/L, 100 mmol/L e 200 mmol/L rispettivamente, e hanno costituito i gruppi di controllo. Sono state fatte tre repliche biologiche per ogni condizione di crescita.
I semi sono stati dunque piantati in vasi contenenti terreno sterilizzato tramite autoclave e costituito da suolo naturale, vermiculite e perlite in rapporto 2:2:1, e sono stati fatti crescere in una serra a temperatura e umidità controllate per 21 giorni irrigandole con acqua con una concentrazione di NaCl uguale a quella del rispettivo trattamento iniziale di ogni gruppo. Da queste piante sono stati ottenuti i materiali usati per le analisi riportate in seguito.
La crescita e la resa della biomassa sono state valutate sulle piante di 21 giorni, e in particolare per la biomassa è stato considerato il peso secco dopo aver sottoposto il materiale a essiccazione per 1 ora a 105°C e poi per 24 ore a 75°C.

2.5 Parametri fisiologici

I parametri della fotosintesi, in particolare il tasso di traspirazione, la conduttanza stomatica e il tasso netto di fotosintesi, sono stati misurati su una foglia completamente cresciuta con uno strumento portatile di misurazione della fotosintesi (LI-6400). La quantità di clorofilla è stata determinata utilizzando foglie fresche omogeneizzate e immerse in acetone (80%) per 48 ore, e misurando l'assorbanza a 663 e 646 nm. Il contenuto relativo di acqua (*relative water content*, o RWC) è stato ottenuto misurando il peso fresco delle foglie appena raccolte, il peso turgido delle foglie messe in acqua sterile per 4 ore e il peso secco dopo averle fatte essiccare completamente, e infine utilizzando la formula RWC(%) = (peso fresco−peso secco)/(peso turgido-peso secco)×100. La perdita di elettroliti (*electrolyte leakage*, o EL) è stata valutata come il rapporto percentuale tra la conducibilità elettrica della soluzione in cui sono state immerse delle foglie tagliate in piccoli pezzi per 24 ore a 25°C, e la conducibilità dei campioni autoclavati per 20 minuti a 120°C.

2.6 Parametri legati allo stress ossidativo

Per valutare la produzione di ROS e lo stress ossidativo nella pianta, nello studio sono stati prima presi in considerazione il perossido di idrogeno (H_2O_2) e lo ione superossido (O_2^-), due delle ROS principali, poi il malondialdeide (MDA), un biomarcatore dello stress ossidativo e della perossidazione lipidica, e infine gli antiossidanti enzimatici e non enzimatici che contrastano la presenza di ROS.
L'analisi quantitativa del contenuto di H_2O_2 e O_2^- è stata condotta omogeneizzando le foglie con acido tricloroacetico (0.1%), centrifugando l'omogeneizzato e trasferendo il surnatante ad un buffer di K_3PO_4 e KI, e infine misurando l'assorbanza a 390 nm. Oltre a ciò si è anche proceduto con un'analisi visiva delle foglie dopo colorazione istochimica che evidenzia l'accumulo di ROS nei tessuti: per visualizzare la presenza di H_2O_2 è stato usato il composto 3,3'-diaminobenzidina (DAB), che reagisce con il perossido di idrogeno in presenza di perossidasi per formare un precipitato di colore marrone, mentre per visualizzare la presenza di O_2^- è stato usato il composto nitro blu tetrazolio (NBT), che reagisce con lo ione superossido formando un precipitato di colore blu. Dopo l'immersione nella soluzione con uno dei due composti a 25°C e al buio per 24 ore, le foglie sono state messe in etanolo (95%) a 80°C per 1 ora per decolorarle, e sono state infine fotografate. La quantità di MDA è stata invece determinata tramite il saggio delle sostanze reattive all'acido tiobarbiturico, che prevede la reazione dell'MDA con esse e una misurazione di assorbanza.

Per quanto riguarda gli enzimi antiossidanti sono stati considerati l'ascorbato perossidasi (APX), la glutatione perossidasi (GPX) e la glutatione reduttasi (GR), e la loro attività è stata misurata tramite kit basati sul saggio immuno-assorbente legato ad un enzima (o *enzyme-linked immunosorbent assay*, ELISA) (Kamai Shu Biotechnology). Sono stati poi presi in esame anche il glutatione (GSH) e il glutatione disolfuro (GSSG), il cui rapporto è un importante indicatore dell'equilibrio redox cellulare in quanto il GSH, la forma ridotta del composto, è un antiossidante non enzimatico ed è essenziale nel regolare il livello di ROS, mentre il GSSG, la forma ossidata dello stesso composto, indica stress ossidativo nella cellula. Le loro concentrazioni sono state ottenute tramite la procedura descritta da Ortiz, V. D. et al. (2019).

2.7 Contenuto di ioni, metaboliti ed ormoni

Il contenuto di calcio, potassio e sodio è stato misurato con uno spettrofotometro di emissione di fiamma, quello di azoto tramite il metodo Kjeldahl e quello di fosforo tramite il metodo descritto da Murphy, J. & Riley, J. P. (1962), sempre a partire da foglie essiccate e digerite con acido solforico.
La quantità di zucchero solubile è stata determinata da foglie fresche con il metodo antrone, un saggio colorimetrico che prevede la misurazione dell'assorbanza a 620 nm. La quantità di prolina è stata determinata da foglie fresche immerse in acido solfosalicilico (3%), filtrate e trattate con ninidrina (2.5%), acido acetico glaciale e calore, e misurando l'assorbanza a 620 nm della soluzione fatta raffreddare e addizionata con toluene.
La produzione degli ormoni IAA e ABA è stata invece misurata grazie a kit basati sulla tecnica ELISA (MLBio ELISA Kit producers) a partire da foglie macinate in ghiaccio e poi incubate a 4°C per 6 ore in un buffer di fosfato, da cui dopo centrifugazione si è ottenuto il surnatante.

2.8 Analisi di trascrittomica

Per la determinazione dei livelli di espressione genica è stata usata la PCR quantitativa, utilizzando l'RNA totale estratto dalle foglie tramite il reagente TRIzol e convertito in cDNA. In particolare si sono misurati i trascritti dei geni *ZmHKT1*, *ZmNHX1*, *ZmNHX2* e *ZmNHX3*, correlati al bilancio ionico, *ZmNCED1*, il gene chiave per la sintesi di ABA, *ZmWRKY58* e *ZmDREB2A*, i geni chiave dei fattore di trascrizione legati alla risposta della pianta allo stress abiotico, e *ZmGR1* e *ZmAPX1*, i geni correlati alla sintesi degli enzimi antiossidanti GR e APX.

3. Risultati e discussione

3.1 Caratteristiche del ceppo Y1

Tramite le analisi dell'rRNA il ceppo batterico Y1 è stato attribuito alla specie *Kocuria rhizophila*. Per quanto riguarda le potenziali caratteristiche di promozione della crescita, il ceppo si è dimostrato capace di solubilizzare il fosfato e di produrre IAA anche in condizioni di stress salino, sebbene ad elevate concentrazioni di NaCl queste capacità erano leggermente ridotte. Non è stata rilevata invece alcuna attività di ACC deaminasi, ma ciò è coerente con il fatto che questo meccanismo non è presente in tutti i PGPR.

3.2 Crescita della pianta

Gli effetti della presenza del ceppo Y1 nella rizosfera del mais riguardo alla crescita in condizioni di stress salino riscontrati nello studio sono molteplici. Innanzitutto l'inoculazione con Y1 ha migliorato il tasso di germinazione: se in assenza di NaCl non c'era differenza tra semi inoculati e non, in presenza di sale c'era invece una significativa differenza nel tasso di germinazione (*figura* 3). Inoltre è stato riscontrato un miglioramento nella crescita dal punto di vista delle dimensioni e della biomassa: le piante inoculate con Y1 e messe in condizioni di stress salino presentavano lunghezza e peso secco sia dei germogli sia delle radici significativamente maggiori rispetto a piante non inoculate.

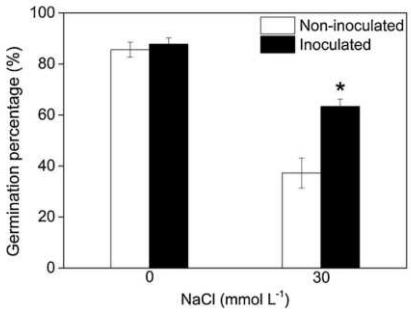

Figura 3. Tasso di germinazione in assenza di NaCl (sinistra) e in presenza di NaCl (destra) (Li, X. et al., 2020)

3.3 Ormoni

Come accennato in precedenza, gli ormoni IAA e ABA hanno un ruolo essenziale nella risposta allo stress salino. I livelli di IAA, il quale è essenziale nello sviluppo della pianta e soprattutto delle radici, solitamente diminuiscono in condizioni di stress salino portando a una crescita compromessa della pianta. Al contrario i livelli di ABA, il quale è implicato nella chiusura stomatica e in generale nelle risposte agli stress, solitamente aumentano in condizioni di stress salino portando ad uno sviluppo ridotto poiché viene data priorità alla sopravvivenza in condizioni avverse.

I PGPR aiutano a mantenere più alta la quantità di IAA e allo stesso tempo a ridurre quella di ABA, in modo da evitare l'attivazione eccessiva di risposte di sopravvivenza che inibiscono la crescita. Questo effetto è stato riscontrato anche nel caso di Y1, il quale ha ridotto in modo statisticamente significativo l'entità delle variazioni dei livelli dei due ormoni nelle piante sottoposte a trattamento con NaCl rispetto a quelle che non avevano alcun PGPR nella loro rizosfera (*figura* 4).
In condizioni normali le piante mantengono autonomamente dei livelli ormonali ottimali, perciò l'influenza del batterio non porta a differenze e quindi a benefici.

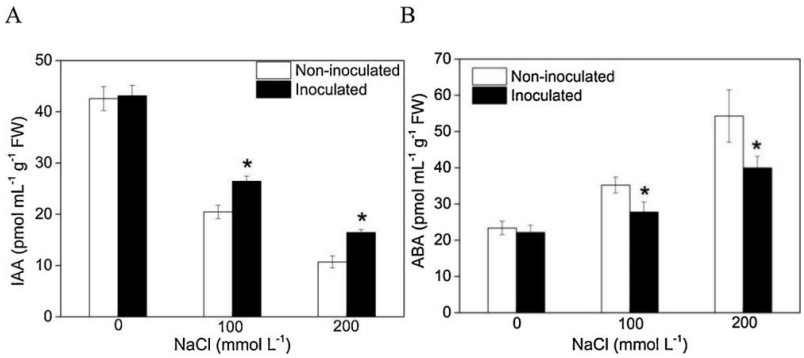

Figura 4. Effetti della presenza di Y1 sui livelli di fitormoni IAA (grafico **A**) e ABA (grafico **B**). Gli asterischi indicano differenze statisticamente significative ($p<0.05$) (Li, X. et al., 2020)

3.4 Stress osmotico

La condizione di stress osmotico viene normalmente mitigata dalla pianta attraverso l'accumulo di osmoliti all'interno delle cellule, e coerentemente a ciò nello studio è stato osservato che in presenza di NaCl il contenuto di prolina e di zuccheri all'interno delle foglie di mais aumentano in modo lineare con quello di sale. Nelle piante inoculate con il ceppo Y1 però l'aumento è significativamente maggiore. La minore perdita di acqua ottenuta grazie alla presenza di questi osmoliti e di altri meccanismi è dimostrata dal contenuto di acqua relativo, o RWC, che è anch'esso maggiore quando è presente Y1.
Per quanto riguarda la fotosintesi e i parametri legati ad essa, la presenza di Y1 ha portato ad un incremento nel tasso netto di fotosintesi, nella quantità di clorofilla, nel tasso di traspirazione e nella conduttanza stomatica rispetto a piante non inoculate, ma il tutto solo in condizioni di ipersalinità: in assenza di NaCl il batterio non ha effetti rilevanti su questi processi fisiologici.

3.5 Stress ionico

Relativamente all'equilibrio ionico della pianta, la presenza di NaCl nel terreno porta a una ridotta concentrazione di fosforo, azoto, calcio e potassio in foglie e radici e invece ad un aumento della quantità di sodio, che come visto in precedenza è dannoso per la pianta. È stato evidenziato che la presenza di Y1 in condizioni di stress salino promuove l'assorbimento dei principali ioni menzionati prima ad eccezione del sodio, il cui assorbimento è diminuito, contribuendo a ristabilire un rapporto più ottimale tra potassio e sodio. Inoltre è aumentata l'espressione dei geni *ZmNHX1*, *ZmNHX2* e *ZmNHX3*, che codificano per proteine con funzione di antiporto Na^+/H^+ che sono coinvolte nel sequestro di sodio nei vacuoli e che quindi influiscono ulteriormente sulla quantità di sodio libero nel citoplasma. Questo aumento di espressione dovuto alla salinità avviene indipendentemente dalla presenza di Y1, ma in piante inoculate ha un'entità significativamente maggiore.
Un altro trasportatore preso in analisi è HKT1, che è fondamentale per la gestione dei livelli di sodio e il mantenimento dell'omeostasi ionica in condizioni di stress salino in quanto riduce al minimo la tossicità del sodio. Al contrario di quanto accade per i tre trasportatori NHX, il livello di espressione del gene di HKT1, *ZmHKT1*, pur aumentando con la concentrazione di NaCl non è influenzato dalla presenza di Y1, infatti i livelli in piante inoculate e non inoculate sono comparabili. Una possibile spiegazione di questa differenza potrebbe essere che l'espressione di HKT1 è regolata da gradienti di concentrazione di sodio specifici o segnali localizzati nei tessuti che non sono influenzati da *K. rhizophila* Y1, mentre geni di trasportatori come quelli della famiglia NHX sono regolati da segnali ormonali e correlati allo stress, che sono ad ampio spettro di azione e più facilmente influenzati dalla presenza del batterio.
Sempre a riguardo dell'equilibrio ionico anche la perdita di elettroliti, che cresce linearmente con la concentrazione di NaCl nel terreno, è significativamente minore in piante che hanno Y1 nella rizosfera.

Un altro importante cambiamento dell'espressione genica influenzato da Y1 riguarda i geni *ZmWRKY58* e *ZmDREB2A*, che codificano due fattori di trascrizione e che dunque regolano a loro volta numerosi geni. Queste due proteine, che appartengono rispettivamente alle famiglie di fattori di trascrizione WRKY e DREB, sono essenziali per la risposta della pianta a diversi tipi di stress abiotico, tra cui quello salino. La loro espressione è incrementata in presenza di *K. rhizophila* Y1, aumentando la tolleranza della pianta a condizioni non ottimali.

3.6 Stress ossidativo

Le analisi riguardanti lo stress ossidativo suggeriscono che *K. rhizophila* Y1 aumenta la risposta di difesa delle piante per la detossificazione dalle ROS: per prima cosa il contenuto di H_2O_2 e O_2^- in piante inoculate è considerevolmente minore rispetto a piante non inoculate messe nelle stesse condizioni di stress salino. Ciò è dimostrato dai dati dell'analisi quantitativa, ma è apprezzabile anche da una meno intensa colorazione istochimica che evidenzia le ROS (*figura* 5). Come per altri valori considerati, la differenza è significativa solo nelle piante trattate con NaCl, mentre quelle in condizioni fisiologiche non sono influenzate positivamente da Y1.

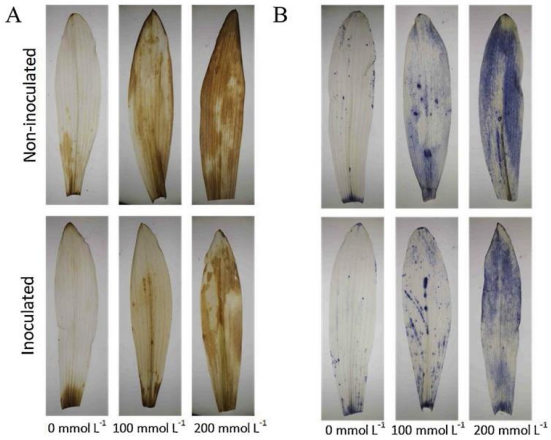

Figura 5. Colorazione istochimca con il composto DAB, che mostra la presenza di H2O2 (**A**) e con il composto NBT, che evidenzia la presenza di O2- (**B**)
(Li, X. et al., 2020)

Relativamente agli enzimi antiossidanti, quindi l'ascorbato perossidasi (APX), la glutatione reduttasi (GR) e la glutatione perossidasi (GPX), l'inoculazione con Y1 ne ha aumentato notevolmente l'attività, e per quanto riguarda APX e GR ne ha anche aumentato il livello di espressione, tutto ciò sempre in condizioni di stress salino e in confronto a piante non inoculate (*figura* 6). Analogamente, i livelli dell'antiossidante non enzimatico glutatione (GSH) e la sua forma ridotta GSSG sono significativamente maggiori in piante inoculate rispetto a piante non inoculate, il che indica una più intensa attività di neutralizzazione delle ROS stimolata dalla presenza del PGPR.
In modo coerente anche la quantità di MDA, uno dei principali prodotti della perossidazione dei lipidi di membrana e quindi un indicatore di danno ai sistemi di membrane della pianta, era ridotta in presenza di Y1.

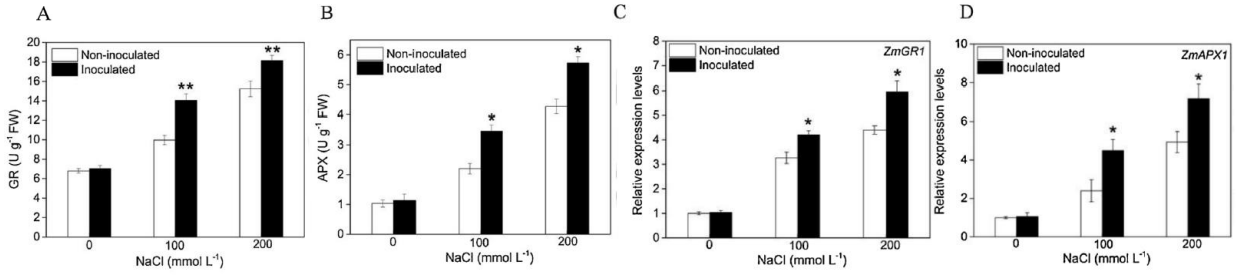

Figura 6. Attività degli enzimi GR (**A**) e APX (**B**) e livelli di espressione dei geni relativi, ZmGR1 (**C**) e ZmAPX1 (**D**). Gli asterischi indicano differenze statisticamente significative ($p<0.05$) (Li, X. et al., 2020)

4. Conclusioni

Per quasi tutti gli aspetti considerati, in mancanza di stress salino la presenza di *K. rhizophila* Y1 non ha effetti significativi nel promuovere la crescita di *Zea mays*. Ciò è probabilmente dovuto al fatto che il PGPR va solo a mitigare la condizione di stress indotta dal sale, agendo ad esempio sullo squilibrio ionico, l'assorbimento di nutrienti, la presenza di ROS e lo scompenso ormonale. In assenza di stress questi meccanismi di compensazione non sono necessari, dunque il batterio ha meno opportunità di contribuire in modo significativo al miglioramento della crescita. I benefici di *K. rhizophila* Y1 quindi sono legati soprattutto allo stress e ciò potrebbe costituire un adattamento simbiotico o di sopravvivenza, ma molti PGPR possono migliorare la crescita delle piante sia in condizioni di stress che fisiologiche a seconda dei loro meccanismi e delle loro caratteristiche specifiche. Complessivamente comunque i risultati ottenuti indicano che la presenza del ceppo Y1 di *K. rhizophila* influisce positivamente sulla tolleranza allo stress salino in *Zea mays* agendo su numerosi processi fisiologici. Gli effetti più importanti sono il miglioramento dell'assorbimento dei nutrienti, la promozione dell'equilibrio ionico, dell'equilibrio e della produzione dei fitormoni, la riduzione dello stress ossidativo e l'attivazione dell'espressione di geni coinvolti nelle risposte allo stress. Ciò lo rende un ottimo candidato per possibili applicazioni nel settore agricolo in quanto PGPR, in particolare in aree che sono soggette a salinità del suolo, e da questo punto di vista sarebbe utile indagare ulteriormente le proprietà del batterio soprattutto in un contesto di comunità, dove le interazioni con altri microrganismi potrebbero influire sui suoi meccanismi di promozione della crescita.

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Abbreviazioni

Composti

ABA = acido abscissico
IAA = acido indol-3-acetico
ACC = acido 1-amminociclopropano-1-carbossilico
MDA= malondialdeide
GSH = glutatione
GSSG = glutatione disolfuro
DAB= 3,3'-diaminobenzidina
NBT= nitro blu tetrazolio

Enzimi

APX = ascorbato perossidasi
GPX = glutatione perossidasi
GR = glutatione reduttasi

Altre abbreviazioni

PGPR = *plant growth-promoting rhizobacteria*
ISR = *induced systemic resistance*, resistenza sistemica indotta
ROS = *reactive oxygen species*, specie reattive dell'ossigeno
ELIZA = *enzyme-linked immunosorbent assay*
RWC = *relative water content*, contenuto relativo di acqua
EL= *electrolyte leakage*, perdita di elettroliti

APPENDICE

A. Articolo di riferimento

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A novel PGPR strain *Kocuria rhizophila* Y1 enhances salt stress tolerance in maize by regulating phytohormone levels, nutrient acquisition, redox potential, ion homeostasis, photosynthetic capacity and stress-responsive genes expression

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ABSTRACT

High salinity is harmful for crop physiology and yield. Accumulating evidences indicate that plant growth promoting rhizobacteria (PGPR) can enhance crop growth and reduce the negative impacts of salt stress through regulation of some molecular, biochemical and physiological features. In the present study, a novel halo-tolerant strain Y1 was isolated from maize rhizosphere soil. Sequence analysis of 16S rRNA gene confirmed its identity as *Kocuria rhizophila*. This strain tolerated up to 10 % NaCl and showed two growth promoting traits like phosphate solubilization and IAA production. The impacts of *K. rhizophila* Y1 on maize (*Zea mays* L.) growth and development with salt treatment (0, 100 and 200 mM NaCl) were further examined. The results showed that inoculation with *K. rhizophila* Y1 strain could protect maize from salt stress by regulating plant hormones (IAA and ABA) levels and improving nutrient acquisition. In detail, inoculation with *K. rhizophila* Y1 significantly improved growth performance, biomass production, seed germination rate, photosynthetic capacity, antioxidant levels, relative water content and chlorophyll accumulation in maize under saline conditions in comparison with non-inoculation treatment. Moreover, strain Y1 inoculated maize showed lower levels of Na^+ and electrolyte leakage under salt treatment compared to non-inoculated ones. Inoculated maize also showed higher transcript levels of genes encoding antioxidants (*ZmGR1* and *ZmAPX1*) and genes involved in salt tolerance (*ZmNHX1*, *ZmNHX2*, *ZmNHX3*, *ZmWRKY58* and *ZmDREB2A*) than in non-inoculated plants. In summary, this study indicated the important contribution of *K. rhizophila* Y1 in mitigating the deleterious effects of salinity on maize growth and development by regulating plant hormones and nutrient acquisition, and thereby maintaining ion homeostasis, improving photosynthetic capacity, enhancing redox potential and stress-responsive genes expression.

1. Introduction

Soil salinity is considered as a serious agricultural problem in the world. Nowadays, about 50 % of irrigated land and 20 % of cultivated land are impacted by various degrees of salinity (Cheng et al., 2012). It was reported that salinity stress could adversely impact plant growth by decreasing nitrogen metabolism, carbon acclimatization and grain yield. Salinity stimulated ionic toxicity and osmotic stress in plants, leading to decrease of plants' ability to uptake nutrients. Furthermore, the elevated Cl^- and Na^+ accumulation in the cytosol had adverse effects on the plant growth and development (Fahad et al., 2015). Salt stresses increased the generation of reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals and superoxide which could cause oxidative stress and negative effect on plant development and yield. Therefore, it is necessary to scavenge ROS for maintenance of normal plant growth. Plants have established various physiological and biochemical mechanisms to cope with environmental stresses (Ahmad et al., 2008). For example, to alleviated the oxidative damages caused by ROS, plants have evolved several self-defense mechanisms, including the induction of non-enzymatic and enzymatic antioxidants and the accumulation of osmolytes (Ahmad et al., 2008). However, the defense mechanisms are not enough to completely eliminate ROS and alleviate oxidative damage in plants under salt stress treatment (Yamaguchi and Blumwald, 2005). Thus, it is still necessary and important to make

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attempts to improve the salt tolerance of crops in an ecofriendly manner.

Although traditional marker-assisted breeding and genetic engineering had been used to improve the salt tolerance of crops and modest results were obtained with these methods (Yamaguchi and Blumwald, 2005). As an alternative to genetic manipulation and traditional breeding, application of salt-tolerant beneficial plant growth-promoting rhizobacteria (PGPR) which inhabit the rhizosphere of various plants, could also enhance the growth of host plants and alleviate salt stress without spoiling the environment (Bhattacharyya and Jha, 2012). PGPR are widely used as inoculants for enhancing the agricultural crops' growth and yield by various mechanisms. These mechanisms are associated with the upregulation of phytohormones such as cytokinins, gibberellins or indole-3-acetic acid (IAA), and an increase in availability of nutrients including siderophore production, biological nitrogen fixation and phosphate solubilization (Bhattacharyya and Jha, 2012). In addition, PGPR were found to synthesize 1-aminocyclopropane- 1-carboxylate (ACC) deaminase which played an important role in the conversion of plant ethylene precursor ACC into α-ketobutyrate and ammonia (Barnawal et al., 2017). Using their ACC deaminase activity, PGPR could prevent growth inhibition of plants caused by ethylene production under salt stress (Barnawal et al., 2017).

Application of PGPB has become a promising approach to alleviate salinity-induced stress in plants. Previous studies showed that treatments of PGPR containing strains of *Bacillus, Azospirillum*, *Agrobacterium*, *Rhizobium*, and *Pseudomonas* species could enhance growth and salt tolerance of their host plants (Bhattacharyya and Jha, 2012). *P. fluorescens* CECT 378 T and *P. fluorescens* biotype F enhanced sunflowers biomass by more than 10 % with NaCl treatment, and the elevated ratio of K^+/Na^+ was also observed (Shilev et al., 2012). Tahir et al. (2019) indicated that consortium of *Bacillus* strains (SR-2-1 and SR-2-1/1) enhanced IAA production in the potato rhizosphere and that ultimately regulated uptake of Ca^{2+}, K^+, Na^+ in potato leading to a higher tuber yield in salt stress conditions compared to control. Ion regulation through modulation of the expression of Na^+/H^+ antiporter (NHX) and high affinity K^+ transporter 1 (HKT1) was reported to be an important mechanism that PGPR utilize to promote plants growth under salinity conditions (Chen et al., 2017). Two bacterial endophytes, *P. migulae* 8R6 and *P. fluorescens* YsS6, isolated from tomato rhizosphere showed some positive PGPR traits, such as phosphate solubilization, IAA production, siderophore production and ACC degradation which might help the host plants to exhibit better under salt stress (Ali et al., 2014). Han and Lee (2005) also indicated that PGPR, such as *Rhizobium* and *Serratia* species, enhanced chlorophyll content and the nutrient uptake in lettuce plants with various degrees of soil salinity treatment. Moreover, El-Esawi et al. (2019) showed that inoculation with *A. lipoferum FK1* enhanced salinity tolerance in *Cicer arietinum* by modulation of stress related genes expression and antioxidant enzyme activities. Kang et al. (2019) showed that ACC deaminase- and IAA-producing halotolerant strain *Leclercia adecarboxylata* MO1 could enhance tomato plants tolerance to salinity stress through modulation of synthesis of plant endogenous organic acids, amino acids and sugar. Previous study indicated that inoculation with *Pseudomonas* sp. strain OFT5 containing ACC deaminase activity could alleviate the adverse effects of salt stress in tomato plants (Win et al., 2018). Sarkar et al. (2018) indicated that a halotolerant strain *Enterobacter* sp. P23 possessing ACC deaminase activity could enhance tolerance in rice plants to salinity stress, and this effect was correlated well with a decrease in salt stress-induced ethylene. The seeds of radish plants inoculated with *B. subtilis* and *P. fluorescens* exhibited significantly increase in photosynthetic pigments, proline, crude protein contents and total free amino acids compared to non-inoculated ones under salinity (Mohamed and Gomaa, 2012).

The genus *Kocuria* was earlier classified in the genus *Micrococcus* proposed by Stackebrandt et al. (1995). This genus contains various halotolerant strains such as *K. rhizophila*, *K. marina* and *K. kristinae* which can grow in media containing various concentrations of NaCl (Kim et al., 2004). *K. rhizophila*, *K. flava*, *K. varians* and some other species of *Kocuria* were showed capacity to produce IAA (Kim et al., 2004). However, these strains have been poorly investigated in detail for their potential as PGPR for agricultural crops, especially under salinity conditions.

Maize (*Zea mays* L.) is one of the most important cultivated cereals in the world, however, maize is also a moderately salt sensitive crop (Zerrouk et al., 2016). Therefore, feasibility studies should probably be performed to identify potential strategies in alleviating the adverse effects of salt stress on maize physiological and biochemical responses. Considering the potential role of PGPR in mitigating salt stresses to plants, in this present study, an IAA-producing strain Y1 was isolated from the rhizosphere of maize grown in Tianjin China. Based on 16S rRNA gene sequence analysis, strain Y1 was classified as a *K. rhizophila* strain. This strain could grow in medium containing 10 % NaCl. We hypothesize that *K. rhizophila* Y1 inoculation can confer salinity tolerance to maize. Thus, this isolate was biochemically characterized in vitro for its other plant growth promoting traits like ACC deaminase activity and phosphate solubilization under salt stress treatment. Moreover, the present investigation studied on the plant growth-promoting attributes of *K. rhizophila* Y1 in alleviating the effects of salt stress on the morphological and biochemical characteristics of maize plants such as seed germination, plant growth, uptake of ions (Ca^{2+}, K^+ and Na^+), nutrient acquisition, chlorophyll content, the accumulation of solutes (proline and soluble sugars), photosynthesis capacity, ROS generation, antioxidant enzymes activities, abscisic acid (ABA) content and the expression levels of genes involved in salt tolerance in maize under salinity conditions by inoculating the selected *K. rhizophila* Y1 strain.

2. Material and method

2.1. Screening of strain with salt tolerance

The soil samples were collected from rhizosphere of maize planted in the field located in the region of Tianjin University, Tianjin, China (39° 06′ 33.24″ N, 117°10′ 18.9″ E). Briefly, the non-rhizosphere soil was removed by pulling up the maize roots and shaking gently. The rhizosphere soil attached to the maize roots was washed down with 50 mL of sterile water three times and collected with a sterile bag. The sample mixtures were filtered with filter paper (No.4, Whatman, England) under aseptic conditions. The soil samples were then transferred to the laboratory for follow-up experiments.

To isolate salt-tolerant rhizosphere bacteria, 5 g of soil samples and 60 mL of sterile water were added to a 250-mL culture flask and the solution was kept on a shaker (160 rpm) for 1 h at 28 ± 2 °C. The 0.1 mL of above suspension was inoculated on LB agar plates supplemented with 5%, 6%, 7%, 8%, 9% and 10 % of NaCl (w/v), respectively. The plates were placed in the constant temperature incubator for 24 h at 28 ± 2 °C. Eighteen single colonies were selected and further purified three times using streak plate method with LB agar plates. Subsequently, the purified colonies were screened for their PGPR traits. Eventually, one bacterial strain with the highest IAA producing and phosphate solubilizing ability named Y1, was further characterized and identified. All the above experiments were carried out under sterile conditions.

2.2. Characterization of the isolates

For 16S rRNA (Partial 16S ribosomal RNA) gene sequence analysis, an Erlenmeyer flask (250 mL) containing 100 mL LB liquid medium was inoculated with 2% (v/v) of bacterial suspension and then incubated at 28 °C in a rotary shaker (160 rpm) for 12 h. The strain was collected by centrifugation at 13, 000 rpm for 10 min. The genomic DNA of the isolate was extracted as described by Li et al. (2019) and then amplified by PCR using the following universal primers: 1492R: 5'-CAC GGA TCC

TAC GGG TAC CTT GTT ACG ACT T-3' and 27 F: 5'-GTG CTG CAG AGA GTT TGA TCC TGG CTC AG-3'. The PCR products were sent to GENEWIZ (Beijing, China) for nucleotide sequencing by Sanger dideoxy sequencing method (Sanger et al., 1977). Multiple 16S rRNA sequence alignments were generated with 16S rRNA sequence of strain Y1 and available 16S rRNA sequences of related species from the NCBI databank. A phylogenetic tree was constructed by MEGA software version 4.0 (Tamura et al., 2007). The 16S rRNA sequence of strain Y1 was deposited in GenBank under the accession number MN013884.

2.3. Phosphorus solubilization assay

Phosphate solubilizing capacity of strain Y1 were measured in culture medium supplement with NaCl (0, 100 and 200 mmol/L) in this study. The evaluation of phosphorus solubilization was first performed on Pikovskaya (PKV) agar medium with a composition of (g/L): $(NH_4)_2SO_4$ 0.6, $MnSO_4$ 0.0001, glucose 10, $ZnCl_2$ 0.2, $FeSO_4$ 0.0001, $Ca_3(PO_4)_2$ 5, $MgSO_4$ 0.1, yeast extract 0.4 and agar 15. The plates were kept in an incubator at 28 ± 2 °C for 7 d. The presence of halo zones formed by the colonies was considered as the phosphate solubilizing capacity of strain Y1 and the size of halo zones could be used to estimate the capacity of phosphate solubilizing (Rani et al., 2018). For the quantitative measurement of phosphate solubilization, molybdenum-antimony anti-spectrophotometric method was used (Kim et al., 2006). Briefly, 1 mL of bacterial inoculum (OD_{600} = 0.8) was added to an Erlenmeyer flask containing 50 mL of PKV broth, then the mixture was cultivated with a shaker (160 rpm) at 28 ± 2 °C for 2 d. After centrifugation at 12 000 rpm for 10 min, the supernatant (2 mL) was obtained, and two drops of 2, 6-dinitrophenol indicator were added to volumetric flask (50 mL), and then the obtained solution was diluted with water to three fifths of the total volume. The solution was adjusted to micro-yellow with the 100 g/L sodium bicarbonate. Then 5 mL of anti-Molybdenum-Antimony chromogenic agent was added to the flask and the total volume was adjusted using sterile water. The absorbance of sample was assayed by ultraviolet spectrophotometer (Shimadzu UV-2550; Shimadzu, Kyoto, Japan) at 700 nm. Uninoculated medium was used as control and a standard curve was determined using pure KH_2PO_4 as standard.

2.4. Determination of indole-3-acetic acid (IAA)

The quantitative assay of IAA was performed with colorimetric method (Glickmann and Dessaux, 1995). The medium had a composition of (g/L): peptone 10, K_2HPO_4 1.15, $MgSO_4{\cdot}7H_2O$ 1.5, glycerol 15 and L-tryptophan (a precursor of IAA) 0.5. The strain was incubated in the medium at 28 ± 2 °C for 7 d with shaking at 160 rpm. Then the supernatant obtained by centrifugation (10 000 rpm for 12 min) was mixed with isochoric Salkowski reagent and maintained for 40 min at 25 °C. The optical density of supernatant was determined by ultraviolet spectrophotometer (Shimadzu UV-2550; Shimadzu, Kyoto, Japan) at 530 nm. Uninoculated medium was used as control and the standard curve was estimated using IAA.

2.5. Assay of ACC deaminase activity in strain

ACC deaminase enzymatic activity of the isolated strain was measured by estimating the amount of α-ketobutyrate produced from the enzymatic cleavage of ACC (Penrose and Glick, 2003). Briefly, a pellet of overnight bacterial culture (supplemented with 5 mM ACC) was collected and washed with Tris−HCl buffer (pH = 7.6). The collected cell pellets were treated with toluene and 20 mL ACC (0.5 mol/L) was added to tolunized cells incubated at 30 °C for 15 min. For the final reaction mixture, 1 mL HCl (0.5 mol/L) and 300 mL 2, 4-dinitrophenylhydrazine were added to ACC-treated tolunized cells and incubated at 30 °C for 30 min; 2 mL NaOH (2 mol/L) was added to the final reaction mixture and the optical density was measured by an ultraviolet spectrophotometer (Shimadzu UV-2550; Shimadzu, Kyoto, Japan) at 540 nm. ACC deaminase enzymatic activity was calculated as the amount of α-ketobutyrate produced per mg of protein per h. The protein was quantified by the Bradford method (1976).

2.6. Preparation of bacterial inoculum and pretreatment of maize seeds

The Erlenmeyer flask (500 mL) containing 200 mL LB liquid medium was inoculated with 2% (v/v) of bacterial suspension and then incubated at 28 °C in a rotary shaker (160 rpm) for 24 h. The strain was collected by centrifugation at 13 000 rpm for 10 min and then suspended in sterile distilled water supplemented with 0.01 mmol/L of $MgSO_4$. The absorbance of suspension was adjusted to OD_{600} = 0.8 (approximate cell density of 1×10^8 CFU). Maize seeds were surface-disinfected by treating with ethanol (75 %) for 30 s and sterilized with sodium hypochlorite solution (10 g/L) for 15 min, and then rinsed with sterile water repeatedly. Sterilized seeds were dipped into sterile water for 12 h, and then immersed with bacterial inoculum for 2 h. These inoculated seeds were used for further characterization.

2.7. Germination rate test

Germination rate of 60 surface sterilized maize seeds was determined in a 15 cm-Petri plate with a piece of filter paper containing 0 mmol/L or 30 mmol/L NaCl solution. Four sets of treatments were carried out as follow: non-inoculated seeds +0 mmol/L NaCl, inoculated seeds +0 mmol/L NaCl, non-inoculated seeds +30 mmol/L NaCl and inoculated seeds +30 mmol/L NaCl. The plates were kept at 25 °C and the germination rate was calculated after 7 days. Each treatment had three replicates.

2.8. Plant material, growing conditions and treatments

Natural soil was collected from maize experimental field located in the region of Tianjin University, Tianjin, China (39° 06′ 33.24″ N, 117° 10′ 18.9″ E). The pots (120 mm × 100 mm × 120 mm) were put into 1.2 kg sterilized (121 °C, 30 min, 100 kPa) soil samples (natural soil: vermiculite: perlite = 2: 2: 1), and 6–7 maize seeds were sown in order that each pot contained at least four plants. Six groups of treatments were carried out as follows: non-inoculated Y1 subjected to 0 mmol/L NaCl stress (N + 0), inoculated Y1 subjected to 0 mmol/L NaCl stress (I + 0), non-inoculated Y1 subjected to 100 mmol/L NaCl stress (N + 100), inoculated Y1 subjected to 100 mmol/L NaCl stress 100 mmol/L NaCl (I + 100), non-inoculated Y1 subjected to 200 mmol/L NaCl stress (N + 200) and inoculated Y1 subjected to 200 mmol/L NaCl stress (I + 200). Pots were irrigated twice a week with sterile water containing 0, 100, 200 mmol/L NaCl and kept in a warm greenhouse. The growth conditions of greenhouse were set as follows: day/night cycle 12 h at 28 °C/12 h at 23 °C, illumination intensity of 4000 lx and a relative humidity of 60 %. Each treatment had three replicates. The plants were harvested for analysis after 21 days of cultivation.

2.9. Growth and biomass yield

After 21 days of cultivation, the plants were collected and washed with sterile water. Shoots height and roots length were measured and recorded. To get dry biomass of roots and shoots, the plants were oven-dried for 1 h at 105 °C and maintain for 24 h at 75 ± 2 °C in order to reach a constant dry weight (DW).

2.10. Photosynthesis parameters, EL, RWC, and chlorophyll content

The photosynthesis parameters including transpiration rate (E), stomatal conductance (gs) and net photosynthesis rate (Pn) were measured in the morning using the adaxial side of segments of a fully-grown leaf with a portable photosynthesis measurement system (LI-

6400, Li-cor, Lincoln, NE, USA). Electrolyte leakage (EL) of samples was estimated according to previous study (Lutts et al., 1996). Briefly, leaves of maize were excised and washed with distilled water. After drying with a filter paper, 2 g FW of leaves were cut into small pieces and then immersed in 10 mL distilled water and incubated at 25 ˚C for 24 h. EC1 of the bathing solution was recorded using an electrical conductivity meter (DDSJ-308A, Shanghai Leici Instrument Factory, Shanghai, China). The samples were then autoclaved at 120 ˚C for 20 min (100 kPa) to release all electrolytes. Samples were then cooled to room temperature and the final EC2 was determined. EL was expressed according to the formula EL = EC1/EC2 × 100. For relative water content (RWC) determination, the entire fresh leaves were collected and its FW was determined immediately. The leaves were put into sterile water for 4 h and then weighed. The weight of leaves was regarded as the turgid weight (TW). Then the sample was dried for determination of its DW. RWC was determined using the formula as indicated previously: RWC (%) = (FW–DW)/(TW-DW) ×100 (Smart and Bingham, 1974). The leaf chlorophyll content was measured using the previous method of Cong et al. (2008). Briefly, the leaves (0.2 g) were immersed and homogenized in 80 % acetone for 48 h. The optical density of supernatant was determined by an ultraviolet spectrophotometer (Shimadzu UV-2550; Shimadzu, Kyoto, Japan) at 663 nm and 646 nm. Chlorophyll (mg/g*fresh weight (FW)) = (7.15 × A663 + 18.71 × A646) × v/1000 × W.

2.11. Histochemical staining and quantitative analysis of H_2O_2 and O_2^-

The accumulation of H_2O_2 and O_2^- in leaves of maize seedlings could be reflected by histochemical staining. The leaves under different treatments were stained and photographed to determine the accumulation of H_2O_2 and O_2^-. Briefly, maize leaves were collected and immersed in plates containing 1 mg/mL solution of 3, 3'- diaminobenzidine (DAB) (pH = 3.9) or 0.1 mg/mL solution of nitroblue tetrazolium (NBT) (pH = 7.6), respectively. The plates were incubated at 25 ˚C for 24 h (in dark) and then the treated leaves were transferred to ethanol solution (95 %) at 80 ˚C for 1 h for decolorization (Shi et al., 2010). Eventually, the stained leaves were photographed for record. The H_2O_2 content and O_2- production in fresh leaves were also quantitatively measured according to previous methods (Velikova et al., 2000).

2.12. Antioxidant enzymes activities, GSH, GSSG, soluble sugar, proline and MDA content

The fresh leaves (0.4 g) were collected and ground on ice supplemented with sodium phosphate buffer. The mixture was centrifuged (13 000 rpm, 4 ˚C, 20 min) to obtain enzyme extracts (supernatant) which was used for enzymes activities and malondialdehyde (MDA) content measurement. The determination of ascorbate peroxidase (APX), glutathione peroxidase (GPX) and glutathione reductase (GR) activities was carried out according to previous study (Li et al., 2019). For the determination of MDA contents, 1 mL of enzyme extracts (supernatant) were added with 3 mL of thiobarbituric acid solution (0.6 %, w/v). The mixture was boiled for 15 min, then cooled to room temperature and centrifuged at 13 000 rpm at 4 ˚C for 10 min. The absorbance of supernatant was determined at 600 nm, 532 nm and 450 nm with a spectrophotometer (Shimadzu UV-2550; Shimadzu, Kyoto, Japan) (Wu et al., 2014). For the determination of reduced glutathione (GSH) and glutathione disulfide (GSSG) contents, the leaves of maize seedlings (0.2 g) were homogenized and deproteinized with perchloric acid (2 mmol/L) and EDTA (2 mmol/L). The supernatant was obtained by centrifugation (13 000 rpm, 4 ˚C, 10 min) and neutralized with KOH (2 mol/L). Total glutathione (T-GSH) and GSSG content were determined in accordance with the method proposed by Ortiz et al. (2019). GSH content was calculated as follow formula: GSH content = T-GSH content - 2 × GSSG content. Proline content in fresh leaves was measured according to the method indicated by El-Esawi et al. (2018a). Briefly, the leaves were immersed into sulfosalicylic acid (3%, w/v) on boiling water for 12 min. The filtrate was mixed with ninhydrin (2.5 %, w/v) and glacial acetic acid. Subsequently, the mixed solution was boiled for 30 min, then cooled to room temperature and toluene was added. Eventually, the absorbance of supernatant (red) collected by centrifugation was recorded by a UV–vis spectrophotometer (UV2550) at 520 nm. L-proline was used to make standard curve and the distilled water was regarded as control. The determination of soluble sugar content in fresh leaves was performed according to the method indicated by Kohler et al. (2009). Briefly, the fresh leaves were immersed into 95 % (v/v) ethanol for 24 h, then 0.2 mL of alcoholic extract and 6 mL of anthrone reagent were mixed and placed in a boiling water bath for 15 min. The absorbance of above cooled mixture was recorded with a spectrophotometer (Shimadzu UV-2550; Shimadzu, Kyoto, Japan) at 620 nm. Serial dilution of glucose was used for standard curve.

2.13. Determination of N, P, Ca^{2+}, K^+, Na^+ and K^+/Na^+

The fresh leaves and roots were harvested and dried to obtain constant-weight and ground with a mortar. The resulting powder was acid digested with H_2SO_4 at 200 ˚C for 4 h. The mixed solutions were then added with H_2O_2 and digested for 1 h. The concentration of ions including (Ca^{2+}, K^+ and Na^+) was determined with a flame spectrophotometer (Jenway, Bibby Scientific Limited, Essex, UK) according to previous methods (Wolf, 1982). K^+/Na^+ ratio was calculated in line with K^+ and Na^+ concentrations. For the determination of the nitrogen (N) and phosphorus (P) content, 0.2 g of oven-dried leaves or roots were grinded and digested in 5 mL H_2SO_4 at 200 ˚C for 4 h, and then 1 mL of H_2O_2 (30 %, w/v) was added and the reaction mixtures were kept for 1 h. Digested solutions were then made up to 40 mL with sterile water. The N content of samples was measured using Kjeldahl method as indicated previously (Bremner, 1960). The P content of samples was determined according to previous study (Murphy and Riley, 1962).

2.14. IAA and ABA measurement in plants

The production of phytohormones including IAA and ABA in leaves was determined using the ELISA (enzyme-linked immunosorbent assay) kit (MLBio ELISA Kit producers, Shanghai, China) according to manufacturer's instruction. Briefly, the leaves were ground on ice supplemented with quartz and sodium phosphate buffer. The mixture was collected and incubated at 4 ˚C for 6 h. The supernatant by centrifugation was collected to measure the contents of IAA and ABA in leaves of maize seedlings.

2.15. Transcription analysis

The levels of related gene expression in maize seedlings inoculated or not inoculated with Y1 were determined by quantitative real-time PCR (RT-qPCR) under NaCl (0, 100 and 200 mmol/L) treatments. The total RNA from maize seedlings was extracted according to previous method (Li et al., 2019). The transcript levels of *ZmHKT1*, *ZmNHX1*, *ZmNHX2* and *ZmNHX3* (genes related to ion balance), *ZmNCED1* (the key gene for ABA synthesis), *ZmWRKY58* and *ZmDREB2A* (the key transcript factor genes related to plant response to abiotic stress), *ZmGR1* and *ZmAPX1* (the genes related to synthesis of antioxidant enzymes GR and APX) were measured. The sequences of primers, which were used for RT-qPCR, were presented in Table 1. The primers were designed to yield fragments of approximately 300 bp. RT-qPCR reactions were performed using the first strand cDNA from leaf tissues as a template. All PCR experiments were performed with SYBR Green Master Mix (PE BioSystems) on an iCycler iQ™5 thermocycler (Bio-Rad, USA) with 40 cycles and an annealing temperature of 55 ˚C was used (in a final volume of 25 μl). Cycle threshold values were determined by Bio-Rad iQ™5 version 2.1 software (Bio-Rad Laboratories) assuming 100 % primer efficiency. The constitutively expressed β-actin gene was

Table 1
The primers used for RT-qPCR detection.

Description	Forward (5'-3')	Reverse (5'-3')
ZmNHX1	TAGGTGGGTGAACGAGTCCACC	GTCTCCCAGTTCAAGTGCGCCG
ZmNHX2	GTCGGGGCGTGAGTATTCACGTTC	CCTGGTTAAGCACCTGTAAGGTGC
ZmNHX3	CGAGCCACATACTTGTGTTCGATG	GTTTCATCCTGGTTAAGCACCTG
ZmHKT1	CAGCTCCTCCTCATGACCCTCCTT	AATCGCCATGGCCGTCCTTGTTG
ZmGR1	GAGTCTCGTGGATTTGGATGGAC	TTTGAAGGCAAATCCAGCGCAGC
ZmAPX1	GACCGTGAGCGCCGAGTACAGC	CCTCCACGGCCACAACTCCCGC
ZmDREB2A	GGCTGAGCGCAACAAGCATTTGG	CCGGGGAAGTTAGTCCGTGCC
ZmWRKY58	GCAGAAGACAGAATCCAGGGTTC	GTTGCTTCTCTTGAACATCTC
ZmNCED1	GCGCAACGGCGCCAACCCCTGC	GTTGTAGACGAGGCCGGCGTTG
Actin	CAGTGGTCGAACAACGGGTATG	CCTGTTCATAATCAAGGGCAACG

used as an internal control. The relative expression levels of target gene were calculated using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001). Three mRNA samples from three independently harvested leaf samples (biological replicates) were analyzed.

2.16. Data analyses

Data analysis was conducted by software IBM SPSS PASW Statistics 18. The significance of the differences between control and treated groups was analyzed using Student's *t*-test or one-way ANOVA followed by Tukey's test (*$P < 0.05$, ** $P < 0.01$). Means and standard errors for all parameters were calculated from at least three replicates. Principal component analysis (PCA) and cluster analysis was performed using the online software MetaboAnalyst 4.0 (http://www.metaboanalyst.ca) (Chong et al., 2018).

3. Results

3.1. Identification and growth-promoting characteristics of the isolate

The strain isolated from maize rhizosphere was able to tolerate salt concentrations up to 10 % (mass percent, g NaCl/100 g water). The colonies were globular, Gram-positive, yellow colored, paired and protruding (Fig. 1A and B), whose growth temperature ranged from 10 to 45 °C and growth pH ranged from 4.0 to 9.0 (data not shown). According to the result of 16S rRNA-sequencing, the sequence of the strain was compared with available 16S rRNA sequences of related species from the NCBI databank. The sequence was submitted to GenBank with the accession number of MN013884. The phylogenetic tree constructed by MEGA 4.0 software showed that *K. rhizophila* Y1 and *Kocuria rhizophila* DSM 11926 shared high homology Fig. 1C).

The isolate was also examined for the plant growth promoting traits in different NaCl concentrations. As shown in Fig. 2A, strain Y1 could grow on Pikovskaya (PKV) agar culture containing NaCl (0 and 200 mmol/L) and formed clear halo zones on plates which were considered as the phosphate solubilizing capacity of strain Y1. Moreover, the P solubilization and IAA activity of strain Y1 were also quantitatively evaluated in nutrient medium as indicated in Material and method section supplemented with different NaCl concentrations (0, 100 and 200 mmol/L). Under 0 or 100 mmol/L NaCl stress, the concentrations of P-solubilization and IAA were 43 μg/mL and 80 μg/mL, respectively. However, the concentrations of P-solubilization and IAA were significantly ($P < 0.05$) decreased under 200 mmol/L NaCl stress compared to 100 mmol/L NaCl treatment (Fig. 2B and C). However, ACC deaminase activity could not be detected in Y1 culture medium supplemented with different concentrations of NaCl (0, 100 and 200 mmol/L) (data not shown).

3.2. Germination rate test

Under no salt stress conditions, germination rates of maize seeds inoculated or non-inoculated with strain Y1 were approximately 87.8 % and 85.6 %, respectively, while no significant ($P < 0.05$) difference was observed between these two groups. Under 30 mmol/L NaCl stress conditions, the higher germination rate of Y1-inoculated maize seeds was presented in comparison with non-inoculated maize seeds. The germination rates of maize seeds inoculated or non-inoculated with strain Y1 were approximately 65.5 % and 38.7 %, respectively (Fig. 3A and B).

3.3. Growth and biomass yield

As shown in Fig. 4A and B, with the treatment of NaCl at 100 and 200 mmol/L, maize seedlings exhibited a reduction in height of shoots and length of roots. The seedlings also showed leaf yellowing symptoms. However, strain Y1 inoculation significantly recovered the inhibition of maize seedlings growth under NaCl stress. A significant increase ($P < 0.05$) by 36.6 % and 49.1 % at I + 100 and I + 200 treatments, respectively, was observed in shoot height compared with controls (N + 100 and N + 200) (Fig. 4A and B). Similarly, the increase in other growth parameters including root length (57.6 % and 39.4 %), shoots dry weight (24.7 % and 50.5 %) and roots dry weight (40.2 % and 98.5 %) was observed in Y1-inoculated maize seedlings at I + 100 and I + 200 treatments in comparison with non-inoculated controls (N + 100 and N + 200) (Fig. 4C and D). However, no significant difference ($P < 0.05$) was observed in all growth parameters between inoculated and non-inoculated groups (I + 0 and N + 0) under non-NaCl stress conditions (Fig. 4C and D).

3.4. Photosynthesis parameters, EL, RWC, and chlorophyll content

Strain Y1 inoculation significantly ($P < 0.05$) enhanced gs, Pn and E values of maize plants under salt-stressed conditions (100 and 200 mmol/L) compared to controls (N + 100 and N + 200), respectively. However, photosynthesis parameters of maize leaves were not significantly ($P < 0.05$) improved at I + 0 treatment when compared to N + 0 treatment (Fig. 5A, B and C). The EL values in leaves of maize seedlings were dramatically ($P < 0.01$) reduced by 34.2 % and 28.5 % at I + 100 and I + 200 treatments, respectively, when compared to non-inoculated controls (N + 100 and N + 200) (Fig. 5D). Under NaCl (100 and 200 mmol/L) stress, RWC and chlorophyll content in Y1-inoculated maize seedlings were markedly ($P < 0.05$) increased compared to non-inoculated ones (Fig. 5E and F). However, in the absence of NaCl stress, the EL values, RWC and chlorophyll contents in Y1-inoculated groups were similar to that of non-inoculated controls.

3.5. The accumulation of H_2O_2 and O_2^-

As shown in Fig. 6, excessive ROS was produced in leaves of maize under NaCl stress. However, the leaves of inoculated maize seedlings exhibited less intense DAB and NBT staining compared to non-inoculated ones under NaCl stress conditions (100 and 200 mmol/L)

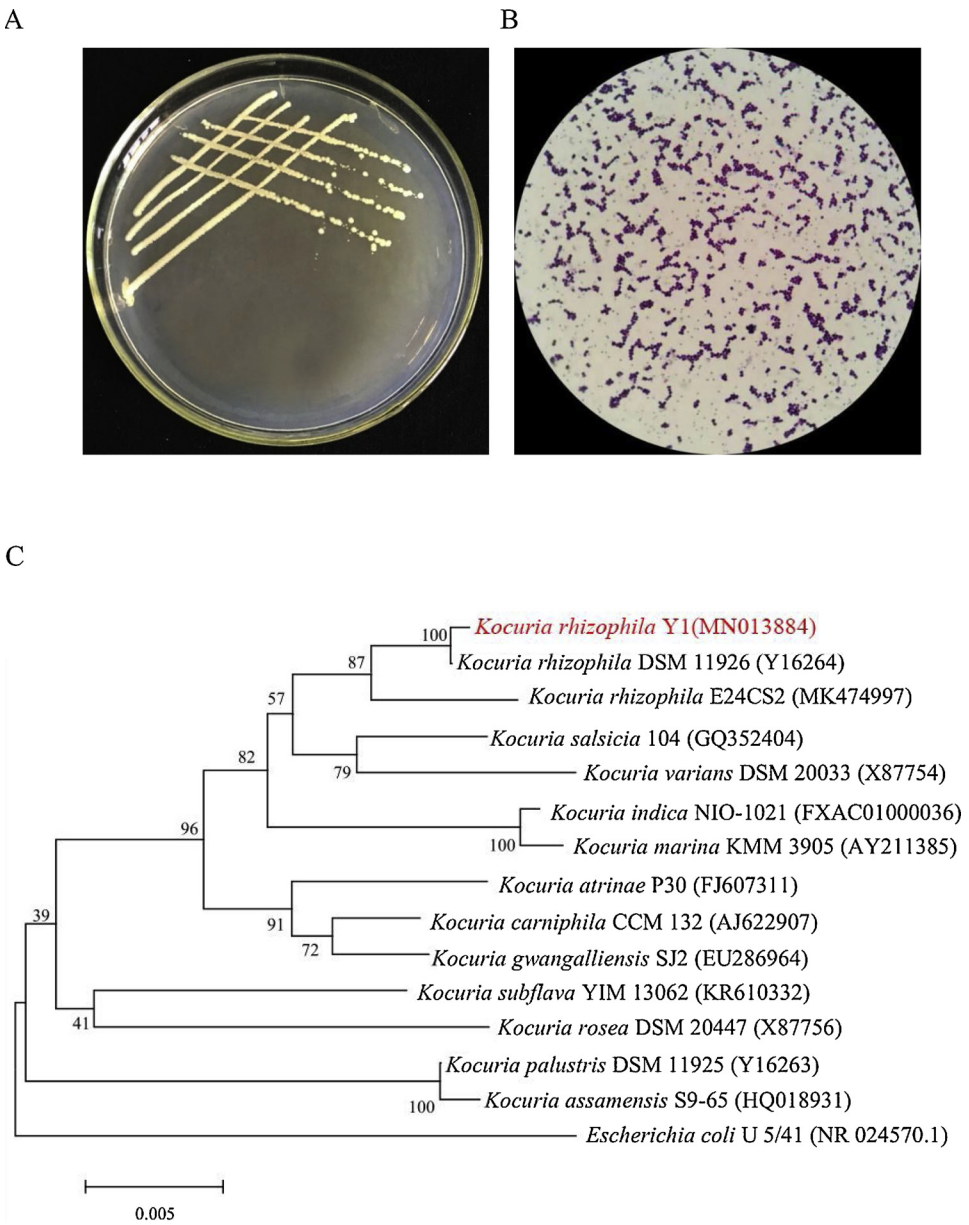

Fig. 1. Characterization of the Y1 strain. A. The morphological characteristics of Y1 colonies. B. Y1 g staining (1000×). C. Phylogenetic tree of the Y1 strain based on 16S rRNA gene sequences.

(Fig. 6A and B). The DAB and NBT staining results revealed that strain Y1 inoculation could reduce the levels of ROS (H_2O_2 and O_2^-) in maize leaves under NaCl stress. To further confirm this result, the quantitative determination of H_2O_2 content and O_2^- production was conducted. Under different concentrations (100 and 200 mmol/L) of NaCl stress, the H_2O_2 content in leaves of inoculated maize seedlings significantly ($P < 0.01$) decreased by 27.3 % and 24.2 % in comparison with non-inoculated groups. The similar decreasing trend appeared in O_2^- content which was dramatically decreased by 34.3 % and 18.8 % compared to non-inoculated groups (Fig. 6C and D). Nevertheless, no significant ($P < 0.05$) difference was observed in H_2O_2 content and O_2- production between inoculated and non-inoculated maize seedlings under non-NaCl stress treatment (Fig. 6C and D).

3.6. Antioxidant enzymes activities, GSH, GSSG, soluble sugar, proline and MDA content

Antioxidant enzymes such as APX, GPX and GR play significant roles in removing of harmful ROS in plants under stress conditions. As shown in Fig. 7A, B and C, the activities of APX, GPX and GR increased dramatically with the rise of NaCl concentration in non-inoculated treatments (I + 0, I + 100 and I + 200). It was worth noting that enzymes (APX, GPX and GR) activities of inoculated maize seedlings were markedly ($P < 0.05$) enhanced compared to non-inoculated ones. Similarly, the GSH and GSSG contents in leaves of inoculated seedlings were also significantly ($P < 0.05$) enhanced compared with non-inoculated plants (Fig. 7D and E). The proline and soluble sugar contents in leaves of seedlings were also determined in this study. As shown in Fig. 7F and G, under NaCl stress conditions, the soluble sugar contents and proline contents were increased significantly compared to non-salt conditions. However, strain Y1 inoculation increased markedly ($P < 0.05$) the proline and soluble sugar contents in maize leaves, when compared to non-inoculated maize under NaCl stress. Those results indicated that strain Y1 inoculation could increase the contents of soluble sugar and proline under NaCl stress. Additionally, MDA was regarded as one of the most significant products of membrane lipid

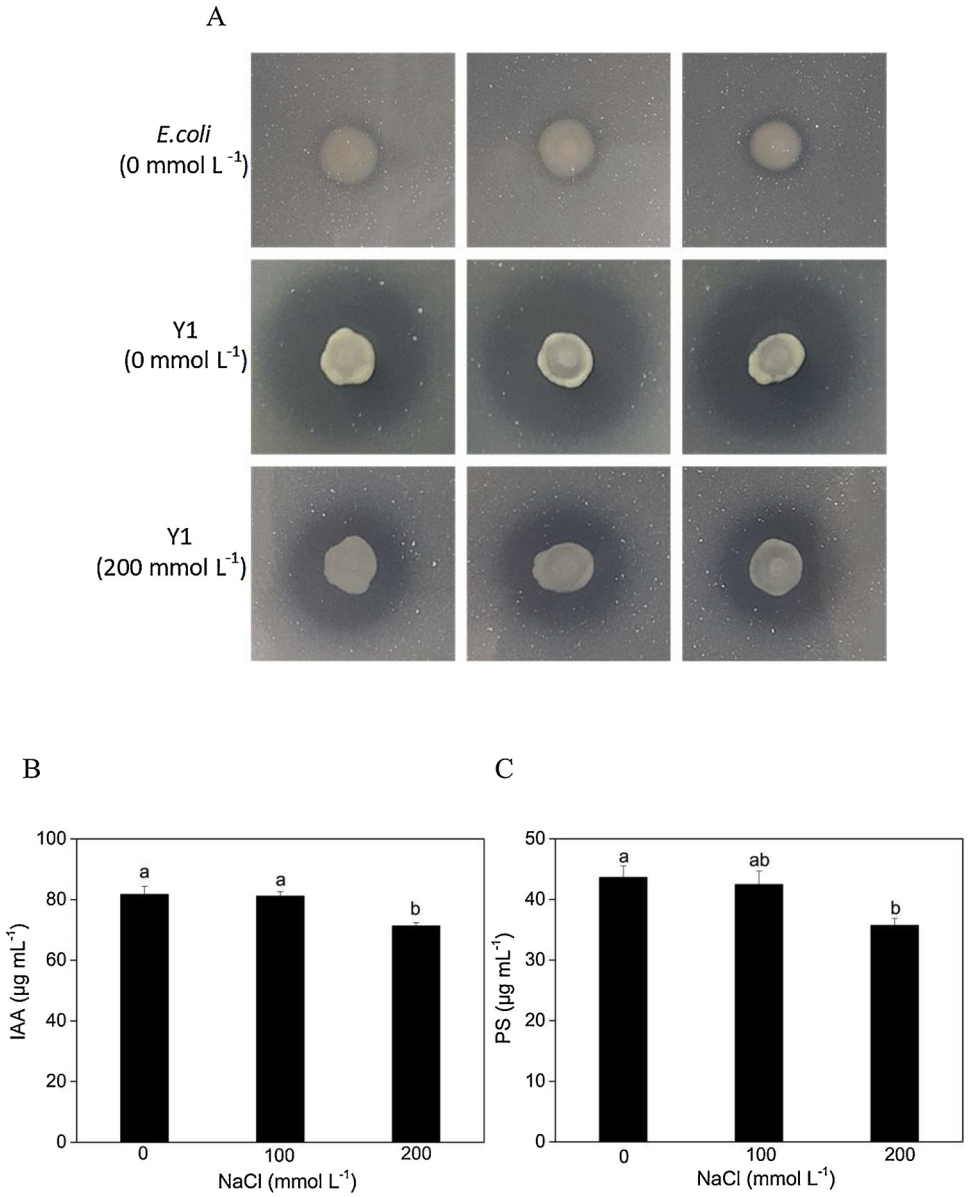

Fig. 2. Plant growth-promoting properties of the strain Y1 under different concentrations of NaCl. A. The size of clear halo zones formed by the *E.coli* and Y1 strain; B. IAA content; C. Phosphate solubilization (PS) production. Different letters indicate significant statistical differences based on one-way ANOVA ($P < 0.05$).

peroxidation, and its production indicated the degree of membrane system damage in plants under stress conditions. Under non-NaCl stress conditions, the MDA content did not differ significantly between the inoculated and non-inoculated maize seedlings. However, the MDA contents in maize leaves were significantly ($P < 0.05$) reduced by 35.8 % and 26.1 % at I + 100 and I + 200 treatments, respectively, in comparison with non-inoculated treatments (Fig. 7H).

3.7. Contents of nutrients, Ca^{2+}, K^+, Na^+ and K^+/Na^+ ratios

To reveal the effects of strain Y1 inoculation on absorption of nutrient elements and main ions by maize seedlings under NaCl stress, P and N content, Ca^{2+}, K^+ and Na^+ levels were measured. A decrease in contents of P, N, Ca^{2+} and K^+ was observed in leaves and roots of maize exposed to NaCl (100 and 200 mmol/L) stress. Nevertheless, strain Y1 inoculation significantly ($P < 0.05$) enhanced contents of P, N, Ca^{2+} and K^+ in comparison with non-inoculated treatments (Table 2). With the treatment of NaCl at the concentrations of 100 and 200 mmol/L, the Na^+ contents in leaves and roots of maize seedlings were markedly higher than non-NaCl treatments. However, the contents of Na^+ were significantly ($P < 0.05$) reduced at I + 100 and I + 200 treatments, when compared to N + 100 and N + 200 treatments (Table 2). Those results indicated that strain Y1 inoculation could promote the absorption of P, N, Ca^{2+} and K^+ and inhibit the absorption of Na^+ by maize seedlings. Moreover, K^+/Na^+ ratio was considered as an indicator of the balance between K^+ and Na^+ absorption by plants. As shown in Table 2, K^+/Na^+ ratio was significantly ($P < 0.05$) enhanced in leaves and roots of inoculated maize seedlings as compared to non-inoculated controls under NaCl (100 and 200 mmol/L) stress. This result further revealed that strain Y1 inoculation could promote absorption of K^+ and inhibit absorption of Na^+ by maize seedlings. Similarly, there was no remarkable difference in above parameters between non-inoculated and inoculated maize seedlings without NaCl stress.

3.8. The contents of IAA and ABA

Endogenous phytohormones including IAA and ABA play critical

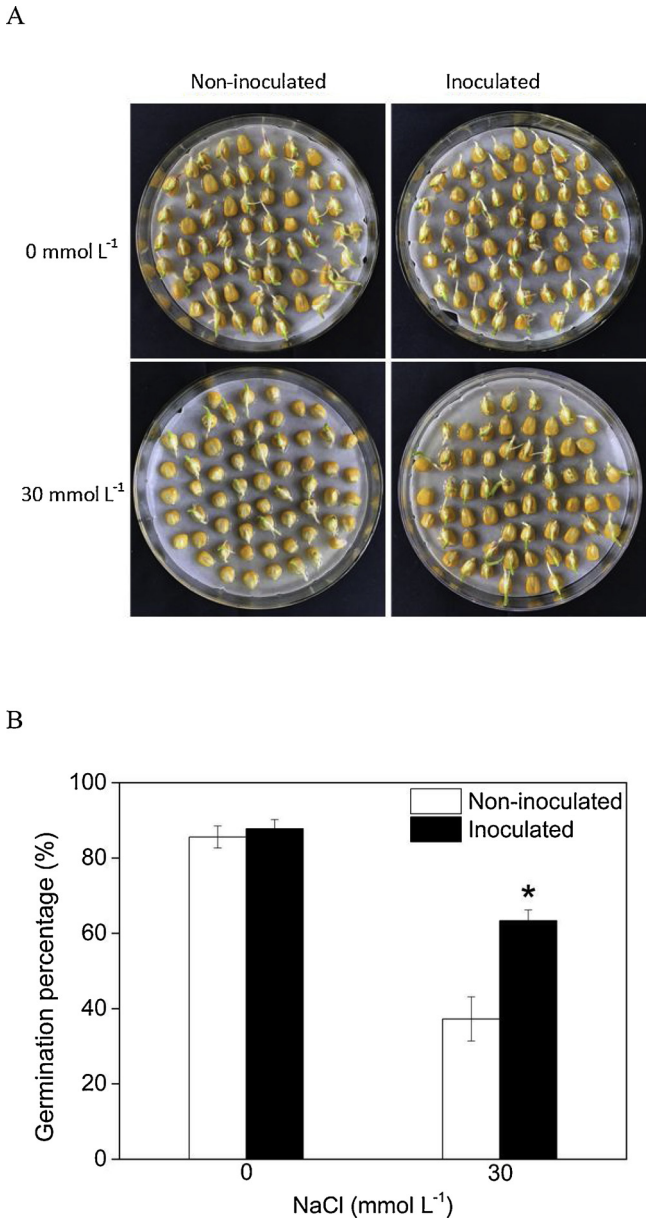

Fig. 3. Germination rates of Y1-inoculated maize seeds under NaCl stress. A. Photograph of maize seeds germination under different conditions. B. Germination rates of non-inoculated and inoculated maize seeds under NaCl (0 and 30 mmol/L) stress. The germination rate was calculated for 7 days. Columns with asterisks (*) represent remarkable differences compared to the control (*$P < 0.05$).

roles in plant response to NaCl stress. As shown in Fig. 8, the production of IAA was reduced and the level of ABA was enhanced in the presence of NaCl stress. However, under different NaCl concentrations (100 and 200 mmol/L), IAA contents in leaves of inoculated-maize seedlings were significantly ($P < 0.05$) increased by 29.2 % and 53.9 %, respectively, in compared to non-inoculated ones (Fig. 8A). In contrary, the contents of ABA in maize leaves was significantly ($P < 0.05$) decreased by 18.2 % and 17.1 %, respectively, under 100 mmol/L and 200 mmol/L NaCl treatments inoculated with strain Y1, when compared to non-inoculated treatments (Fig. 8B). In the absence of NaCl stress, no significant variation of IAA and ABA contents in maize leaves between non-inoculated and inoculated treatments was observed. The above results outlined the role of strain Y1 in enhancing IAA content and reducing ABA content in maize under NaCl stress.

3.9. Expression of relative genes in maize seedlings under different conditions

As showed in Fig. 9A, B, C and D, under NaCl stress, the expression levels of *ZmHKT1*, *ZmNHX1*, *ZmNHX2* and *ZmNHX3* were upregulated under NaCl stress in comparison with controls. Furthermore, strain Y1 inoculation markedly ($P < 0.05$) enhanced the expression levels of *ZmNHX1*, *ZmNHX2* and *ZmNHX3* genes under NaCl (100 and 200 mmol/L) stress conditions, when compared to the non-inoculated groups. However, the expression levels of *ZmHKT1* were not increased significantly in the above conditions. *ZmWRKY58* and *ZmDREB2A* are involved in plant responses to various abiotic stresses and possibly play a role in plants' ability to tolerate salt stress. Meanwhile, the upregulation of *ZmGR1* and *ZmAPX1* genes expression could enhance the removal of ROS by increasing enzymes (GR and APX) activities. Our study indicated that the expression levels of *ZmWRKY58*, *ZmDREB2A*, *ZmGR1* and *ZmAPX1* were significantly ($P < 0.05$) upregulated at I + 100 and I + 200 treatments in comparison with N + 100 and N + 200 ones (Fig. 9E, F, G and H). NCED is regarded as a critical regulatory enzyme in the ABA biosynthesis pathway. This study indicated that strain Y1 inoculation reduced ABA content markedly in comparison with non-inoculated treatments under salt stress treatment. Similarly, the expression levels of *ZmNCED1* were also significantly decreased by 22.4 % and 16.3 % in *K. rhizophila* Y1-inoculated maize under 100 and 200 mmol/L salt stress, respectively, compared to non-inoculated ones (Fig. 9I).

3.10. Cluster and PCA analysis of all parameters

Cluster analysis of all parameters revealed that all samples clustered into three major clusters (Fig. 10A). The changes of the parameters are further observed in the heatmap and indicated that the different treatment groups could be separated based on these parameters (Fig. 10A). The PCA analysis of all parameters revealed the effects of salt stress and *K. rhizophila* Y1 inoculation on maize plants (Fig. 10B). It was found that PC1 accounted for 91.5 % of the variance and that PC2 accounted for 5.1 % of the variance. The I + 100, I + 200, N + 100 and N + 200 treatment group was clearly differentiated from other groups which might mainly be attributed to the changes of parameters including ABA content, IAA level, EL, RWC and GSSG values. The I + 0 group could not be clearly differentiated from N + 0 treatment group, meaning that there was no remarkable difference between non-inoculated and inoculated maize seedlings without NaCl stress.

4. Discussion

In the present study, a PGPR strain Y1 capable of effectively producing IAA and solubilizing calcium phosphate under salt stress treatment was isolated and identified as *K. rhizophila* (Fig. 1). The species of this genus are aerobic, non-encapsulated, gram-positive, and coccoid belonging to the order of *Actinomycetales*. Members of the genus *Kocuria* were collected from various sources, such as air, freshwater, the rhizosphere of plants, seawater and marine sediment (Kaur et al., 2011). *K. rhizophila* DC2201 was tolerant to certain selected organic chemicals and capable of growing and reproducing in various stressful conditions (Fujita et al., 2006). DeRito et al. (2005) indicated that the species of the genera *Kocuria* was considered as the main potential phenol-degraders in soil with phenol as the spiked contaminant. Karn et al. (2011) reported that the strain *Kocuria* sp. CL2 could remove about 60 % of pentachlorophenol (PCP) from the sludge within two weeks. The genus *Kocuria* also includes some halotolerant strains including *K. marina* and *K. kristinae* which can grow in medium containing up to 10 % NaCl in growth media (Kim et al., 2004). The actinomycete isolate *Kocuria* sp. LSM1-65 showed the siderophore producing capability (Haiyambo et al., 2015). Previous report revealed that the inoculation of wheat by ACC deaminase-producing *K. rhizophila* was a promising

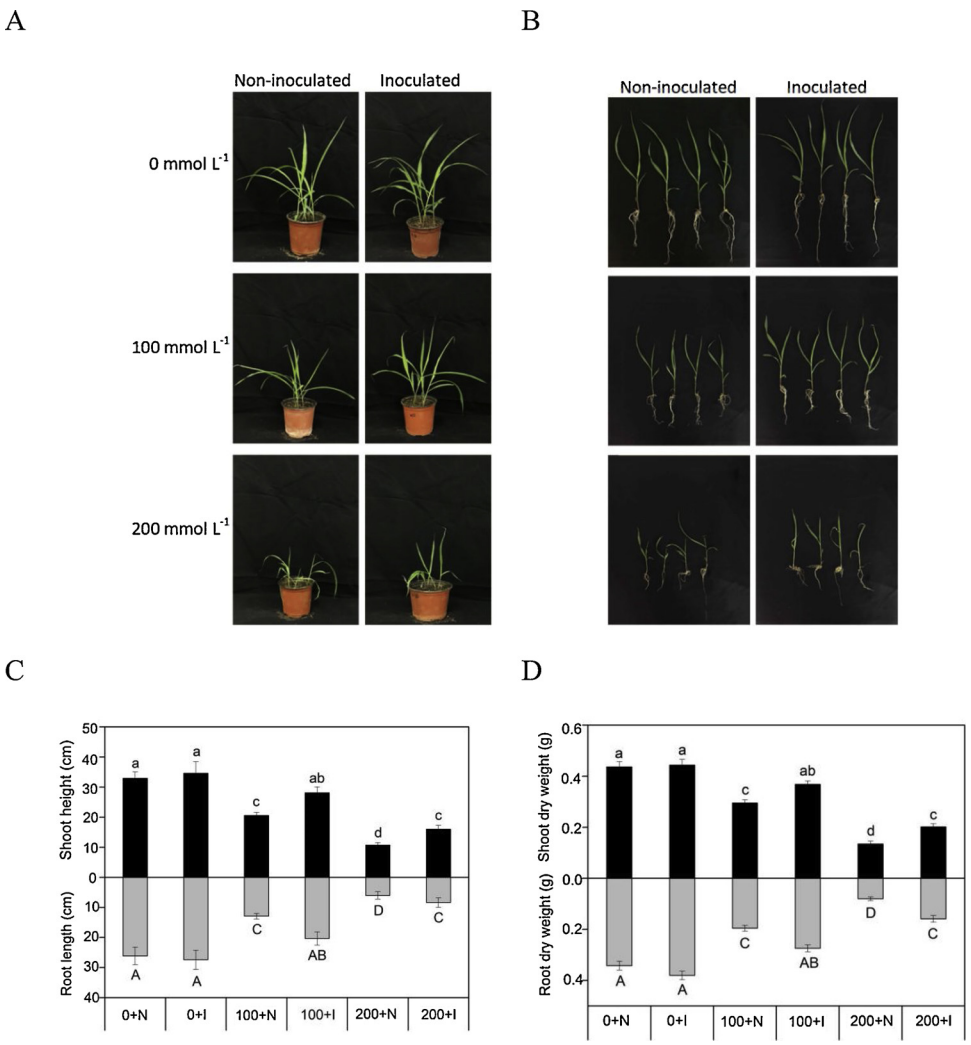

Fig. 4. A. Photograph of inoculated maize seedlings and non-inoculated maize seedings irrigated with different concentrations (0, 100 and 200 mmol/L) of NaCl solution for twice a week. B. Photograph of uprooted maize seedlings. C. Effect of strain Y1 on shoots height and roots length of maize under NaCl (0, 100 and 200 mmol/L) stress. D. Effect of strain Y1 on FWs of shoots and roots under NaCl (0, 100 and 200 mmol/L) stress. Six groups of treatments were carried out as follows: non-inoculated Y1 subjected to 0 mmol/L NaCl stress (N + 0), inoculated Y1 subjected to 0 mmol/L NaCl stress (I + 0), non-inoculated Y1 subjected to 100 mmol/L NaCl stress (N + 100), inoculated Y1 subjected to 100 mmol/L NaCl stress 100 mmol/L NaCl (I + 100), non-inoculated Y1 subjected to 200 mmol/L NaCl stress (N + 200) and inoculated Y1 subjected to 200 mmol/L NaCl stress (I + 200). Different letters represent significant statistical differences based on one-way ANOVA ($P < 0.05$).

approach for the enhancement of wheat growth and salt stress tolerance (Afridi et al., 2019). *K. turfanensis* strain 2M4 was found to be a phosphate solubilizer, siderophore producer and IAA producer, which was isolated from the rhizosphere soil of halotolerant plant *Suaeda fruticosa* (Goswami et al., 2014). Similarly, this study indicated that *K. rhizophila* Y1 could effectively producing IAA and was capable of solubilizing calcium phosphate (Fig. 2). Although the above documented evidences indicated that some species belonging to *Kocuria* genus showed suitable potential to be used as PGPR, to the best of our knowledge, this is the first report showing that *Kocuria* genus can be used as PGPR to enhance the photosynthesis and growth of maize plant due to the production of IAA and phosphate solubilization under salt tress treatment.

PGPR can enhance plants tolerance to various stresses through its direct growth-promoting effects on plants (Bhattacharyya and Jha, 2012). In our study, we observed that the maize inoculated with *K. rhizophila* Y1 showed better growth prospects in comparison with non-inoculated plants. For example, strain Y1 alleviated the adverse effect of salt stress on shoots and roots growth as compared with controls (Fig. 4). The inoculation with *K. rhizophila* Y1 could also increase the chlorophyll contents in maize compared on non-inoculation control (Fig. 5). Our study was consistent with previous study that inoculation with PGPR stimulated the synthesis of chlorophyll and therefore caused an obvious enhancement in the growth of radish plants (*Raphanus sativus*) (Mohamed and Gomaa, 2012). This study showed that the inoculation of maize with *K. rhizophila* Y1 markedly alleviated the toxic effects of salt stress on the photosynthetic performance (Fig. 5). Consistently, Mayak et al. (2004) indicated that tomato plants inoculated with a PGPR *Achromobacter piechaudii* ARV8 enhanced plant photosynthesis rate under saline conditions. This study also showed that the leaf RWC in Y1-inoculated maize seedlings was markedly increased compared to non-inoculated ones, which might contribute to the enhanced photosynthesis rate in the strain Y1-inoculated maize. The close relationship between RWC and photosynthetic rate in plants have also been studied. For example, Lawlor (2002) reported that the reduction of RWC strongly reduced photosynthesis and transpiration in plants.

PGPR traits, such as IAA synthesis, nitrogen fixation, and phosphate solubilization, had positive effects on plant growth by influencing plant development and increasing nutrient availability (Bhattacharyya and Jha, 2012). This study indicated that *K. rhizophila* Y1 was able to solubilize tricalcium phosphate and synthesize IAA (Fig. 2). Therefore, growth promotion by the strain *K. rhizophila* Y1 might be mediated by these PGPR traits. It was suggested that the phytohormone IAA played important roles in promotion of plant growth (Fahad et al., 2015). Experiment conducted on soybean plants indicated that the accumulation of IAA markedly reduced with salt stress treatment, however, the IAA content was significantly increased in soybean plant with inoculation of PGPR (Kumari et al., 2015). It was indicated that wheat plants inoculated with *Pseudomonas* species and *B. cereus* showed high IAA content in their leaves (Ul Hassan and Bano, 2015). Previous reports also showed that rice plants inoculated with osmotolerant bacteria exhibited higher seed germination rates with salt stress treatment compared to control. This might be due to the reason that the inoculated bacteria could increase rice IAA content which played an essential role in rice seed germination (Etesami et al., 2014). Our study

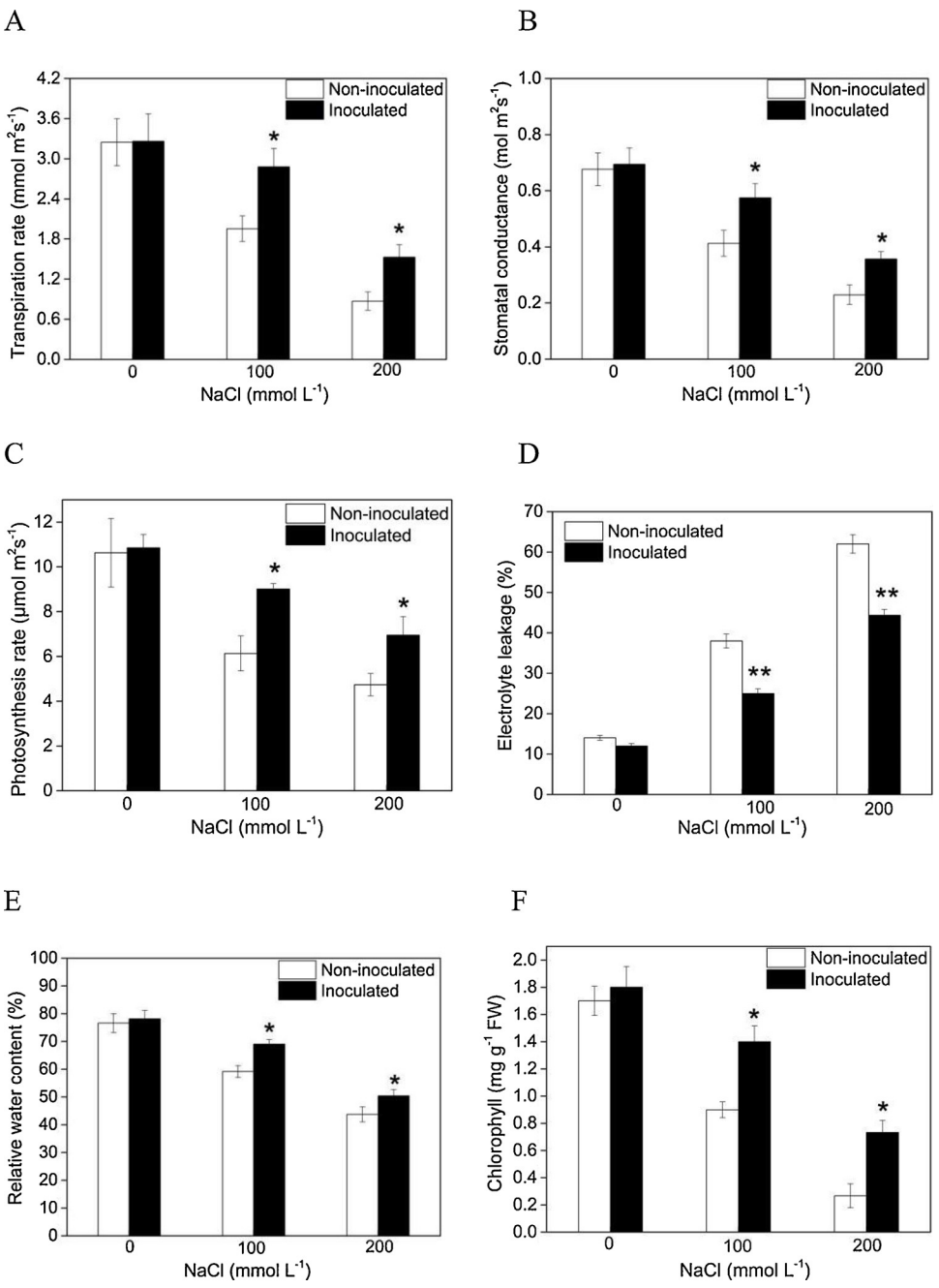

Fig. 5. A-C. Influence of strain Y1 on photosynthesis parameters of maize seedlings including stomatal conductance (gs), net photosynthesis rate (Pn) and transpiration rate (E) under NaCl (0, 100 and 200 mmol/L) stress. D. Electrolyte leakage (EL) of strain Y1 inoculated and non-inoculated maize seedlings under NaCl stress. E. Relative water content (RWC) of leaves in strain Y1 inoculated and non-inoculated maize seedlings under NaCl stress. F. Chlorophyll contents of non-inoculated and inoculated maize seedlings under NaCl conditions. Columns with asterisks (* and **) represent remarkable differences compared to the control (**$P < 0.01$ and *$P < 0.05$).

also indicated that *K. rhizophila* Y1 inoculation increased the seed germination rate of maize (Fig. 3). This can also be explained in view of the bacterial capability to produce IAA, which was adsorbed on the surface of seeds and being used to promote seed germination (Bhattacharyya and Jha, 2012).

Phosphorous ranks next to nitrogen among essential plant nutrients in importance because of its essential role in the plant life processes (Yang et al., 2009). However, a very low availability and mobility for P uptake by plants was observed due to the high sorption affinity of P to soil particles. It was also indicated that phosphate was the primary limiting factor for plant growth, especially under salinity conditions. Numerous reports indicated that phosphate content could be enhanced in plant inoculated with phosphate solubilizing bacteria (PSB) (Yang et al., 2009). PSB was known to fix soil P and enhance solubilization of applied phosphates, leading to higher crop yields and plant tolerance to various abiotic stresses. The principal member of PSB was distributed in genus *Acinetobacter*, *Pseudomonas* and *Bacillus* (Zaidi et al., 2009). In this investigation, the strain *K. rhizophila* Y1 was considered as phosphate solubilizer and the solubilization of phosphate might help maize to overcome the nutrient deficiency and hormonal imbalance under saline conditions (Fig. 2). Consistently, potato plants inoculated with PGPR showed enhanced pigment synthesis and increment of nutrient uptake, particularly of P and N (Dawwam et al., 2013), and subsequently increased plant growth was observed. *A. calcoaceticus* inoculated *Cucumis sativus* also exhibited higher P content in comparison with non-inoculated controls under saline conditions (Kang et al., 2014).

Salt tolerance was related to plants' capacity to promote K^+ influx and avoid Na^+ accumulation (Fahad et al., 2015). A number of previous reports indicated that salt stress could elevate the ratios of Na^+/K^+ and Na^+/Ca^{2+} in plants, which were somewhat more susceptible to nutritional disorders and osmotic stress (Fahad et al., 2015). PGPR could serve to maintain high ratio of K^+/Na^+ and ion homeostasis in plant shoots through increasing Na^+ exclusion by roots and decreasing

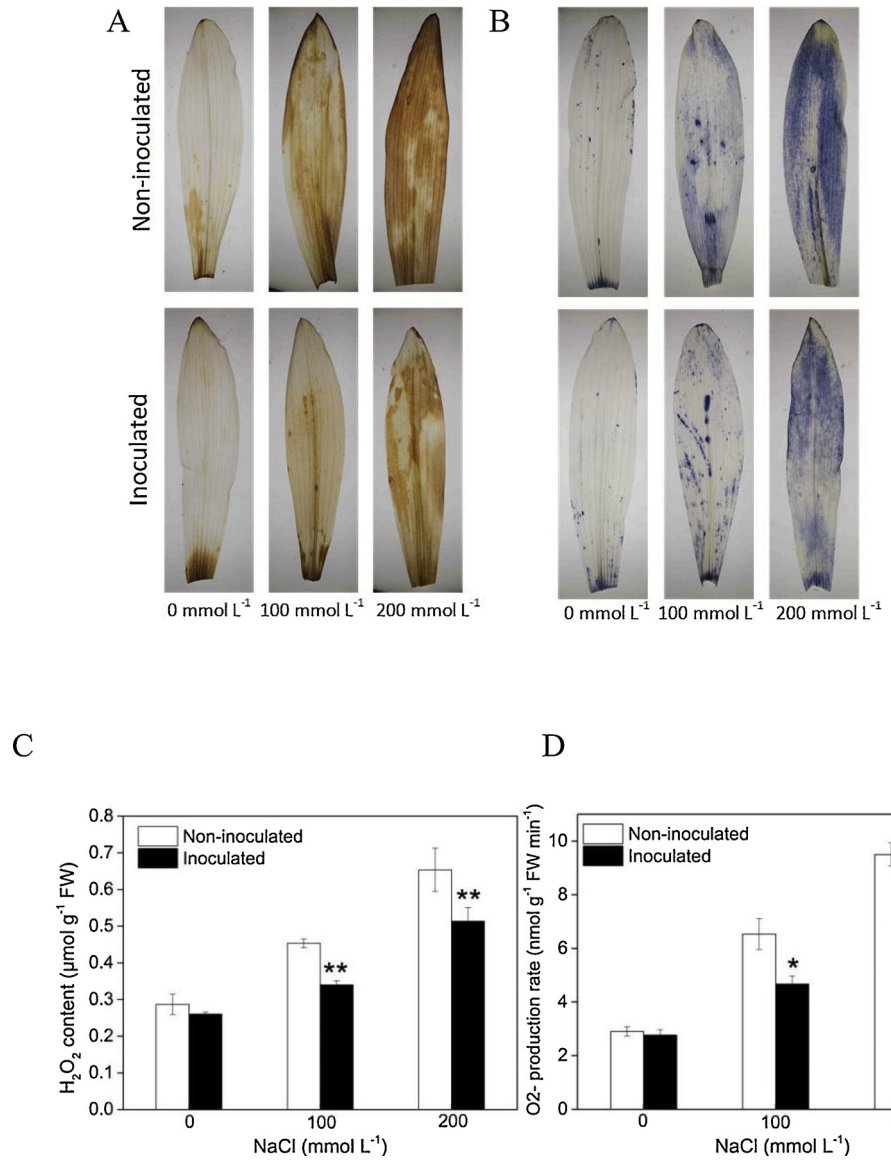

Fig. 6. The accumulation of H_2O_2 (A and C) and O_2^- (B and D) in leaves of maize seedlings with or without Y1 inoculation under NaCl stress. A. Diaminobenzidine (DAB) staining. B. Nitroblue tetrazolium (NBT) staining. C. H_2O_2 content; D. O_2- production. Columns with asterisks (* and **) represent remarkable differences compared to the control (**$P < 0.01$ and *$P < 0.05$).

Cl^+ and Na^+ levels in leaves (Kang et al., 2014). This study showed that salinity elicited a decrease in Ca^{2+} and K^+ levels and an increase in Na^+ content with or without strain treatment. However, the *K. rhizophila* Y1-inoculated maize showed marked increase in K^+ uptake and decrease in Na^+ levels in comparison with non-inoculated controls, thus increased ratios of K^+/ Na^+ in maize plants under salinity stress was observed (Table 2). Similarity, Niu et al. (2016) indicated that *B. subtilis* (GB03) inoculation significantly decreased Na^+ and increased K^+ accumulation in halophyte grass *Puccinellia tenuiflora* under saline conditions. Our results were also in accordance with the findings of He et al. (2018) who reported a marked decrease in shoot Na^+ concentration and a significant increase in shoot K^+ concentration, and therefore an increase in the ratio of K^+/Na^+ was exhibited in *Haloxylon ammodendron* with *Pseudomonas* sp. M30-35 (characterized as a PGPR) inoculation under salinity stress.

With the salt treatment, the negative effects of Na^+ could be alleviated in plants by Cl^- and Na^+ ion exclusion mechanisms, such as recycling Na^+ from shoot to root, sequestering Na^+ into vacuoles, exporting Na^+ out of cells and restricting Na^+ uptake from soil (Fahad et al., 2015). *Arabidopsis* Na^+/H^+ antiporters (NHX5 and NHX6) could sequester Na^+ into vacuoles and expel them out of plant cells (Bassil et al., 2011). In the current study, *K. rhizophila* Y1 inoculation markedly mitigated the Na^+ levels and upregulated the *NHX* genes expression in maize under saline conditions, suggesting that *K. rhizophila* Y1 might decrease Na^+ content in maize plants through sequestering this ion into vacuoles, expelling it out of cells, thereby ameliorating Na^+ toxicity and enhancing salinity tolerance (Fig. 9B, C and D). Previous study indicated that sodium transporter NHX could be regulated by IAA in *Arabidopsis* (Chen et al., 2010). El-Esawi et al. (2018b) indicated that *S. liquefaciens* KM4 inoculation significantly enhanced the maize biomass correlated with the upregulation of *NHX1* gene under salt stress treatment. Furthermore, Chen et al. (2016) also indicated that the *B. amyloliquefaciens* SQR9-inoculated maize showed enhanced salt tolerance by upregulation of *NHX1* and *HKT1* genes expression. Bharti et al. (2016) showed that the inoculation of wheat seedlings with *Dietzia natronolimnaea* showed less oxidative damage compered to non-inoculation control, through improvement of some important transport proteins genes expression, such as *NHX*, *HKT*, *HAK* and *SOS*, which were related to the compartmentalization and exclusion of toxic sodium ions. However, no significant change in the expression of *ZmHKT1* gene

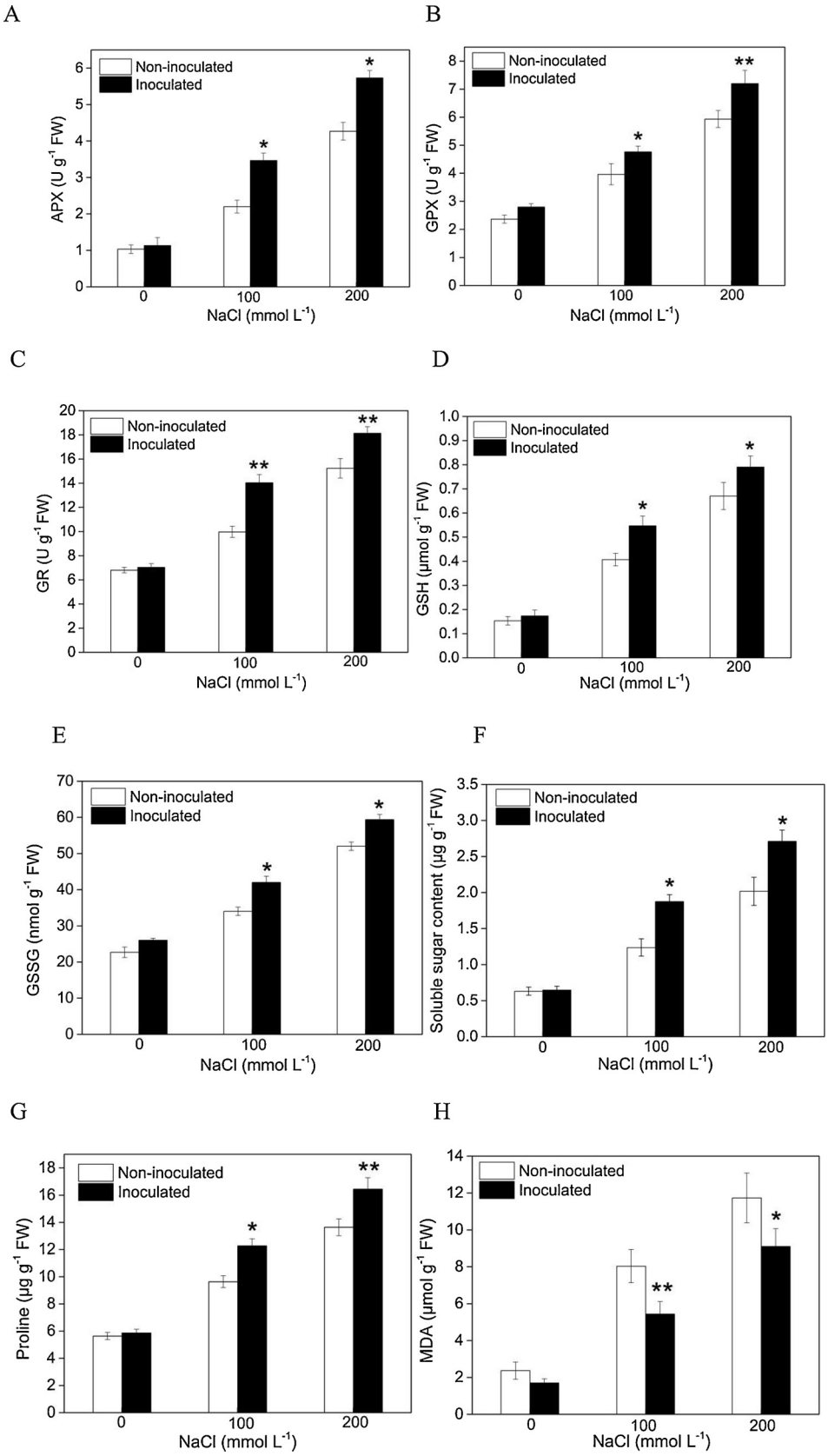

Fig. 7. A-C. Antioxidant enzymes including APX, GPX and GR activities of maize seedlings with or without Y1 inoculation under NaCl stress. Effects of Y1 inoculation on GSH (D), GSSG (E), soluble sugar (F), proline (G) and MDA (H) contents of maize seedlings grown in pots for 21 days under NaCl (0, 100 and 200 mmol/L) stress. Columns with asterisks (* and **) represent remarkable differences compared to the control (**$P < 0.01$ and *$P < 0.05$).

Table 2
Effects of strain Y1 inoculation on nutrient elements and essential ions absorption by maize seedlings grown in pots for 21 days under NaCl (0, 100 and 200 mmol/L) conditions. Six groups of treatments were carried out as follows: non-inoculated Y1 subjected to 0 mmol/L NaCl stress (N + 0), inoculated Y1 subjected to 0 mmol/L NaCl stress (I + 0), non-inoculated Y1 subjected to 100 mmol/L NaCl stress (N + 100), inoculated Y1 subjected to 100 mmol/L NaCl stress 100 mmol/L NaCl (I + 100), non-inoculated Y1 subjected to 200 mmol/L NaCl stress (N + 200) and inoculated Y1 subjected to 200 mmol/L NaCl stress (I + 200). Different letters represent significant statistical differences based on one-way ANOVA ($P < 0.05$).

Groups	N content (mg g^{-1} DW)		P content (mg g^{-1} DW)		Ca^{2+} content (mg g^{-1} DW)		K^+ content (mg g^{-1} DW)		Na^+ content (mg g^{-1} DW)		K^+/Na^+ ratio	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
0+N	32.03 ± 0.75a	24.93 ± 1.42a	22.33 ± 1.07a	18.56 ± 1.40a	1.64 ± 0.06a	1.18 ± 0.08a	12.84 ± 0.53a	8.75 ± 0.52a	3.41 ± 0.08d	2.18 ± 0.09d	3.77 ± 0.22a	4.01 ± 0.08a
0+I	32.50 ± 1.42a	26.00 ± 1.37a	22.67 ± 0.84a	19.45 ± 1.23a	1.68 ± 0.03a	1.24 ± 0.12a	13.71 ± 0.58a	9.15 ± 0.59a	3.26 ± .045d	2.11 ± 0.07d	4.26 ± 0.42a	4.33 ± 0.14a
100+N	24.57 ± 0.59c	15.53 ± 0.45c	13.60 ± 0.97b	10.49 ± 1.12c	0.90 ± 0.03b	0.57 ± 0.03b	7.49 ± 0.22b	4.86 ± 0.32b	7.40 ± 0.32b	5.41 ± 0.17b	1.02 ± 0.07c	0.90 ± 0.05c
100+I	28.23 ± 0.73b	20.43 ± 1.18b	19.00 ± 0.78a	15.49 ± 1.16b	1.24 ± 0.05a	0.81 ± 0.08a	8.90 ± 0.25a	6.19 ± 0.32a	5.32 ± 0.28c	4.14 ± 0.14c	1.68 ± 0.04b	1.50 ± 0.06b
200+N	17.10 ± 0.72d	9.40 ± 0.58d	7.33 ± 0.48c	5.41 ± 0.59d	0.55 ± 0.03c	0.18 ± 0.03c	4.99 ± 0.14c	1.52 ± 0.23c	12.64 ± 0.54a	10.41 ± 0.58a	0.40 ± 0.02d	0.15 ± 0.02e
200+I	22.73 ± 1.07c	13.07 ± 0.32c	10.77 ± 0.59b	8.21 ± 0.89c	0.75 ± 0.03b	0.33 ± 0.05b	6.49 ± 0.26b	2.85 ± 0.23b	8.80 ± 0.30b	8.27 ± 0.40b	0.74 ± 0.04c	0.35 ± 0.03d

was observed in maize inoculated with *K. rhizophila* Y1 under saline conditions, which was contrary with the report by Zhang et al. (2008) that *B. subtilis* GB03-inoculated *A. thaliana* showed enhancement of salt tolerance by regulation of *HKT1* gene expression (Fig. 9A).

Scavenging of salt induced ROS is a vital defense mechanism in plants by using various interconnected enzymatic and non-enzymatic antioxidants. GSH is a crucial scavenger of ROS in plants and its content is commonly increased under salt stress treatment in order to detoxify the accumulated ROS and alleviate the damage to cellular membrane damage caused by ROS (Ahmad et al., 2008). In this study, *K. rhizophila* Y1 inoculation markedly increased the activity of enzymatic antioxidants (GR, APX and GPX) and the content of non-enzymatic antioxidant GSH in maize under saline conditions in comparison with non-inoculation controls (Fig. 7). *K. rhizophila* Y1 inoculation also enhanced the expression of genes encoding antioxidant enzyme (*ZmGR1* and *ZmAPX1*) under saline conditions, which was in concordance with the induced antioxidant enzyme activities (Fig. 9G and H). These results suggested that *K. rhizophila* Y1 could stimulate the plant defense response to detoxify ROS and enhanced the growth of maize seedlings. This study was consistent with previous reports by Hashem et al. (2016) who showed that antioxidant activity was enhanced in PGPR-inoculated plants. Also, Nunkaew et al. (2014) reported that inoculation with 5-aminolevulinic acid producing bacteria could reduce the generation of H_2O_2, and increase the activities of antioxidant enzymes such as catalase (CAT), APX, superoxide dismutase (SOD) and GR in salt stressed rice plants. Moreover, the increased expression of various antioxidant enzymes genes such as *GR*, *GPX*, *CAT*, *SOD* and *APX* was observed in *D. natronolimnaea*-inoculated wheat which contributed to increased tolerance of wheat to salinity stress (Bharti et al., 2016). However, PGPR could also reduce antioxidant enzymes activity in PGPR-inoculated plants under saline conditions (Upadhyay et al., 2012). A similar study was reported by Han and Lee (2005) that decreased GR and APX activity was observed when lettuce plants were inoculated with PGPR (*Rhizobium* sp. and *Serratia* sp.) under salinity stress.

Proline functions as an osmoprotectant and plays important roles in the antioxidant defense system. Proline accumulation was involved in conferring protection from salt stress-induced damage in plants (Fahad et al., 2015). Salt stress could increase foliar proline content, especially in plants inoculated with the PGPR (Akram et al., 2016). In the present study, an increase in proline accumulation was observed under saline conditions and *K. rhizophila* Y1-inoculated maize leaves showed higher proline content than non-inoculated plants (Fig. 7G). To date, there are some evidences of close correlation between proline accumulation and the induction of salt stress tolerance by PGPR, however, the available results are still controversial. A study conducted by Hamdia et al. (2004) showed that proline accumulation increased in plant under saline condition, but this accumulation was declined by inoculation with PGPR. Also, Rojas-Tapias et al. (2012) indicated that inoculation with strain *Azotobacter* markedly reduced the proline content in maize leaves.

ABA is involved in various plant physiological processes including stomatal aperture control and seedling growth as well as plants' responses to environmental stresses such as salt and drought (Fahad et al., 2015). In this study, the ABA content and the *ZmNCED1* gene expression in maize with *K. rhizophila* Y1 inoculation markedly decreased under saline conditions in comparison with the non-inoculation control (Figs. 8B and 9I), which was similar with previous study that the accumulation of ABA was decreased in *Glycine max* inoculated with *Aspergillus flavus* CSH1 compared to control (Lubna et al., 2018). However, the mechanism of microbe-plant interactions related to ABA pathway is still needed to be investigated. Recently, Shahzad et al. (2016) indicated that *B. amyloliquefaciens* RWL-1-inoculated rice plants showed enhanced salt tolerance through increased ABA accumulation which was contrary to the present study. *Arabidopsis* with *P. indica* inoculation also showed enhanced ABA content, which was in turn

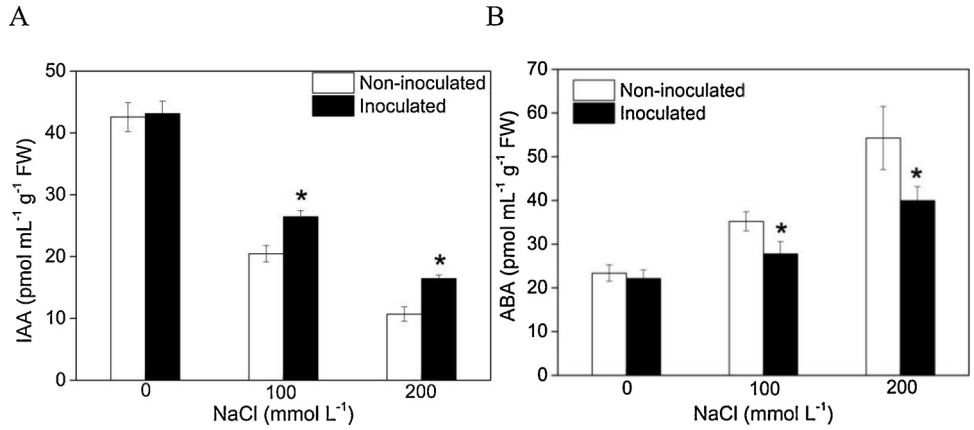

Fig. 8. Effect of strain Y1 inoculation on endogenous phytohormones of maize seedlings grown in pots for 21 days under NaCl (0, 100 and 200 mmol/L) stress conditions. A. IAA content; B. ABA content. Columns with asterisks (*) represent remarkable differences compared to the control (*$P < 0.05$).

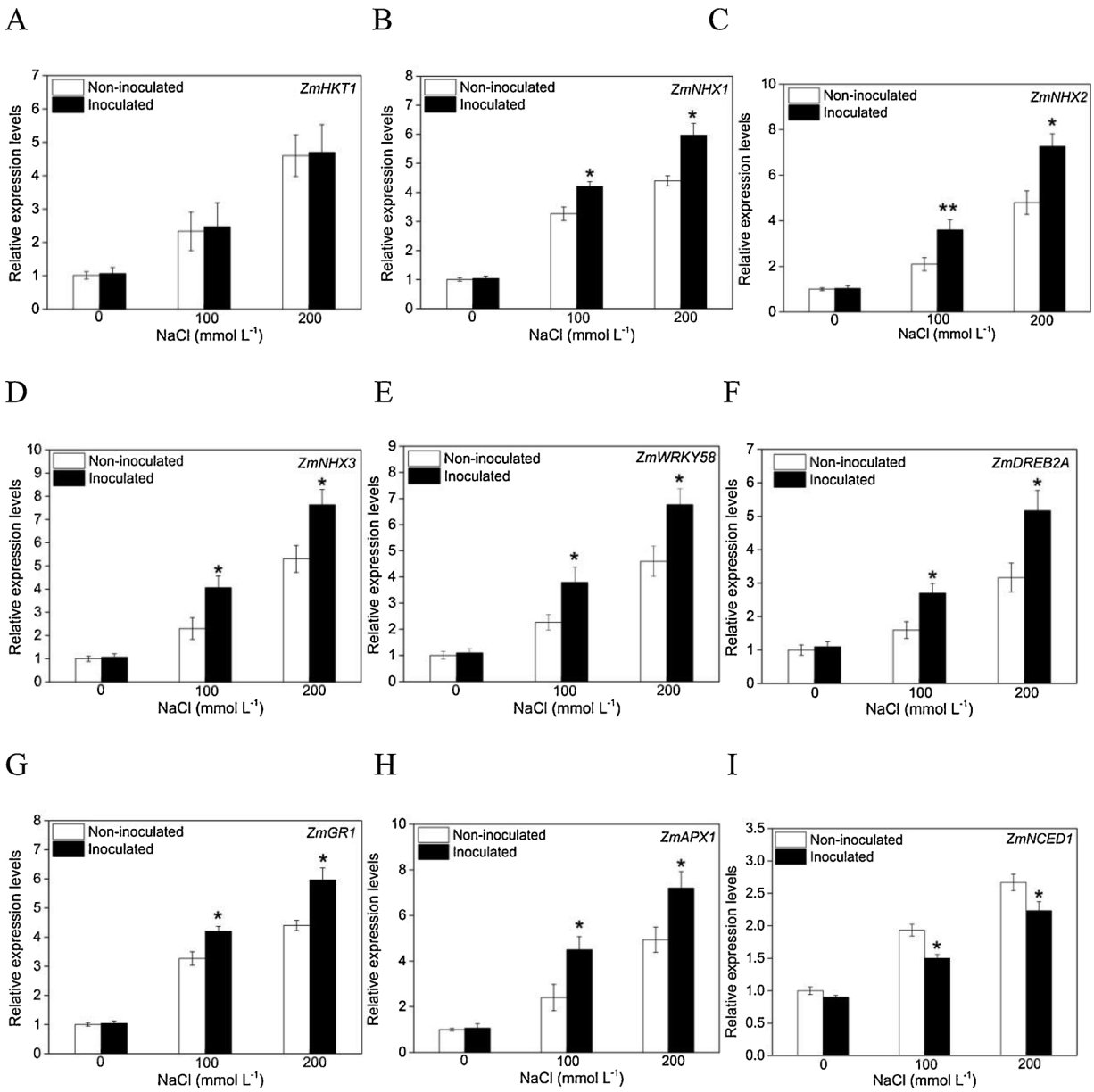

Fig. 9. Effect of strain Y1 inoculation on expression of genes related to salt tolerance in maize seedlings under NaCl (0, 100 and 200 mmol/L) stress conditions. A-D. Expression levels of *ZmHKT1*, *ZmNHX1*, *ZmNHX2* and *ZmNHX3*; E and F. Expression levels of *ZmWRKY58* and *ZmDREB2A*; G and H. Expression levels of *ZmGR1* and *ZmAPX1*; I. Expression levels of *ZmNCED1*. Columns with asterisks (* and **) represent remarkable differences compared to the control (**$P < 0.01$ and *$P < 0.05$).

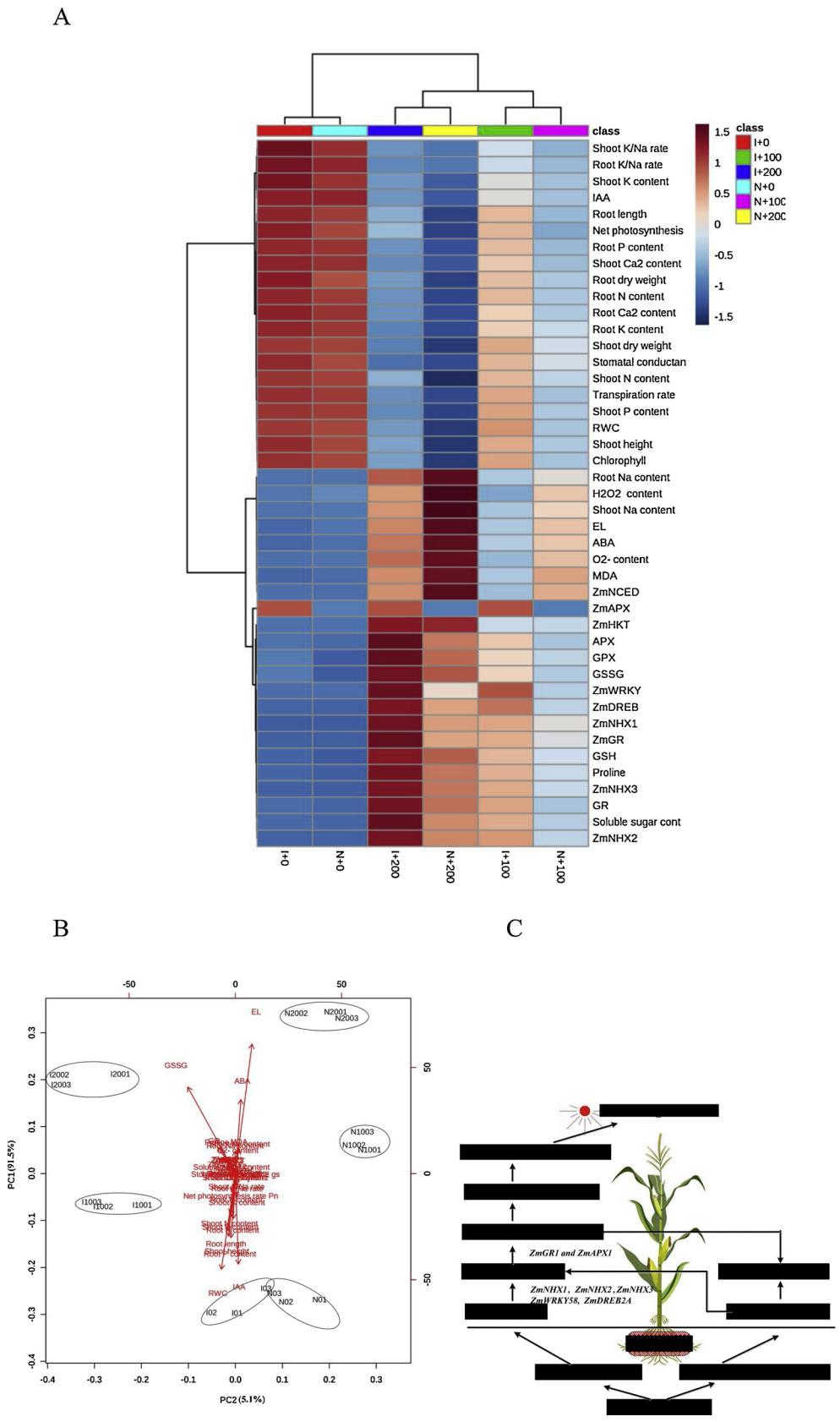

Fig. 10. A: The cluster analysis of all parameters in maize plants with or without the *K. rhizophila* Y1 inoculation under salt stress. B: PCA analysis (bi-pot, PC1 vs. PC2); C: The proposed pathways of salinity tolerance in which *K. rhizophila* Y1 is involved. Arrows represent the plant biochemical and physiological parameters impacted by *K. rhizophila* Y1 showed two growth-promoting traits like phosphate solubilization and IAA production which could improve plant growth and seed germination under salinity stress.

capable of mediating K^+ efflux coupled to Na^+ influx (Peskan-Berghöfer et al., 2015).

DREBs play key roles in enhancement of salt stress tolerance in plants and can regulate the stress-inducible genes expression in an ABA-independent manner. Overexpression of *DREBs* genes showed more tolerance of transgenic plants to freezing, heat, salt and drought stress conditions (Lata and Prasad, 2011). This study showed an increase of *ZmDREB2A* gene expression in *K. rhizophila* Y1-inoculated maize under saline conditions compared to non-inoculated control plants (Fig. 9F). Consistently, inoculation of salt-treated wheat with *Arthrobacter protophormiae* (SA3) and *D. natronolimnaea* (STR1) significantly improved *TaDREB2* gene expression compared to control plants (Barnawal et al., 2017). It is also known that WRKY transcript factors are involved in plant abiotic stress tolerance. Wang et al. (2018) indicated that *ZmWRKY106* acted as a positive factor and participated in various abiotic stress response pathways in plants under drought stress. The transgenic rice overexpressing a maize *WRKY58* gene showed enhancement of drought and salt tolerance (Cai et al., 2014). The increased *WRKY* gene expression in response to salt stress treatment was indicated previously in various plants (Chen et al., 2012). The upregulation of *ZmWRKY58* gene expression in maize plants inoculated with *K. rhizophila* Y1 was observed in this study in comparison with non-inoculated maize under saline conditions (Fig. 9E). This study was consistent with previous study that *GmWRKY54* gene expression was elevated in soybean plants with salt treatment. Moreover, the expression of this salt-related gene was enhanced in soybean inoculated with *B. firmus* SW5, thereby an improved salt tolerance was observed in soybean through accumulating antioxidants, alleviating the adverse effect of ROS and increasing the protein biosynthesis (El-Esawi et al., 2018a).

5. Conclusions

In this study, a novel halo-tolerant *K. rhizophila* Y1 was isolated from maize rhizosphere soil. This strain was able to tolerate NaCl concentrations up to 10 % and showed two growth-promoting traits like phosphate solubilization and IAA production. Inoculation with *K. rhizophila* Y1 strain could protect maize from salt stress. The alleviation of salt stress in maize inoculated with *K. rhizophila* Y1 could be considered as the integration of multiple physiological process including improving plant mineral nutrition, increasing plant antioxidative capacity and regulating plant growth by plant hormones (IAA and ABA) or activating ion transporters genes expression that led to an increased ratio of K^+/Na^+ in maize plants (Fig. 10C). In summary, this report showed that *K. rhizophila* Y1 could alleviate adverse effects on maize caused by salt stress. We conclude that *K. rhizophila* Y1 could be used as an ecofriendly PGPR based on its potent plant-protecting and plant growth-promoting activities under saline conditions. This isolate might be useful in formulating new inoculants with combinations of different mechanisms of action, leading to a more efficient application for salt stress adaption and thereby to improve cropping systems.

Authors contributions

X Li and P Sun performed the experiments and wrote the article; Y Zhang, C Jin and X Li analyzed the data and performed statistical analyses; C Jin contributed to experiment design and manuscript editing; C Guan designed and coordinated the experiments, interpreted the results, revised and improved the manuscript; C Guan is responsible for the manuscript as a whole.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

CRediT authorship contribution statement

Xiaozhou Li: Investigation, Validation, Writing - review & editing. **Pei Sun:** Investigation, Methodology, Writing - original draft. **Yanan Zhang:** Methodology, Validation, Writing - review & editing. **Chao Jin:** Investigation, Methodology, Writing - review & editing. **Chunfeng Guan:** Funding acquisition, Project administration, Supervision, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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APPENDICE

B. Attività di tirocinio

Durante la mia esperienza presso il laboratorio della prof.ssa Baldan all'Orto Botanico di Padova ho seguito principalmente un progetto di ricerca che si incentra sullo studio della pianta di pomodoro (*Solanum lycopersicum*) e l'effetto che ha su di essa la presenza di particolari batteri nella rizosfera. Nel laboratorio viene utilizzata una particolare varietà di pomodoro detta Micro-Tom per le sue dimensioni ridotte, che la rendono utile alla ricerca in quanto necessita di spazi più limitati e che si è affermata come sistema modello di studio per le piante orticole.

Preparazione dei semi

I semi sono stati ottenuti da pomodori maturi e lasciati essiccare per qualche giorno. Per lavorare *in vitro* i semi vanno sterilizzati in candeggina 5%, lavati in acqua deionizzata e lasciati germinare in acqua di rubinetto autoclavata per un paio di giorni. Da qui in avanti le condizioni di lavoro devono essere mantenute in sterilità lavorando sotto cappa biologica. Per favorirne la germinazione, i semi vengono posti su piastre quadrate in terreno solido Murashige & Skoog ½ agar.

Inoculo delle radici

La soluzione per l'inoculo batterico è stata preparata usando un campione di batteri (*B. licheniformis*) conservato a -80°C che è stato disciolto in terreno PCA liquido, e poi lasciato crescere in agitazione a 28°C per 24h.

Dopo aver misurato la densità ottica del terreno in cui erano stati lasciati crescere i batteri e averlo diluito per ottenere una quantità di 10^6 CFU, la soluzione è stata centrifugata per sostituire il terreno PCA con quello MS ½. Sono stati selezionati i semi con radichetta ben visibile e messi in nuove piastre in modo da avere 5 piastre con 5 piante ciascuna che costituiscono i replicati biologici. Le radici sono state bagnate con 20 µl di soluzione di batteri in MS½, e le piastre chiuse sono state posizionate in camera di crescita a (26°C, 70% umidità). Dopo 7 giorni le piante presentavano un apparato radicale sviluppato, ed era anche visibile ad occhio nudo la zona in cui avevano proliferato i batteri, (*figura* 1).

Figura 1. Piastra con piante di 7 giorni inoculate con *B. Licheniformis*

Estrazione dell'RNA

I tessuti vegetali di questa seconda fase sono stati ottenuti da piante inoculate tramite la procedura esposta in precedenza con *Bacillus subtilis*, e cresciute per 3 e 7 giorni dalla germinazione. Per procedere con analisi dei metaboliti e di espressione genica tramite sequenziamento si è effettuata la frammentazione e la liofilizzazione dei tessuti, in modo che potessero essere spediti a ditte specializzate in questo tipo di analisi. Una parte dei tessuti frammentati in azoto liquido con mortaio e pestello invece è stata usata per la quantificazione dell'RNA, il quale è stato estratto mediante il protocollo di estrazione con CTAB, cloroformio-isoamilalcol e litio-cloruro.

Una volta ottenuto l'RNA, per la quantificazione e il controllo della purezza è stato usato lo strumento NanoDrop, e i dati ottenuti sono riportati nella *tabella 1*. I quantitativi di RNA sono leggermente minori per le piante di 3 giorni, in particolare per le radici, e mentre il rapporto di assorbanza 260/280, che indica eventuali contaminazioni di proteine, è buono per tutti i campioni, il rapporto 260/230, che indica eventuali contaminazioni da solventi organici, è leggermente basso per alcuni campioni delle piante di 3 giorni.

	Giorno 3					
Campione	R1	R2	R3	S1	S2	S3
Concentrazione (ng/ µl)	66,3	114,5	443,4	1177,0	749,9	1139,1
A 260/280	2,196	2,209	2,207	2,077	2,197	2,186
A 260/230	2,649	1,994	2,257	1,511	1,999	2,184

	Giorno 7					
Campione	R1	R2	R3	S1	S2	S3
Concentrazione (ng/ µl)	312,0	394,4	536,7	1192,5	1482,8	1377,6
A 260/280	2,172	2,201	2,208	2,187	2,199	2,199
A 260/230	2,130	2,151	2,158	2,108	2,321	2,341

Tabella 1. Valori di quantità e purezza dell'RNA ottenuti tramite lo strumento NanoDrop.

I campioni R provengono dalle radici (root), mentre i campioni S provengono dalla parte aerea del germoglio (shoot). I valori evidenziati in rosso indicano campioni con una quantità ridotta di RNA, mentre quelli evidenziati in giallo indicano possibili contaminazioni da solventi organici.

Per il controllo della qualità invece è stata fatta una elettroforesi su gel di agarosio, il cui risultato è mostrato nella *figura* 3, assieme alla scala di peso molecolare per l'RNA in *figura* 2. Dall'immagine del gel si può notare che due su tre campioni di radici delle piante di 3 giorni contengono poco RNA, confermando i risultati dell'analisi quantitativa. Inoltre in generale i campioni di piante di 3 giorni presentano bande, corrispondenti all'rRNA (28s, 18s e 5s), poco definite ed uno *smear* più evidente, il che è indice di degradazione e dunque di bassa qualità. Al contrario i campioni delle piante di 7 giorni presentano delle bande definite e uno *smear* uniforme e meno intenso, che corrisponde agli mRNA del tessuto.
Di conseguenza per l'analisi di espressione genica che prevede il sequenziamento dell'RNA sarà preferibile usare le piante di 7 giorni piuttosto che 3, in quanto permettono di estrarre una quantità maggiore di RNA e più pulito. La degradazione potrebbe comunque essere stata causata da errori dell'operatore durante l'estrazione, dunque si prevede di ripetere l'esperimento per verificare tali risultati.

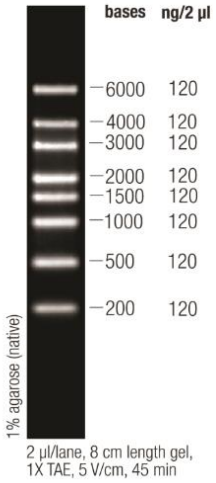

Figura 2. RNA ladder usato nell'esperimento (Thermo Scientific RiboRuler High Range RNA Ladder)

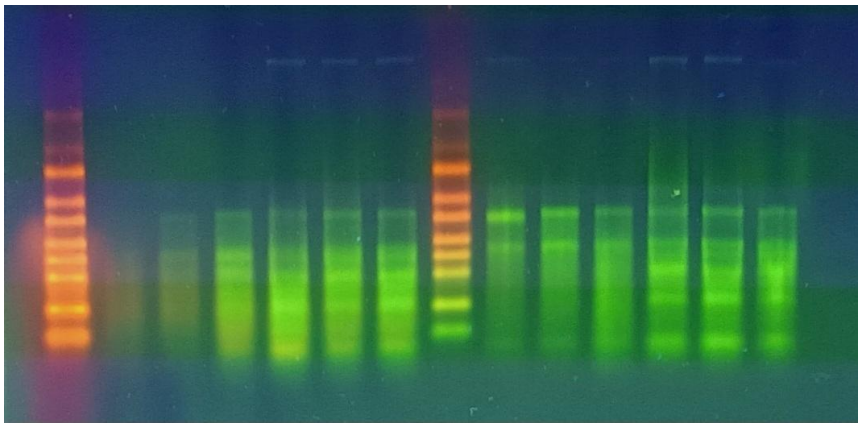

Figura 3. Risultato dell'elettroforesi dell'RNA su gel di agarosio.

Partendo da sinistra le corsie corrispondono a: RNA ladder, campioni del giorno 3 (R1, R2, R3, S1, S2, S3), RNA ladder e campioni del giorno 7 (R1, R2, R3, S1, S2, S3).