# Rhizobacteria-induced systemic tolerance against drought stress in *Sorghum bicolor* (L.) Moench

**René Carlson[a], Fidele Tugizimana[b], Paul A. Steenkamp[b], Ian A. Dubery[b], Ahmed Idris Hassen[c] and Nico Labuschagne[a*]**

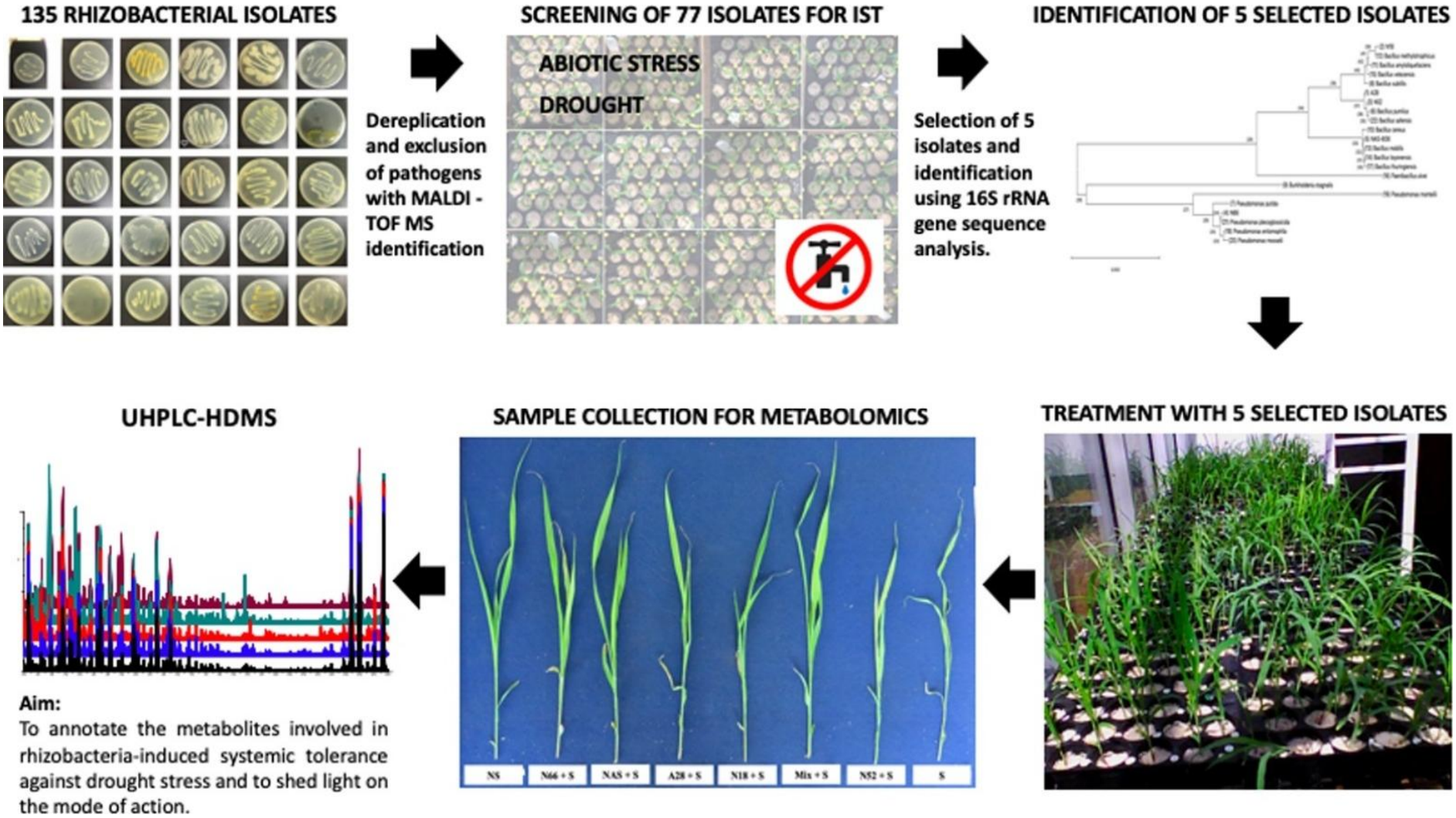
[a]Department of Plant and Soil Sciences, Faculty of Natural and Agricultural Sciences, University of Pretoria, Private Bag X20, Hatfield, Pretoria, 0028, South Africa; renecarlson@gmail.com (R.C.); nico.labuschagne@up.ac.za (N.L.).
[b]Centre for Plant Metabolomics Research, Department of Biochemistry, Faculty of Science, University of Johannesburg, P.O. Box 524, Auckland Park, Johannesburg, South Africa; idubery@uj.ac.za (I.A.D.); ftugizimana@uj.ac.za (F.T.); psteenkamp@uj.ac.za (P.A.S.).
[c]Agricultural Research Council, Plant Health and Protection, Private bag X134, Queenswood, 0121, Pretoria, South Africa; HassenA@arc.agric.za (A.I.H.).

*** Correspondence:**
Nico Labuschagne
Department of Plant and Soil Sciences, Natural and Agricultural Science Building, University of Pretoria, Private Bag X20, Hatfield, 0028, South Africa; nico.labuschagne@up.ac.za.

## Abstract

Induction of systemic tolerance in sorghum [*Sorghum bicolor* (L.) Moench] against drought stress was studied by screening a large collection of rhizobacterial isolates for their potential to exhibit this essential plant growth-promoting trait. This was done by means of a greenhouse assay that measured the relative change in both plant height and -biomass (roots and shoots) between rhizobacteria-primed *versus* non-primed (naïve) plants under drought stress conditions. In order to elucidate the metabolomic changes in *S. bicolor* that conferred the drought stress tolerance after treatment (priming) with selected isolates, untargeted ultra-high performance liquid chromatography-high definition mass spectrometry (UHPLC-HDMS)-based metabolomics was carried out. Intracellular metabolites were methanol-extracted from rhizobacteria-primed and naïve *S. bicolor* roots and shoots. Extracts were analysed on a UHPLC-HDMS system and the generated data were chemometrically mined to determine signatory metabolic profiles and bio-markers related to induced systemic tolerance. The metabolomic results showed significant treatment-related differential metabolic reprogramming between rhizobacteria-primed and naïve plants, correlating to the ability of the selected isolates to protect *S. bicolor* against drought stress. The selected isolates, identified by means of 16S rRNA gene sequencing as members of the genera *Bacillus* and *Pseudomonas*, were screened for 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity by means of an *in vitro* assay and the presence of the *acdS* gene was subsequently confirmed by PCR for strain N66 (*Pseudomonas* sp.). The underlying key metabolic changes in the enhanced drought stress tolerance observed in rhizobacteria-primed *S. bicolor* plants included (1) augmented antioxidant capacity; (2) growth promotion and root architecture modification as a result of the upregulation of the hormones gibberellic acid, indole acetic acid and cytokinin; (3) the early activation of induce systemic tolerance through the signalling hormones brassinolides, salicylic acid and jasmonic acid and signalling molecules sphingosine and psychosine; (4) the production of the osmolytes proline, glutamic acid and choline; (5) the production of the epicuticular wax docosanoic acid and (6) ACC deaminase activity resulting in lowered ethylene levels. These results unravelled key molecular details underlying the PGPR-induced systemic tolerance in sorghum plants, providing insights for the plant priming for abiotic stress.

**Graphical abstract**



**Keywords:** Induced systemic tolerance, ACC deaminase activity, Drought stress, PGPR, Metabolomics, UHPLC-HDMS.

**Abbreviations:** ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylate; AOX, antioxidants; BPI, base peak intensity; ESI, electrospray ionization; GA, gibberellic acid; HCA, hierarchical clustering analysis; HD, high definition; LSD, least significant difference; MALDI, matrix-assisted laser desorption/ionization; MF, Molecular formula; MOA, mode of action; MS, mass spectrometry; OPLS-DA, orthogonal partial least square-discriminant analysis; PCA, principal component analysis; PCR, polymerase chain reaction; PGPR, plant growth-promoting rhizobacteria; QC, quality control; ROS, reactive oxygen species; Rt, retention time; RWC, relative water content; SAF, stress alleviation factor; TOF, time of flight; UHPLC, ultra-high performance liquid chromatography; VIP, variable importance in projection.

## 1 Introduction

With the alarming rate at which the global population is growing, overcoming the effects of climate change and its associated threat to food security is crucially important in current research endeavors (Carvalho, 2006). To address these needs, research focus is progressively moving towards the investigation of more sustainable and environmentally-friendly methods to increase food production (Gliessman, 1995; Horrigan et al., 2002; Reganold et al., 1990). Beneficial microbes are known for their capacity to protect crops, enhance yields and improve soil quality (Baker, 1991; Pieterse et al., 2014; Sturz and Christie, 2003). The application thereof in mainstream agriculture has become imperative in the move towards sustainability and has, become a regular topic in peer-reviewed research (Finkel et al., 2017; Y. Kang et al., 2016; Schisler et al., 2004; Singh et al., 2017; Stewart, 2001; Welbaum et al., 2004).

The growth promotion activity by root colonising plant growth-promoting rhizobacteria (PGPR) is well documented (Bashan and De-Bashan, 2010; DeBrito Alvarez et al., 1995; Ghyselinck et al., 2013; Kloepper, 1994). These PGPR promote plant growth directly or indirectly, through a variety of mechanisms (Bashan and De-Bashan, 2010). The direct mechanisms of plant growth promotion consist of the production of phytohormones and the ability to render nutrients to a more plant-available form; whereas the indirect modes of action (MOA) consist of the ability to suppress plant pathogens and reduce disease pressure. In addition to this, PGPR have the ability to activate plant defenses against biotic- and abiotic stress, by eliciting physical and/or chemical changes *in planta* that offer protection (Lucas et al., 2014). The capacity of PGPR to elicit plant defenses against biotic stress is known as induced systemic resistance (Harish et al., 2008; Walters and Fountaine, 2009). Similarly, a PGPR-induced response to abiotic stress factors, such as drought, salt, and extremes in temperature, has been described as a form of induced systemic tolerance (Naseem and Bano, 2014; Ngumbi and Kloepper, 2016; Yang et al., 2009). This PGPR-induced tolerance of plants against abiotic stress is of critical importance in mitigating the negative impact of climate change in crop production and research in this area is of vital importance.

The plant's drought response caascade comprise of the activation of a complex signalling network, initially involving cytosolic $Ca^{2+}$ and $H^{+}$ ions, reactive oxygen intermediates and the signaling hormones ethylene, jasmonic acid and salycilic acid that triggers physiological responses such as loss of turgor pressure, closure of stomata and a reduction in leaf area (Baxter et al., 2014). This reduction in leaf area lowers photosynthesis capacity, which negatively impacts root biomass and yield. When drought conditions persist it can ultimately lead to leaf

senescence and plant death (Kaushal and Wani, 2016). Methods used to measure drought in plants include relative water content (RWC) and infrared thermography (Jones et al., 2009; Ngumbi and Kloepper, 2016; Seelig et al., 2008). The capacity of plants to protect themselves against drought stress involves physical, biochemical and molecular modifications. These adaptations consist of physical changes (changes in root and shoot growth) and chemical modulations (augmented phytohormone-, antioxidant- and osmolyte levels). *S. bicolor* is considered as having a relatively high tolerance to drought. This is credited to the active regulation of stomatal opening and closure, enabling the maintenance of photosynthesis at low water potentials, modification of root architecture and osmotic adjustment (Tari et al., 2013).

There are several reports for the ability of PGPR to enhance the plant's capacity to tolerate abiotic stress, also referred to as induced systemic tolerance (Ngumbi and Kloepper, 2016; Yang et al., 2009). Induced systemic tolerance is induced by PGPR-derived elicitors such as phytohormones, volatiles, enzymes, antioxidants (AOX) and exopolysaccharides (Sandhya et al., 2009). The MOA involved in rhizobacteria-induced drought stress tolerance are grouped into three main categories namely: (1) osmotic adjustment, (2) augmented antioxidant metabolism and (3) changes in plant growth and root architecture (Grover et al., 2011; Ngumbi and Kloepper, 2016; Yang et al., 2009). In a study done by Dugas *et al.*, (2011), investigating induced systemic tolerance in *S. bicolor*, PGPR-derived cytokinin acted as an elicitor of induced systemic tolerance through the regulation of abscisic acid (ABA) levels, which resulted in the augmentation of osmolytes (proline and choline) and phytohormones (ethylene, gibberellins, jasmonic acid, brasinosteroids, cytokinin and auxins).

The augmentation of phytohormone levels in response to treatment with PGPR is important in the induction of tolerance against drought stress, either as a result of an early signalling response or through the manipulation of stomatal opening and closure and plant growth e.g. changes in leaf area and modified root architecture (Farooq et al., 2009; Sukumar et al., 2013). The phytohormones that bestow drought tolerance by modifying plant growth are: ABA, auxin and gibberellic acid (GA). ABA is known for its role in stomatal opening and closure, thus minimising water-loss that occurs through transpiration and is regulated by PGPR-derived cytokinin. Through its effect on phytohormone levels PGPR thus aid in the tolerance of plants against drought stress by maintaining RWC, plant growth and ultimately yield. Phytohormones act as signalling molecules in the early activation of the induced systemic tolerance cascade. These signalling phytohormones include jasmonic acid (JA), salicylic acid (SA) (Horváth et al., 2007), ethylene (Glick, 2005) and brassinolide (Pieterse et al., 2009). JA- and SA are known to enhance plant defenses against both- and abiotic stress conditions. However, ethylene result in early senescence and ultimately plant death (Glick, 2005). Some PGPR with 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity are able to inhibit the synthesis of ethylene. The enzymatic activity of ACC deaminase results in lower ethylene levels by cleaving its precursor ACC to α-ketobutyrate and ammonia, thus enhancing tolerance against environmental stress (Glick and Bashan, 1997).

During normal growth conditions the reduction-oxidation equilibrium is maintained in plant cells by quenching reactive oxygen species (ROS) through the synthesis of AOX. This equilibrium is challenged under conditions of plant stress, when the plant is unable to maintain the production of AOX, resulting in oxidative damage (Noctor et al., 2015). The capacity of plants to quench these ROS through the production of AOX is thus directly linked to their tolerance of drought and other oxidative stresses (Gill and Tuteja, 2010). PGPR are known to enhance their own and the plant's capacity to produce AOX under oxidative stress that result from conditions of biotic- and abiotic stress (Ngumbi and Kloepper, 2016; Yang et al., 2009).

One of the most important strategies of plants to tolerate drought stress is the ability to accumulate solutes that lower cellular water potential, thus enabling the maintenance of cellular turgor. These solutes, also known as osmolytes, consist of non-protein amino acids, compounds that contain ammonium, inorganic ions, organic acids, polyols and sugars (Birben et al., 2012; Gill and Tuteja, 2010). PGPR confer drought stress tolerance in plants by increasing the plant's capacity to produce osmolytes (Ngumbi and Kloepper, 2016; Yang et al., 2009).

Sorghum [*Sorghum bicolor* (L.) Moench] is an important source of food, feed and forage (Rooney et al., 2007) and thus of significant important in food security and sustainable livelihoods in developing countries (Mareya et al., 2019). This study is aimed at investigating the potential of rhizobacteria to protect *S. bicolor* against drought stress. In order to gather information that could potentially shed light on the MOA involved in rhizobacteria-induced drought stress tolerance an ultra-high performance liquid chromatography-high definition mass spectrometry (UHPLC-HDMS)-based metabolomics investigation was carried out and reported herein. Metabolomics has emerged as an indispensable methodological approach to interrogate cellular biochemistry at a global level. It can be described as a multidisciplinary science that aims to define the entire complement of metabolites within a biological matrix of interest. Furthermore, metabolite profile patterns can provide a holistic biochemical phenotype of a cell or tissue under defined physiological conditions (Tugizimana et al., 2013).

## 2 Materials and methods

### 2.1 Dereplication and exclusion of possible animal and plant pathogens

A group of 135 rhizobacterial isolates was obtained from the University of Pretoria's PGPR collection. These rhizobacteria were isolated from the rhizosphere of seven grass species namely *Sporobolus fimbriatus*, *Stipagrotis zeyheri* subsp. *zericans*, *Themeda triandra Eragrostis biflora*, *Arstida canescens* subsp. *canescens*, *Eragrostis* spp. and *Cyprus esculantus* L. that were collected from selected sites at the Nylsvlei Nature Reserve in South Africa (Hassen, 2007). The selected rhizobacterial isolates were subjected to matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) identification in order to exclude replicates and human- and plant pathogens. The method described by Ghyselinck et al., (2011, 2013) was followed.

### 2.2 Greenhouse screening for induced systemic tolerance against drought stress

#### 2.2.1 Sorghum cultivation

*Sorghum bicolor* seed (cultivar Sweet NS 5655) was obtained from Advance Seed (Krugersdorp, South Africa). The seeds were sterilised successively in 70% ethanol (5 min), 1% sodium hypochlorite (1 min) and rinsed five times with sterile $dH_2O$. The seeds were subsequently transferred to Petri dishes containing filter paper moistened with sterile $dH_2O$ and allowed to germinate for 48 h at 25 °C. The germinating seeds were inspected daily for any bacterial and fungal growth and contaminated germlings were discarded. The *S. bicolor* germlings were directly planted into plastic seedling trays filled with washed, autoclaved (120 °C for 20 min), pure silica sand. The trays consisted of 30 × 50 mL cells per tray and were sterilised with 10% sodium hypochlorite. Each cell was filled with exactly 40mL of washed, autoclaved and dried pure silica sand. The plants were watered every second day with 10mL sterilized $dH_2O$. The plants received a general water soluble fertilizer [Multifeed (Nulandis, South Africa)] at a rate of 4.5g $10L^{-1}$ of water once per week. No pesticides or fungicides were

needed. The greenhouse temperature was maintained at between 20 °C and 30 °C and the relative humidity fluctuated between 40% and 60%. At harvest, the fresh and dry weights of both roots and shoots were measured and samples for metabolomic analysis, were taken. The experimental design consisted of three independent biological repeats.

### 2.2.2 Rhizobacteria inoculum preparation and treatment

After excluding the replicates and pathogens from the initial collection of 135 new rhizobacterial isolates, the remaining 77 isolates were screened for their potential to induce systemic tolerance against drought stress in *S. bicolor* plants. The group of 77 isolates consisted of 74 new isolates (Hassen, 2007), 2 strains in the process of being commercialized by the University of Pretoria (*Paenibacillus alvei* NAS-6G6 and *Paenibacillus alvei* T29) and 1 commercial strain (RhizoVital® *Bacillus amyloliquefaciens*, Andermatt Biocontrol AG, Switzerland). Each rhizobacterial isolate/strain were maintained at -72 °C on Microbeads® (Davies diagnostics, Randburg, South Africa). The 74 isolates and 3 strains were streaked onto Nutrient agar and a 1 w old culture of each was inoculated into Nutrient broth and incubated in a rotary shaker at 25 °C and 150 rpm for 48 h. Each bacterial suspension was subsequently centrifuged in 50 mL capacity sterile plastic tubes at 2000 rpm for 10 min. The resulting pellet was then re-suspended in quarter strength sterile Ringer's solution to give a final concentration of $10^8$ cfu $mL^{-1}$. Three weeks after planting of the sorghum seed, each plant (one plant per pot) was treated with 1 mL of a $10^8$ cfu $mL^{-1}$ cell suspension of the respective rhizobacterial isolates/strains. A total of three biological replicates were included, consisting of 10 sorghum seedlings per replicate and thus 30 sorghum seedlings per treatment.

### 2.2.3 Drought stress

Four days after treatment (priming) of the plants with the respective rhizobacterial isolates/strains, the *S. bicolor* plants were subjected to drought stress once weekly for 3 successive weeks. Plants were monitored hourly until sufficient wilting was noticed in non-rhizobacteria-treated (naïve) plants at which point all pots were watered with 10mL sterilized $dH_2O$. The non-stressed control plants were not subjected to drought stress.

### 2.2.4 Assessment of drought stress tolerance

At 6 w post planting, *S. bicolor* plants were assessed for drought stress tolerance. Plant heights were recorded just before harvest. The plants were then carefully removed by watering each pot to field capacity with tap water, to loosen the roots in order to avoid breakage. The roots were then carefully washed to remove any remaining sand particles and dried with tissue paper. The biomass of both roots and shoots were recorded. Fresh weights were taken directly after harvest, whereas dry weights were taken after the plant material was dried in an oven at 40 ºC for 48 h.

### 2.2.5 Statistical analysis of growth parameters

The data were subjected to analysis of variance and means were compared using Tukey's least significance determination (LSD) test at a significance level of $p < 0.05$.

### 2.2.6 Selection of the rhizobacterial isolates

Based on the results of the various rhizobacterial treatments, the individual rhizobacterial isolates were scored according to their ability to induce systemic tolerance against drought stress in *S. bicolor* plants. This was done by calculating a stress alleviation factor (SAF) incorporating the percentage increase in (1) plant height, (2) root- and (3) leaf biomass obtained by treatment with each rhizobacterial isolate/strain against the stress control (untreated plants receiving drought stress). Rhizobacterial isolates were selected according to their SAF score. The SAF for induced systemic tolerance (ISTolerance) was formulated for the current study and calculated for each rhizobacterial isolate with the equation:

$$\mathbf{SAF\_ISTolerance} = \frac{\left(\frac{Ih - Ch}{Ch} \times 100\right) + \left(\frac{Ir - Cr}{Cr} \times 100\right) + \left(\frac{Is - Cs}{Cs} \times 100\right)}{3}$$

where: I = rhizobacterial isolate; C = untreated control receiving drought stress; h = plant height; r = root dry mass; s = shoot dry mass.

## 2.3 Identification of the selected rhizobacterial isolates

Genomic DNA was extracted from five selected rhizobacterial isolates using a Promega Wizard genomic DNA purification kit (Promega Corporation, USA) following manufacturer's instructions. The 16S ribosomal RNA region was amplified by the polymerase chain reaction (PCR) was performed using primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') corresponding to *E. coli* numbering 8 - 27 (Lane, 1991) and 1485R (5'-TAC GGT TAC CTT GTT ACG AC-3') corresponding to *E. coli* numbering 1489 - 1508 (Embley et al., 1988). The PCR was performed with OneTaq Quick-Load 2X Master Mix containing 1 × standard PCR buffer, 1.8 mM $MgCl_2$, 0.5 μM of each primer, 0.2 μM of each dNTP, 0.3 U OneTaq® DNA Polymerase (New England Biolabs, USA). To obtain the 16S rRNA (27F/1485R) amplicons, 35 × PCR cycles were performed that include initial denaturation at 94 °C for 3 min, denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 °C for 1 min and second extension at 72 °C for 5 min. The PCR product was separated by electrophoresis through 1% agarose gel, purified and sequenced (Inqaba Biotechnical Industries, South Africa). The resulting sequences were edited on BioEdit program version 7 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) after which they were aligned using the Clastal W alignment tool. The MEGA 7 software was then used to construct maximum likelihood phylogenetic trees based on bootstrap analysis of 1000 replicates to calculate the statistical significance of the branches of the phylogenetic tree (Kumar et al., 2018). The nucleotide sequence of the 16S rRNA of each isolate was deposited in the GenBank at the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/WebSub/) with accession numbers MK855498 to MK855502.

## 2.4 Screening for ACC deaminase activity

### 2.4.1 *In vitro* assay

The selected rhizobacterial isolates were grown on DF salts minimal medium (Dworkin and Foster, 1958) containing ACC as source of nitrogen. ACC deaminase activity was confirmed when growth was noticed (Penrose and Glick, 2003). Plates were incubated at 28 °C for 7 days,

growth was checked daily and final readings were taken after 7 days. The assay was repeated three times, each consisting of three replicates per isolate.

### 2.4.2 Isolation of the gene encoding 1-aminocyclopropane-1-carboxylate deaminase by PCR amplification

In order to determine the presence of the ACC deaminase gene *acdS*, DNA was extracted from the five selected rhizobacterial isolates using a Promega Wizard genomic DNA purification kit (Promega Corporation, USA) and the PCR was performed using synthetic oligonucleotides F1936 (forward) 5'-GHG AMG ACT GCA AYW SYG GC-3' and F1939 (reverse) 5'-GAR GCR TCG AYV CCR ATC AC-3' as primers (Blaha et al., 2006). The PCR was performed with OneTaq Quick-Load 2X Master Mix containing 1 × standard PCR buffer, 1.8 mM $MgCl_2$, 0.5 µM of each primer, 0.2 µM of each dNTP, 0.3 U OneTaq® DNA Polymerase (New England Biolabs, USA). To obtain the *acdS* (F1936/F1939) amplicons, 35 × PCR cycles were performed that include an initial denaturation at 95 °C for 3 min, denaturation at 95 °C for 1 min, annealing at 62 °C for 1.5 min, extension at 72 °C for 1 min and second extension at 72 °C for 5 min. The PCR product was separated by electrophoresis through 1% agarose gel, purified and sequenced (Inqaba Biotechnical Industries, South Africa).

## 2.5 Metabolomics study

To investigate the effects of the selected rhizobacterial isolates on the metabolome of *S. bicolor* plants, a metabolomics study was done by making use of UHPLC-HDMS. This was done in order to shed light on the possible MOA involved in rhizobacteria-induced drought stress tolerance.

### 2.5.1 Assessment of drought stress tolerance

Sorghum (*S. bicolor*) was cultivated as outlined in section 2.2.1 and the inoculum was prepared from each of the 5 selected rhizobacterial isolates and used to treat the *S. bicolor* seedlings as outlined in section 2.2.2. Four days after treatment (priming) of the plants with the selected rhizobacterial isolates, the plants were subjected to drought as described under section 2.2.3. A total of three biological replicates were included, consisting of 20 sorghum seedlings per replicate and thus 60 sorghum seedlings per treatment. Samples for the metabolomics analysis were taken 24 h post initial drought consisting of half of the seedlings (10 seedlings per replicate or 30 seedlings per treatment). The 30 remaining plants were subjected to drought stress once weekly for 3 successive weeks and harvested at 6 w post planting to assess drought stress tolerance. Parameters included RWC, plant height, root length, fresh and dry biomass of both roots and shoots. The RWC was calculated as outlined by Seelig et al., (2008), the only exception being that dry weight was obtained after oven-drying samples at 40 °C for 48 h. A total of three biological replicates were included, consisting of 20 sorghum seedlings per replicate and thus 60 sorghum seedlings per treatment. The data were subjected to analysis of variance and means were compared using Tukey's LSD test at a significance level of $p < 0.05$.

### 2.5.2 Metabolite profiling

For the metabolite profiling the same methods as described by Carlson *et al.*, (2019) was used. The sample collection, metabolite extraction, UHPLC-HDMS- and data analysis, metabolite annotation and metabolic pathway analysis for this study is described under supplementary

material. Fresh samples for UHPLC-HDMS analysis was taken 24 h post drought, which coincided with 5 d post treatment with each of the selected rhizobacterial isolates. The samples were collected from roots and shoots of drought stressed and non-stressed *S. bicolor* plants of which each group was primed with each of the selected rhizobacterial isolates or left naïve (untreated control). The batch consisted of a total of 72 samples comprising 2 tissues (roots and shoots), 12 treatments [rhizobacterial treatment (6) *vs.* stress (2)], 3 biological replicates and each sample was injected 3 times (a total of 216 injections) to account for any technical variability. It is important to note here that in addition to metabolites originating from *S. bicolor*, root samples also contained metabolites originating from the different rhizobacterial treatments, whereas leaf samples only contained metabolites originating from *S. bicolor*.

# 3 Results

## 3.1 Dereplication and exclusion of possible animal and plant pathogens

One hundred and thirty five rhizobacterial isolates were identified by means of MALDI-TOF MS. Fifty eight rhizobacterial isolates were identified as possible replicates or pathogens and were excluded from the group. The remaining 77 rhizobacterial isolates were subsequently included in the greenhouse screening for their potential to induce systemic tolerance in *S. bicolor* against drough stress (under supplementary material Table S1).

## 3.2 Greenhouse screening for induced systemic tolerance against drought stress

The rhizobacterial isolates A28, N18, N52 and N66 were selected according to their performance in alleviating stress in *S. bicolor* plants as measured by their SAF score (under supplementary material Table S1). The semi-commercial rhizobacterial strain NAS-6G6 (*Paenibacillus alvei*) was included as reference. Some of the other rhizobacterial isolates with high SAF_ISTolerance scores were not selected because of either low reliability of the MALDI TOF identification, lack of information on the clinical significance of some species (e.g. *Pseudomonas rhodesiae*) and potential risk of toxin production (e.g. *Bacillus cereus*).

## 3.3 Identification of the selected rhizobacterial isolates

The identity of selected rhizobacterial isolates and their evolutionary relatedness with known bacterial species was elucidated by nucleotide sequence analysis of the 16S ribosomal RNA (rRNA). Prior to the analysis of the nucleotide sequences and phylogenetic tree construction, BLAST similarity search of the 16S rRNA at the National Centre for Biotechnology Information (NCBI) database using BLASTN (Zhang et al., 2000) was conducted that resulted in $\geq$ 99% similarity with strains of *Bacillus* and *Pseudomonas* spp. This was followed by construction of a Maximum Likelihood (ML) phylogenetic tree with the inclusion of 20 reference strains from the NCBI data base library. The analysis of the nucleotide sequence alignment and the ML phylogenetic tree together with the BLAST result putatively identified the four selected rhizobacterial isolates as: *Bacillus* sp. strain N18, *Bacillus* sp. strain N52, *Bacillus* sp. strain A28 and *Pseudomonas* sp. strain N66 (Figs. 1 and 2).

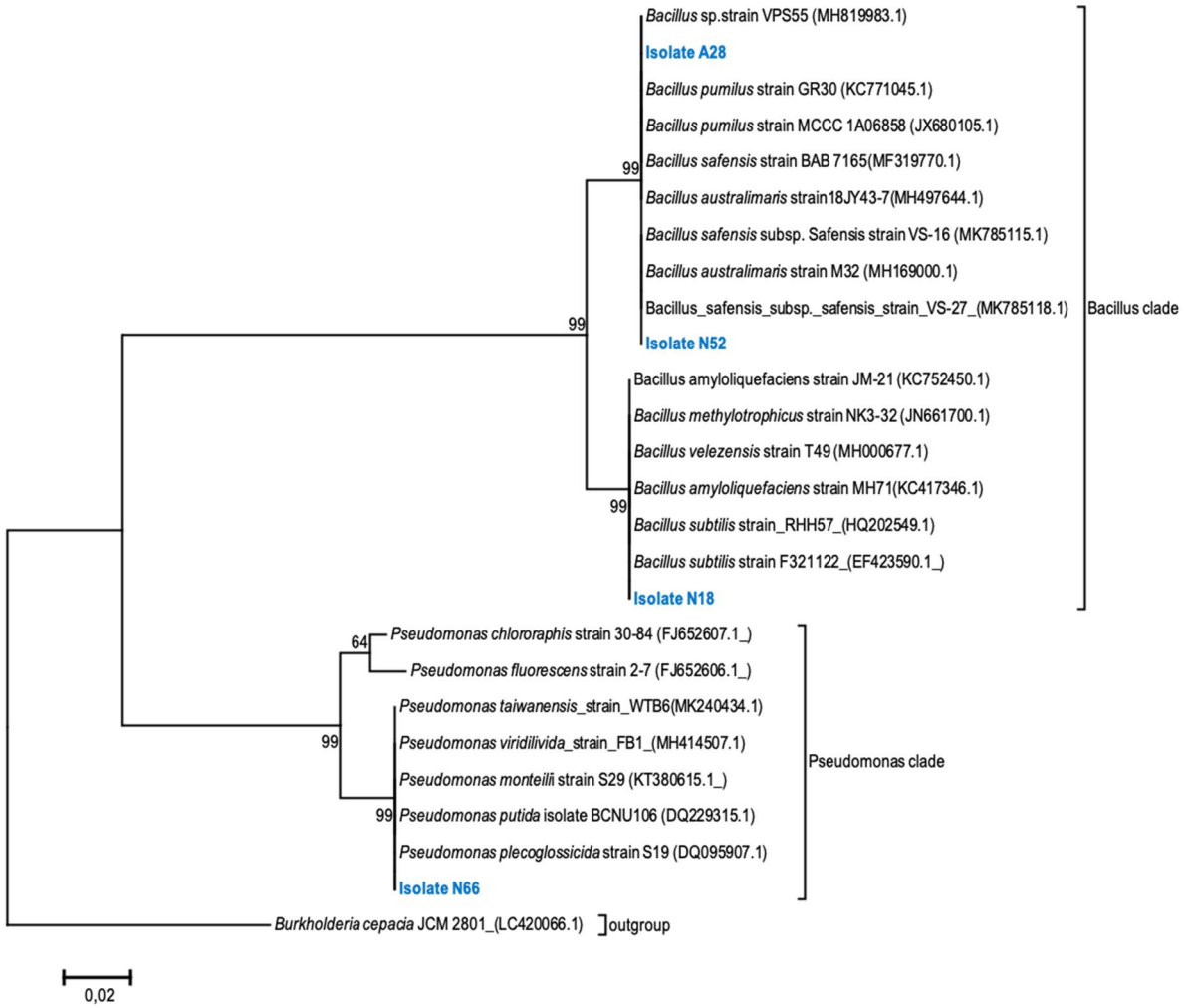



**Fig. 1** Phylogenetic tree derived from the aligned 16S rRNA nucleotide sequences of four effective isolates in this study (in blue) and reference strains from the Genebank. The evolutionary history was inferred by using the Maximum Likelihood (ML) method based on the Jukes-Cantor model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (0.02). The Evolutionary analyses were conducted in MEGA7.

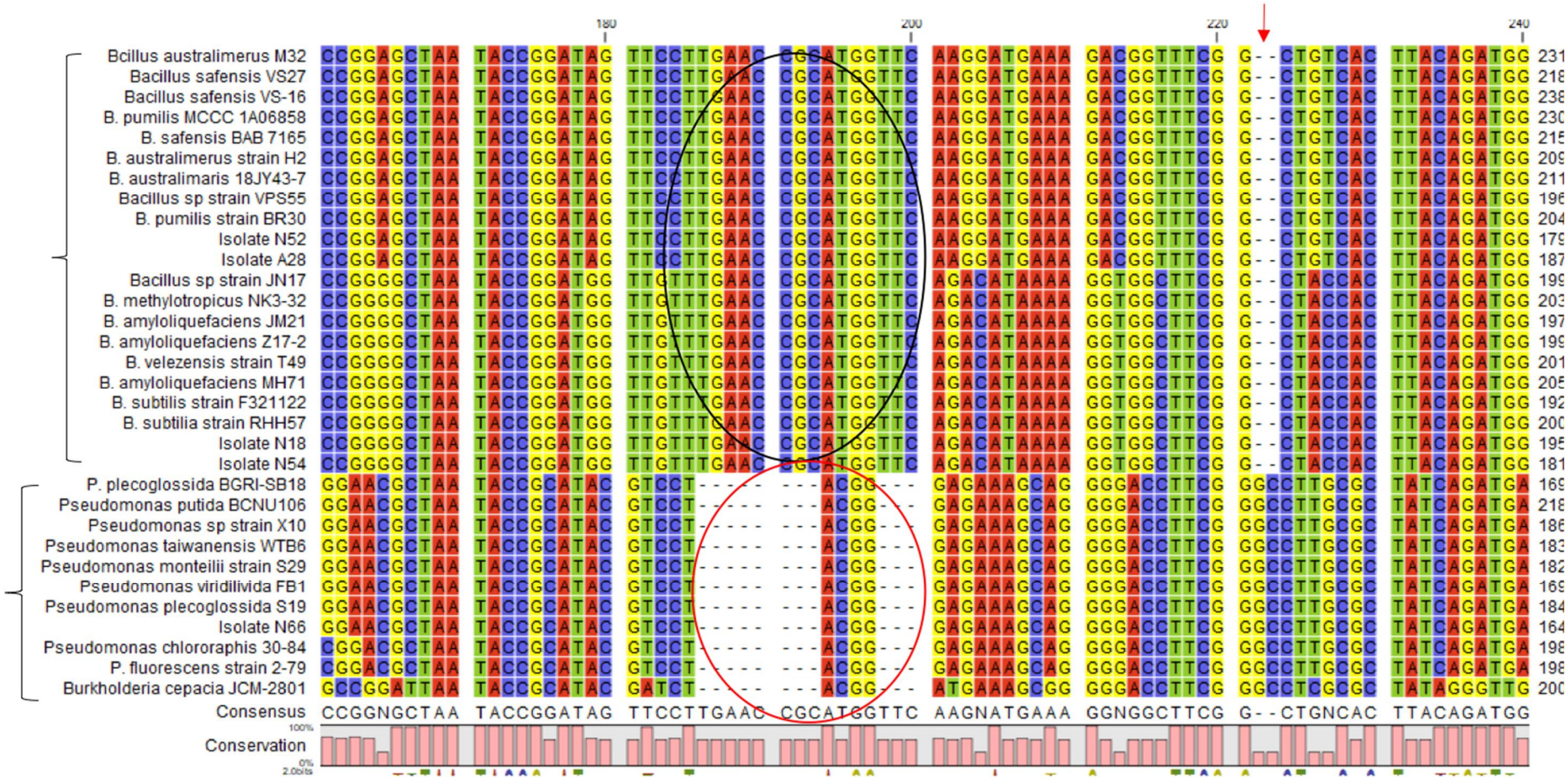



**Fig. 2** CLC Genomic Workbench aligned sequences of the 16S rRNA of the PGPR isolates in this study and 27 reference strains from the NCBI Genebank. Note that isolate N66 identified as *Pseudomonas* sp. share similar alignment region with all the reference *Pseudomonas* spp. having some deletion events of the ancestral (consensus) sequences TGAAC CGC & TTC encircled in red). On the other hand these nucleotides are conserved in isolates ( N52, A28, N18 and all the *Bacillus* reference strains ( circle in black). All the *Bacillus* group and the consensus sequence show deletions at position 222-223 (red arrow) where as there is a GC insertion at the same position for all the *Pseudomonad* group including isolate N66.

### 3.4 Screening for ACC deaminase activity

The results of the screening for ACC deaminase activity and confirmation of the presence of the *acdS* gene is summarized under supplementary material Table S1. Only *Pseudomonas* sp. strain N66 was able to grow successfully on DF salts minimal medium containing ACC as the only source of nitrogen. The presence of the *acdS* gene was confirmed for *Pseudomonas* sp. strain N66 by the electrophoretic separation of *acdS* amplicons and formation of a PCR band of expected size (558 bp) as shown in Fig. 3. Rhizobacterial isolates A28, N52, N18 and NAS-6G6 did not yield any bands indicating that the *acdS* gene was absent in these rhizobacterial isolates.

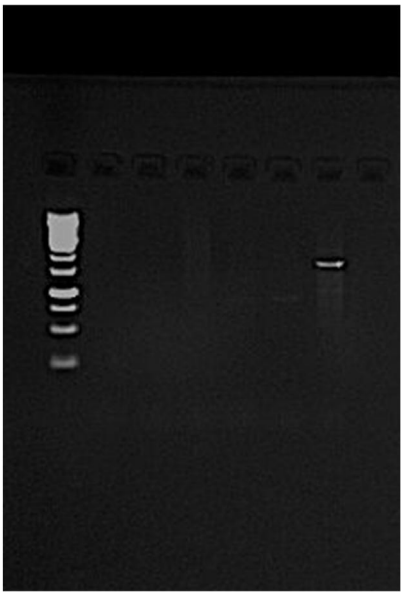


**Fig. 3** Electrophoretic gel displaying the band/amplicon (558 bp) corresponding to *acdS* transcripts obtained for *Pseudomonas* sp. strain N66 but absent in the other four rhizobacterial isolates assessed in the current study.

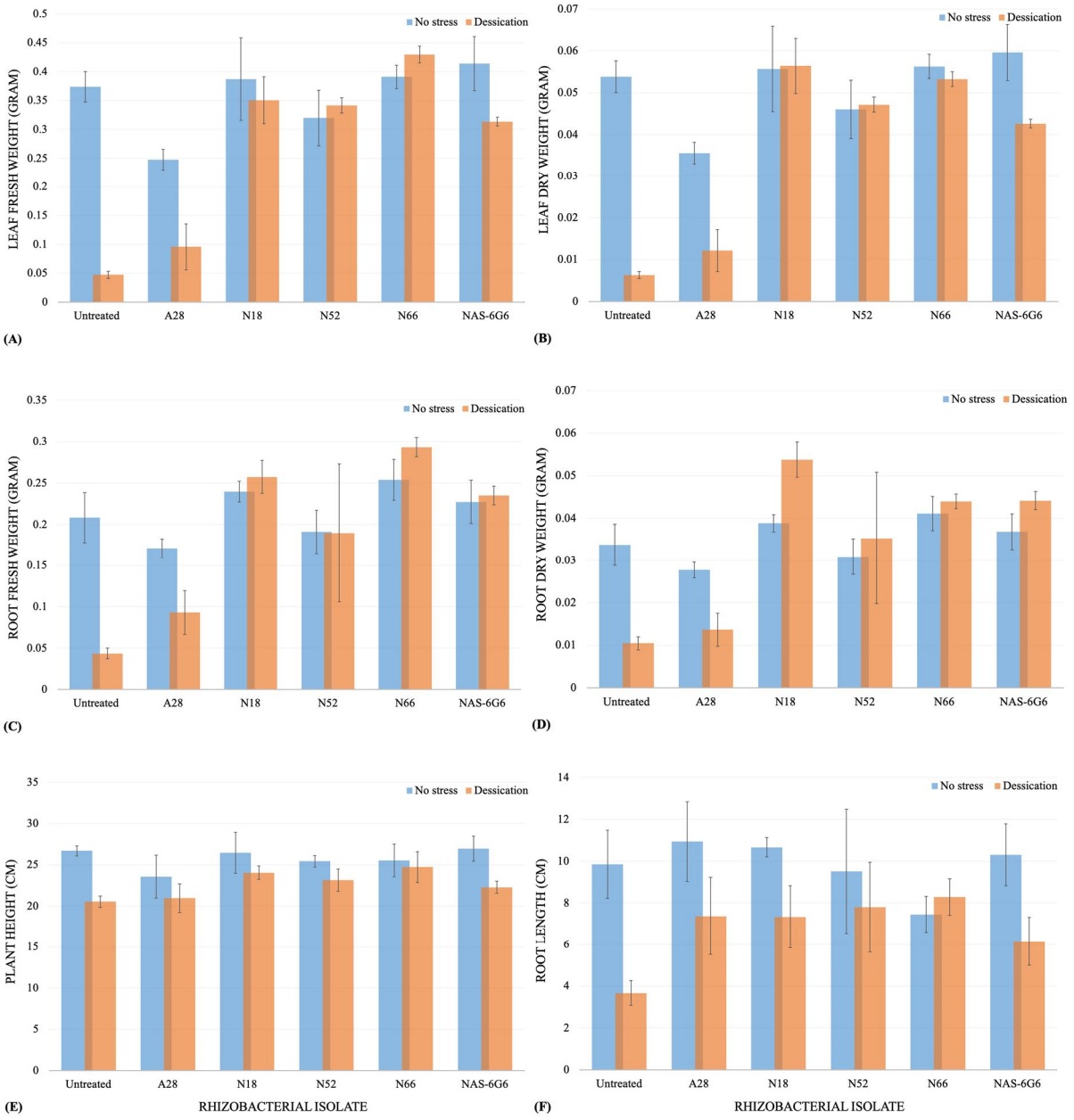



**Fig. 4** Plant growth promotion and induction of drought stress tolerance in *S. bicolor* after treatment with the selected rhizobacterial isolates for induced systemic tolerance as indicated by (A and B) leaf fresh and -dry weight respectively, (C and D) root fresh and -dry weight respectively, (E) plant height and (F) root length.

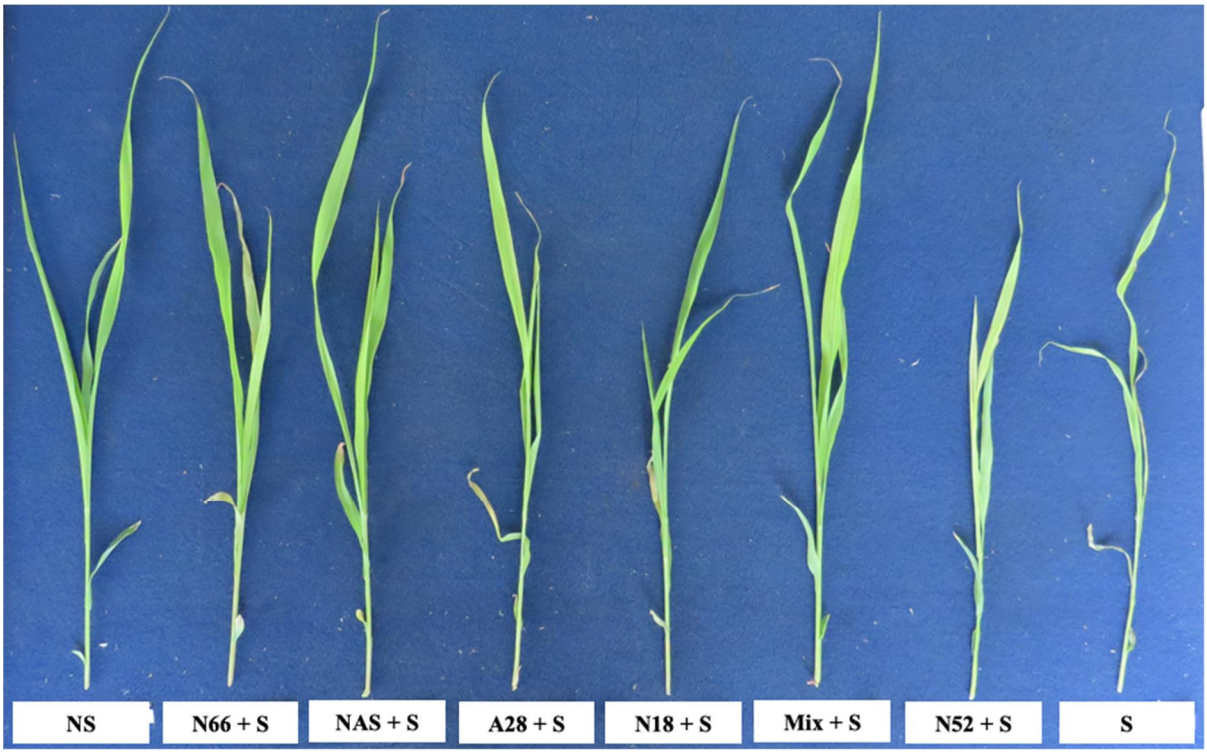



**Fig. 5** Effect five of the selected rhizobacterial isolates on *S. bicolor* shoot growth under drought stress conditions (growth chamber experiment assessing induced systemic tolerance), where NS: untreated control receiving no drought stress; N66+S: treatment with N66 and drought stress; NAS+S: treatment with NAS-6G6 and drought stress; A28+S: treatment with A28 and drought stress; N18+S: treatment with N18 and drought stress; Mix+S: treatment with all five of the selected rhizobacterial isolates and drought stress; N52+S: treatment with N52 and drought stress; and S: untreated stress control (drought stress alone).

## 3.5 Metabolomics study

### 3.5.1 Assessment of drought stress tolerance

The drought stress tolerance results of *S. bicolor* seedlings treated with the selected rhizobacterial isolates are shown in Fig. 4 (data given in Table S2 under supplementary material) and the visual effects on *S. bicolor* shoot growth are shown in Fig. 5. Treatment effects were statistically significant ($p < 0.05$) for all parameters assessed, except for root to shoot ratio and RWC. When the untreated control treatments (drought stress *vs.* no stress) are compared, it is clear that the drought stress resulted in significant lowering of leaf and root weights, plant height and root length (Fig. 4). Treatment with each of the 5 selected rhizobacterial isolates offered protection by enhancing drought stress tolerance in *S. bicolor* plants when compared to that of the untreated stress control. *Pseudomonas* sp. strain N66 was the top-performer i.t.o. fresh biomass, plant height, root length and root to shoot ratio, whereas isolate N18 was the top performer in terms of dry biomass and maintaining RWC.

### 3.5.2 Metabolite profiling

Visual inspection of the base peak intensity (BPI) chromatograms showed evidently differential peak population (presence, intensities) in rhizobacteria-primed *S. bicolor* plants *versus* the untreated controls, reflecting differential metabolite composition (and profiles)

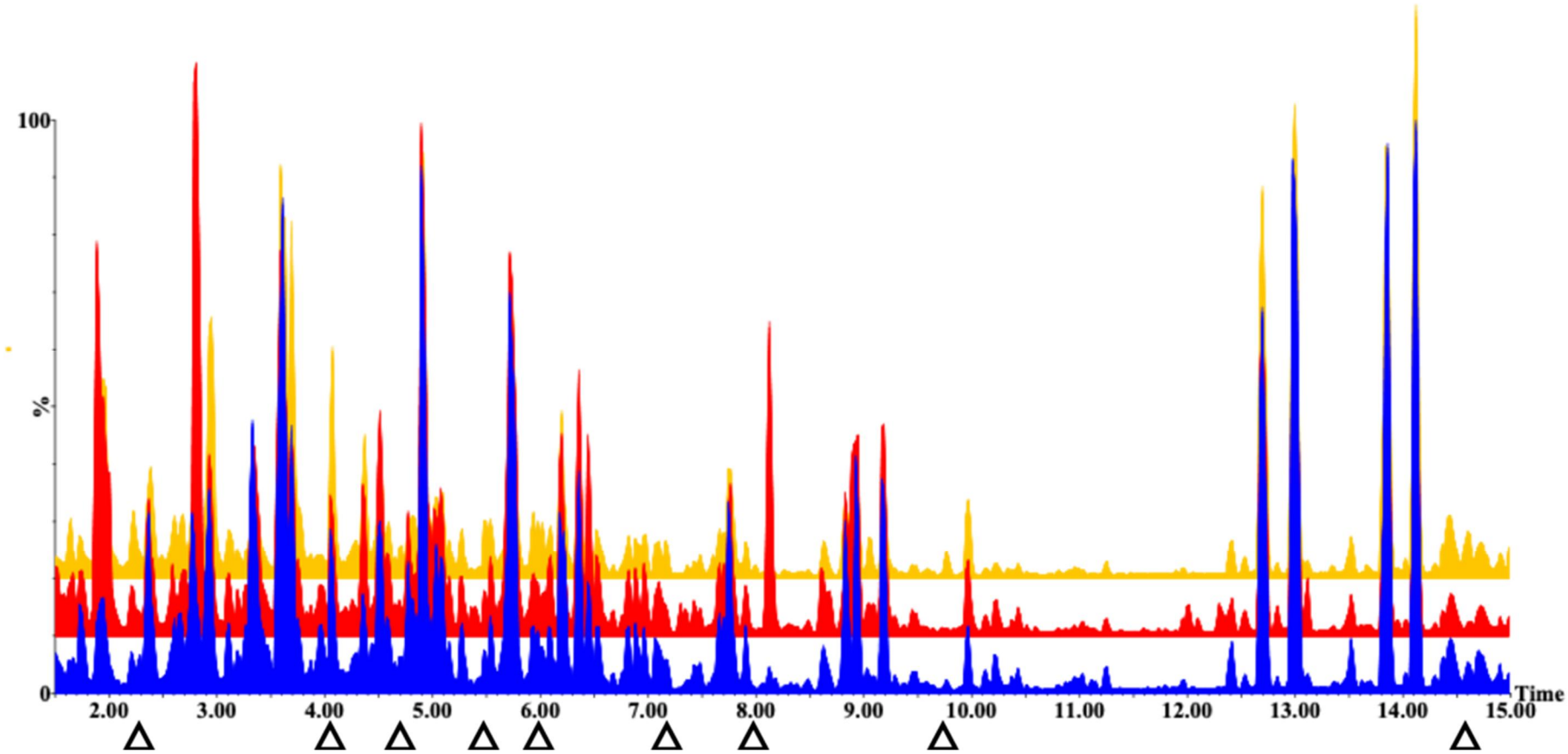



**Fig. 6** UHPLC-HDMS BPI chromatograms of ESI-positive data obtained from *S. bicolor* shoots, indicating the metabolomic profiles of treatments (from bottom to top): (1) untreated (naïve) control receiving no drought stress (blue); (2) untreated (naïve) stress control receiving drought stress alone (red); (3) treatment with rhizobacterial isolate N66 and drought stress (yellow). The triangles below the x-axis indicate the peaks of features that were upregulated in N66-primed *S. bicolor* shoots (yellow) *vs.* those left naïve (red) under drought stress conditions.

under drought stress conditions. Examples of the BPI chromatograms of ESI-positive data obtained from the shoots and roots of *S. bicolor* plants primed with *Pseudomonas* sp. strain N66 are shown in Figs. 6 and S1 (supplied under supplementary material) respectively (those for A28, N52, N18 and NAS-6G6 are not shown). Similarly that of the ESI-negative data are shown in the supplementary material as Figs. S2 and S3 respectively. Thus, in order to elucidate informative description of specific metabolic features related to these observed differential chromatographic profiles, data mining and comparative chemometric analyses were performed as described under the experimental section. The chemometric analyses that were employed included unsupervised methods namely principle component analysis (PCA) and hierarchical clustering analysis (HCA) and a supervised approach orthogonal partial least square-discriminant analysis (OPLS-DA).

The PCA and HCA provided an explorative overview of the data, pointing to structures within the data, which reflected the metabolic changes in *S. bicolor* plants in response to (1) treatment with all five of the selected rhizobacterial isolates and (2) drought stress. The PCA and HCA computed for the ESI-positive data are shown in Figs. 7 and S4 (supplied under supplementary material) for shoots and roots respectively, whereas that of the ESI-negative data are shown in the supplementary material as Figs. S5 and S6. The close clustering of the quality control samples in the PCA scores space, indicate the stability of the samples and analysis, and the quality and reliability of the generated data. The PCA models showed both rhizobacterial treatment-related clustering (Figs. 7 A) and drought stress-related clustering (Figs. 7 B). The HCA, on low-dimensional data generated from the PC analyses, was represented graphically on dendrograms. The latter provided visual evaluation of distinct subspaces in the 'metabolite space', corresponding to different treatments; thus complementing the sample observed in the PCA.

For biological characterization and interpretation of these informative metabolite profiles described by explorative modelling: PCA and HCA results, a supervised method namely OPLS-DA was applied. Infographics from OPLS-DA models of the ESI-positive data of shoots and roots are shown in Figs. 8 and S7 (supplied under supplementary material) respectively; and models for the ESI-negative data of shoots and roots are shown in the supplementary material as Figs. S8 and S9 respectively. The OPLS-DA allowed sample classification and the identification of the metabolite features underlying the discrimination between classes or groups (Tugizimana et al., 2013). The computed and validated OPLS-DA models (CV-ANOVA $p < 0.05$) used in the current study (here again examples only given for *Pseudomonas* sp. strain N66) were perfect binary classifiers (Fig. 8 A) and had no signs of possible overfitting, as indicated by cross-validation, and none of the permutated models (n = 100) performed better than the original models in separating classes (Fig. 8 B). For selection of 'variables', i.e. discriminating metabolite features with unique Rt-*m/z* values, OPLS-DA loadings S-plots (Fig. 8 C) were evaluated: this loading plot aids in identifying variables which differ between groups, i.e. the discriminating features. Variables that combine high model influence (covariation) with high reliability (correlation), i.e. variables at the far ends of the S-plot, are statistically relevant as potential discriminant variables to be selected (Tugizimana et al., 2013). To avoid variable selection bias, the variable importance in projection (VIP)-plots were generated (Fig. 8 D) and only the variables (from S-plots) with the VIP score exceeding 1.0 were retained. As mentioned in the experimental section, the statistically selected variables (from S-plots) were then annotated to MSI level-2 and are reported in Table 1.

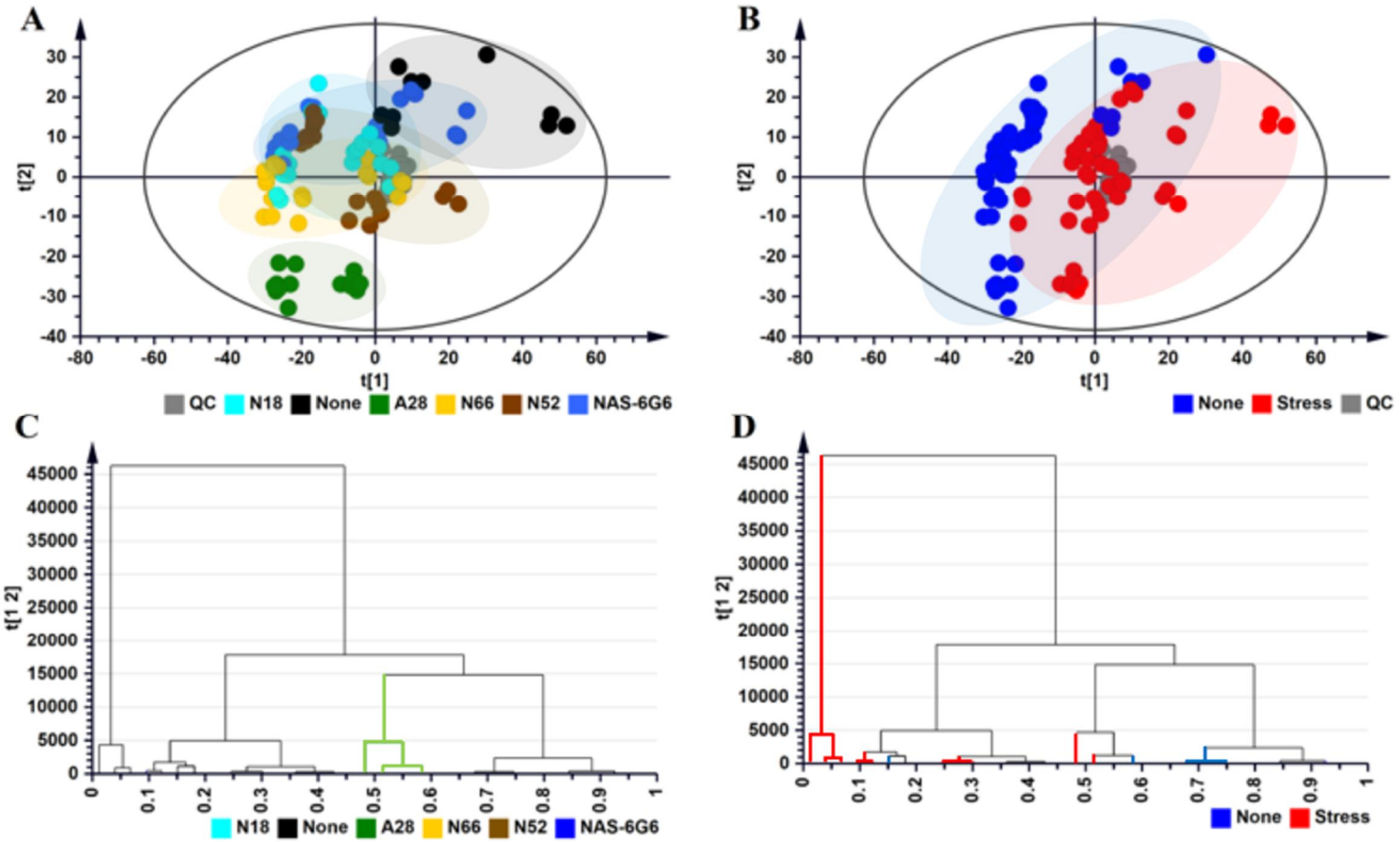



**Fig. 7** PCA score / scatter plot of *S. bicolor* shoot samples computed from ESI-positive data representing the first two PCs of a 13-component PCA model. The model explains 60.8% variation in the Pareto-scaled data ($R^2X$ = 0.608) and 46.9% predicted variation according to cross-validation ($Q^2$ =0.469). (A and B) represents the same PCA scores plot with (A) showing the rhizobacteria treatment-related clustering and (B) showing the drought stress-related clustering. (C and D) HCA dendrogram corresponding to (A and B). Legend: (A and C) QC: Quality control samples (grey); N18: treatment with rhizobacterial isolate N18 (light blue); None: untreated control (black); A28: treatment with rhizobacterial isolate A28 (green); N66: treatment with rhizobacterial isolate N66 (yellow); N52: treatment with rhizobacterial isolate N52 (brown); NAS-6G6: treatment with semi-commercial strain NAS-6G6 (dark blue) and (B and D) None: no-drought stress (blue); Stress: drought stress (red).

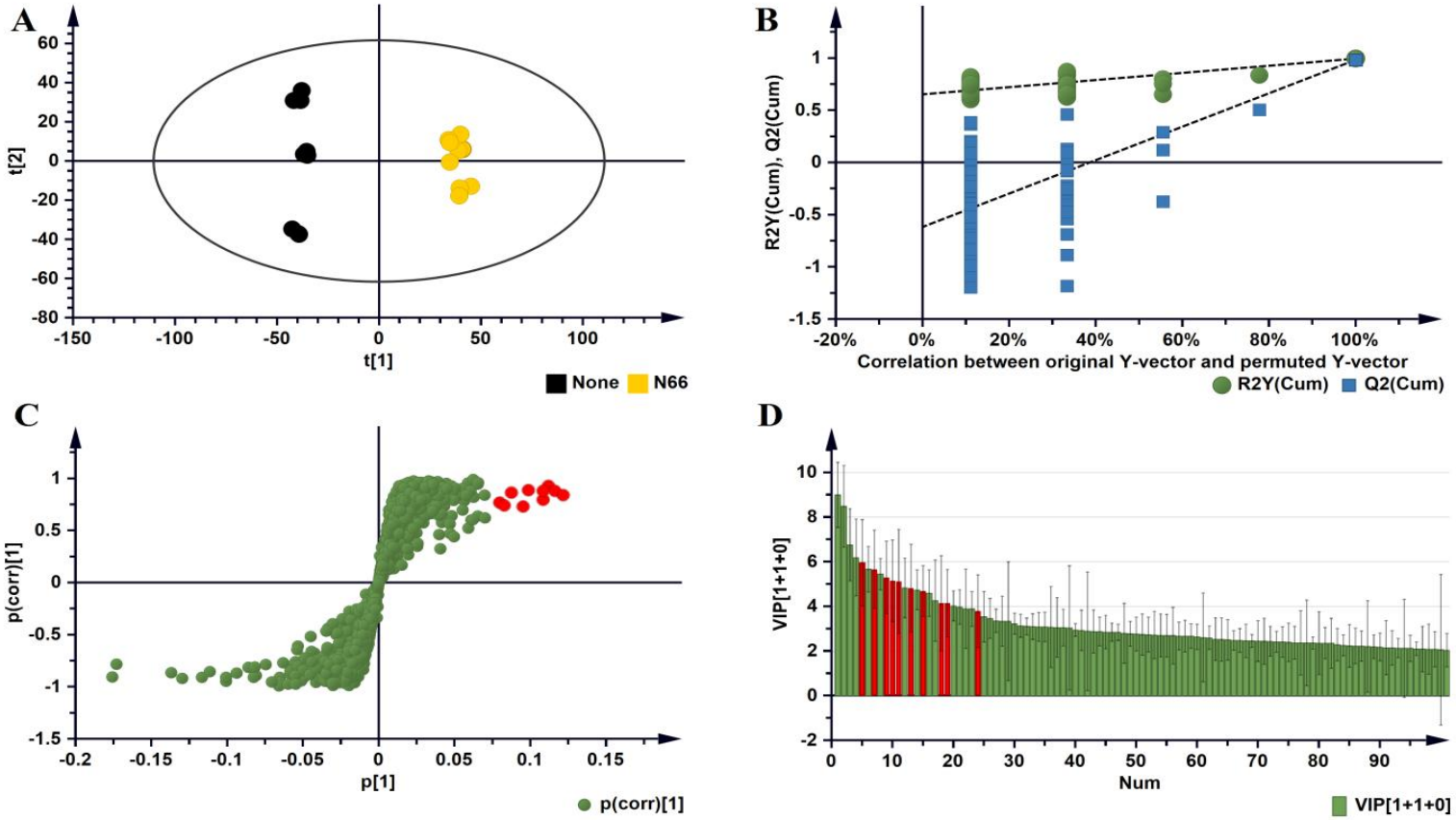



**Fig. 8** OPLS-DA modelling of *S. bicolor* shoot samples and variable/feature selection ESI-positive data. (A) A typical scores scatter plot for the OPLS-DA model separating drought stressed plants that were (1) left untreated (None) *vs* (2) N66-treated (N66) (1 + 1 + 0 components, $R^2X = 0.593$, $Q^2 = 0.982$, CV-ANOVA $p < 0.05$). In the scores plot, it is evident that the two groups are clearly separated: None *vs* N66. (B) A typical response permutation test plot (n = 100) for the OPLS-DA model in (A); the $R^2$ and $Q^2$ values of the permutated models correspond to *y*-axis intercepts: $R^2 = (0.0, 0.56)$ and $Q^2 = (0.0, -0.56)$; (C) An OPLS-DA loadings S-plot for the same model in (A); variables situated in the extreme end of the S-plot are statistically relevant and represent prime candidates as discriminating variables/features. (D) A VIP plot for the same model; pointing mathematically to the importance of each variable (feature) in contributing to group separation in the OPLS-DA model. (C and D) Examples of the variables that were significantly upregulated in primed plants are highlighted in red.

**Table 1**. Summary of metabolites significantly upregulated in the roots and shoots of rhizobacteria-treated *S. bicolor* plants under drought stress conditions *versus* those left naïve (untreated stress control). Discriminating metabolites were identified based on OPLS-DA S-plots.

| | | | | | | | | Metabolite fold change (fc) and *p*-value of primed *versus* naïve drought stressed plants | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | N66 | | NAS-6G6 | | A28 | | N18 | | N52 | |
| | Metabolite | *m/z* | RT (min) | Tissue | ESI mode | MF | Metabolic pathway / IST MOA | fc | *p* | fc | *p* | fc | *p* | fc | *p* | fc | *p* |
| 1 | 1-Organyl-2-lyso-sn-glycero-3-phosphocholine | 542.32 | 13.95 | Roots | Pos | $C_{26}H_{50}NO_7P$ | Amine / Osmolyte | 3.31 | 2E-02 | 4.96 | 1E-05 | 6.79 | 2E-05 | 3.53 | 5E-03 | - | - |
| 2 | 5-L-Glutamyl-Taurine | 288.05 | 8.87 | Shoots | Neg | $C_7H_{14}N_2O_6S$ | Amino acid | 2.95 | 7E-12 | 3.19 | 6E-10 | - | - | 2.78 | 2E-11 | 2.35 | 1E-07 |
| 3 | N2-Succinyl-L-Arginine | 311.08 | 5.04 | Shoots | Neg | $C_{10}H_{18}N_4O_5$ | Amino acid / Signalling | 3.00 | 2E-02 | 2.46 | 4E-02 | - | - | 2.27 | 3E-02 | - | - |
| 4 | 2-(3-Carboxy-3-aminopropyl)-L-Histidine | 323.10 | 2.52 | Shoots | Neg | $C_{10}H1_6N_4O_4$ | Amino acid | 6.16 | 1E-08 | 5.77 | 1E-09 | 4.25 | 1E-08 | 7.73 | 5E-09 | 6.82 | 1E-10 |
| 5 | L-Tyrosine | 182.08 | 3.42 | Shoots | Pos | $C_9H_{11}NO_3$ | Amino acid / Precursor | 5.44 | 4E-05 | 7.52 | 9E-09 | 3.63 | 3E-02 | 4.34 | 2E-03 | 3.06 | 7E-05 |
| 6 | Glutathione | 466.00 | 3.74 | Shoots | Neg | $C1_0H_{16}N_3O_6S$ | Amino acid / AOX | 5.47 | 2E-04 | 4.98 | 2E-08 | 4.50 | 2E-06 | 4.72 | 2E-03 | 4.00 | 4E-03 |
| 7 | N6,N6,N6-Trimethyl-L-Lysine | 315.11 | 1.91 | Shoots | Pos | $C_9H_{20}N_2O_2$ | Amino acid / AOX | 3.34 | 1E-03 | 4.03 | 1E-05 | 2.57 | 2E-09 | 2.26 | 2E-09 | 2.34 | 2E-04 |
| 8 | N6-Methyl-L-Lysine | 245.09 | 5.59 | Roots | Pos | $C_6H_{14}NO_6P$ | Amino acid / AOX | 3.74 | 6E-04 | 4.24 | 1E-05 | 8.93 | 6E-11 | 4.91 | 3E-07 | 2.53 | 1E-04 |
| 9 | 5-Hydroxyindoleacetylglycine | 272.07 | 8.67 | Roots | Pos | $C_{12}H_{12}N_2O_4$ | Amino acid / Carbon support | 3.22 | 2E-03 | 3.23 | 2E-04 | 3.02 | 2E-04 | 4.34 | 9E-07 | - | - |
| 10 | 5-Hydroxy-L-tryptophan | 287.06 | 4.41 | Roots | Neg | $C_{11}H_{12}N_2O_3$ | Amino acid / Precursor | 5.27 | 1E-02 | 5.27 | 1E-02 | 4.91 | 2E-03 | 3.13 | 3E-02 | 3.71 | 4E-02 |
| 11 | 4-Hydroxyphenylacetylglutamic acid | 280.08 | 3.13 | Shoots | Neg | $C_{13}H_{15}NO_6$ | Amino acid / Osmolyte | 4.14 | 3E-06 | - | - | 3.23 | 1E-10 | 3.02 | 2E-09 | 3.74 | 2E-06 |
| 12 | L-Proline | 303.09 | 1.73 | Shoots | Pos | $C_5H_9NO_2$ | Amino acid / Osmolyte | 3.05 | 8E-03 | - | - | 3.81 | 3E-08 | 3.15 | 2E-05 | 2.24 | 4E-05 |
| 13 | Hydroxyproline | 297.11 | 4.91 | Roots | Pos | $C_5H_9NO_3$ | Amino acid / Osmolyte | 5.04 | 4E-03 | - | - | 7.17 | 2E-03 | 6.39 | 5E-06 | - | - |
| 14 | Docosanedioate | 427.26 | 14.24 | Roots | Pos | $C_{22}H_{40}O_4$ | Cutin, suberine & wax / cuticular reinforcement | 5.87 | 3E-04 | 3.16 | 3E-05 | 3.34 | 1E-02 | 6.77 | 2E-04 | - | - |

**Table 1** *Cont.*

| | Metabolite | *m/z* | RT (min) | Tissue | ESI mode | MF | Metabolic pathway / IST MOA | Metabolite fold change (fc) and *p*-value of primed *versus* naïve drought stressed plants | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | N66 | | NAS-6G6 | | A28 | | N18 | | N52 | |
| | | | | | | | | fc | *p* | fc | *p* | fc | *p* | fc | *p* | fc | *p* |
| 15 | 13'-Apo-beta-Carotenone | 384.14 | 5.00 | Shoots | Neg | $C_{18}H_{26}O$ | Carotenoid / AOX | 6.08 | 5E-08 | 4.47 | 2E-08 | 2.92 | 1E-04 | 4.53 | 2E-04 | 3.26 | 8E-08 |
| 16 | Shikimic acid | 173.05 | 2.88 | Shoots | Neg | $C_7H_{10}O_5$ | Flavonoid / AOX | 10.29 | 2E-09 | 13.80 | 9E-11 | 12.23 | 8E-10 | 13.38 | 4E-10 | 9.35 | 3E-05 |
| 17 | Naringenin 7-O-beta-D-glucoside | 435.13 | 3.60 | Roots | Pos | $C_{21}H_{22}O_{10}$ | Flavonoid / AOX | 3.42 | 3E-02 | 3.65 | 3E-03 | 7.44 | 2E-07 | 5.53 | 5E-03 | - | - |
| 18 | 3',4',5'-Pentahydroxyflavanone | 303.05 | 4.79 | Roots | Neg | $C_{15}H_{12}O_7$ | Flavonoid / AOX | 2.72 | 8E-07 | 2.56 | 1E-05 | 2.33 | 1E-04 | 2.65 | 4E-06 | - | - |
| 19 | Hydroquinone-O-beta-D-glucopyranoside | 361.05 | 3.96 | Shoots | Neg | $C_{12}H_{16}O_7$ | Flavonoid / AOX | 3.82 | 2E-03 | - | - | 3.53 | 7E-04 | 3.48 | 2E-03 | - | - |
| 20 | Hesperetin 7-O-glucoside | 463.12 | 1.72 | Shoots | Neg | $C_{22}H_{24}O_{11}$ | Flavonoid / AOX | 3.58 | 1E-05 | - | - | 3.83 | 3E-09 | 3.45 | 6E-06 | 2.38 | 8E-05 |
| 21 | Caffeoylshikimate | 357.06 | 9.20 | Roots | Neg | $C_{16}H_{16}O_8$ | Flavonoid / AOX | 2.60 | 6E-05 | 2.17 | 1E-02 | 2.60 | 6E-05 | - | - | - | - |
| 22 | Caffeoylquinate | 355.10 | 4.27 | Shoots | Pos | $C_{16}H_{18}O_9$ | Flavonoid / AOX | 2.23 | 3E-02 | 2.69 | 1E-03 | 2.39 | 2E-02 | 2.63 | 4E-04 | 2.20 | 8E-03 |
| 23 | Trans-5-O-(4-coumaroyl)-D-Quinate | 361.09 | 4.46 | Shoots | Pos | $C_{16}H_{18}O_8$ | Flavonoid / AOX | 2.61 | 4E-03 | - | - | 2.83 | 3E-03 | 3.28 | 2E-05 | 2.71 | 2E-02 |
| 24 | 1-O-Sinapoyl-beta-D-glucose | 409.11 | 4.16 | Shoots | Pos | $C_{17}H_{22}O_{10}$ | Flavonoid / AOX | - | - | 2.99 | 4E-05 | 3.44 | 4E-06 | 2.67 | 2E-04 | 2.11 | 2E-02 |
| 25 | Luteolin 7-O-neohesperidoside | 610.18 | 1.85 | Shoots | Neg | $C_{27}H_{30}O_{15}$ | Flavonoid / AOX | 2.46 | 3E-04 | 3.54 | 3E-06 | 2.09 | 2E-03 | 2.86 | 2E-04 | - | - |
| 26 | Luteolin 7-O-(6''-malonylglucoside) | 535.11 | 4.33 | Shoots | Pos | $C_{24}H_{22}O_{14}$ | Flavonoid / AOX | 5.71 | 2E-02 | 5.51 | 3E-02 | 23.02 | 9E-07 | 2.32 | 1E-02 | - | - |
| 27 | Naringin | 665.15 | 5.59 | Shoots | Pos | $C_{27}H_{32}O_{14}$ | Flavonoid / AOX | 4.08 | 8E-03 | - | - | 14.49 | 6E-06 | 5.83 | 2E-05 | - | - |
| 28 | Hesperetin | 303.09 | 8.12 | Roots | Pos | $C_{16}H_{14}O_6$ | Flavonoid / AOX | 4.05 | 4E-03 | 4.14 | 1E-04 | 4.34 | 1E-03 | 2.96 | 1E-03 | 2.89 | 1E-02 |
| 29 | 3,7-Di-O-methylquercetin | 331.08 | 2.63 | Roots | Pos | $C_{17}H_{14}O_7$ | Flavonoid / AOX | 15.52 | 3E-02 | 14.97 | 8E-03 | - | - | 15.14 | 2E-03 | - | - |

**Table 1** *Cont.*

| | Metabolite | *m/z* | RT (min) | Tissue | ESI mode | MF | Metabolic pathway / IST MOA | Metabolite fold change (fc) and *p*-value of primed *versus* naïve drought stressed plants | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | N66 | | NAS-6G6 | | A28 | | N18 | | N52 | |
| | | | | | | | | fc | *p* | fc | *p* | fc | *p* | fc | *p* | fc | *p* |
| 30 | (9Z,12Z,15Z)-Octadecatrienoic acid | 872.52 | 5.49 | Shoots | Pos | $C_{41}H_{80}NO_8P$ | Lipid / JA precursor | 2.81 | 1E-05 | 2.24 | 6E-04 | 3.00 | 8E-04 | 3.08 | 4E-04 | 3.28 | 4E-07 |
| 31 | 13(S)-Hydroperoxylinolenate | 331.19 | 13.29 | Roots | Neg | $C_{18}H_{30}O_4$ | Lipid / JA precursor | 12.08 | 4E-05 | 10.12 | 2E-07 | 23.61 | 3E-08 | 9.66 | 5E-06 | 7.93 | 4E-03 |
| 32 | Phosphatidylserine | 546.28 | 11.69 | Roots | Pos | $C2_3H_{44}NO_7P$ | Lipid / JA precursor | 5.52 | 1E-07 | 4.40 | 8E-08 | 2.54 | 3E-06 | 3.41 | 3E-06 | - | - |
| 33 | 3,4-Dihydroxyphenylacetate | 191.03 | 8.00 | Shoots | Pos | $C_8H_8O_4$ | Phytohormone / Growth regulation | 3.50 | 6E-10 | - | - | 2.17 | 2E-07 | 3.22 | 9E-11 | 2.30 | 8E-06 |
| 34 | Indole-3-acetic acid | 208.06 | 4.17 | Shoots | Pos | $C_{10}H_6O_4$ | Phytohormone / Growth regulation | - | - | 2.20 | 7E-04 | 3.50 | 4E-07 | 2.41 | 2E-04 | - | - |
| 35 | Gibberellin A1 | 385.11 | 4.74 | Shoots | Neg | $C_{19}H_{24}O_6$ | Phytohormone / Growth regulation | - | - | 2.05 | 9E-05 | 2.98 | 1E-08 | 2.05 | 6E-05 | - | - |
| 36 | Kinetin-7-N-glucoside | 466.00 | 3.58 | Shoots | Neg | $C_{16}H_{19}N_5O_6$ | Phytohormone / Growth regulation | 3.31 | 5E-03 | 2.83 | 3E-03 | 3.15 | 6E-04 | 2.35 | 4E-02 | 3.14 | 3E-03 |
| 37 | Gibberellin A29 | 413.12 | 8.87 | Roots | Neg | $C_{19}H_{22}O_6$ | Phytohormone / Growth regulation | - | - | 2.25 | 3E-02 | 2.89 | 4E-03 | 2.80 | 1E-03 | - | - |
| 38 | Gibberellin A34 | 421.00 | 1.73 | Shoots | Pos | $C_{19}H_{22}N_6O_4$ | Phytohormone / Growth regulation | 4.24 | 5E-09 | 3.52 | 7E-09 | 4.65 | 6E-09 | 3.85 | 3E-09 | 3.62 | 3E-09 |
| 39 | Salicin | 354.09 | 6.01 | Shoots | Neg | $C_{13}H_{18}O_7$ | Phytohormone / Signalling | 2.16 | 3E-05 | - | - | - | - | 2.24 | 7E-07 | 2.11 | 3E-04 |
| 40 | 26-Hydroxybrassinolide | 512.36 | 13.08 | Roots | Neg | $C_{28}H_{48}O_7$ | Phytohormone / Signalling | - | - | 7.62 | 7E-05 | 5.21 | 8E-03 | 5.49 | 1E-02 | 4.02 | 5E-02 |
| 41 | (-)-Methyl Jasmonate | 299.08 | 2.10 | Roots | Pos | $C_{13}H_{20}O_3$ | Phytohormone / Signalling | 6.63 | 1E-02 | 2.82 | 5E-02 | 4.59 | 1E-02 | 10.02 | 3E-03 | - | - |
| 42 | Ethylene thiourea | 103.03 | 1.90 | Shoots | Pos | $C_3H_6N_2S$ | Phytohormone / Stress signalling | 0.31 | 8E-07 | 0.38 | 3E-06 | 0.68 | 9E-03 | 0.32 | 2E-07 | - | - |
| 43 | Glucosylsphingosine | 589.29 | 11.77 | Roots | Pos | $C_{24}H_{47}NO_7$ | Sphingolipid / Signalling | 10.53 | 5E-08 | 7.19 | 6E-08 | 6.50 | 2E-06 | 4.86 | 2E-07 | - | - |
| 44 | Psychosine | 588.29 | 11.78 | Roots | Pos | $C_{24}H_{47}NO_7$ | Sphingolipid / Signalling | 6.84 | 5E-07 | 4.31 | 2E-10 | 3.97 | 9E-07 | 3.14 | 1E-11 | - | - |
| 45 | Loganin | 457.13 | 4.17 | Shoots | Neg | $C_{17}H_{26}O_{10}$ | Terpenoid / AOX | 3.86 | 1E-03 | 3.41 | 2E-05 | 3.93 | 3E-04 | 3.75 | 4E-04 | 4.05 | 3E-04 |

**Table 1** *Cont.*

| | | | | | | | | Metabolite fold change (fc) and *p*-value of primed *versus* naïve drought stressed plants | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | N66 | | NAS-6G6 | | A28 | | N18 | | N52 | |
| | Metabolite | *m/z* | RT (min) | Tissue | ESI mode | MF | Metabolic pathway / IST MOA | fc | *p* | fc | *p* | fc | *p* | fc | *p* | fc | *p* |
| 46 | Secologanin | 433.13 | 3.60 | Roots | Neg | $C_{17}H_{24}O_{10}$ | Terpenoid / AOX | - | - | 2.03 | 3E-03 | 2.82 | 6E-06 | 2.03 | 2E-03 | 2.23 | 1E-02 |
| 47 | Demethylmenaquinol | 557.29 | 11.04 | Shoots | Pos | $C_{30}H_{46}O_2$ | Terpenoid / AOX | 19.44 | 7E-07 | 10.85 | 1E-09 | 6.34 | 2E-05 | 11.20 | 3E-05 | 12.19 | 3E-06 |
| 48 | 2,3-Dimethyl-6-phytyl-1,4-benzoquinol | 541.30 | 11.45 | Roots | Neg | $C_{28}H_{48}O_2$ | Terpenoid / AOX | 4.96 | 2E-06 | 3.84 | 7E-09 | 3.17 | 4E-07 | 2.81 | 4E-06 | 2.13 | 3E-02 |
| 49 | Ubiquinol | 291.10 | 5.59 | Roots | Pos | $C_{14}H_{20}O_4$ | Terpenoid / AOX | 3.30 | 6E-05 | 2.29 | 3E-02 | 7.18 | 7E-09 | 4.20 | 3E-07 | - | - |
| 50 | Riboflavin | 399.20 | 1.62 | Roots | Pos | $C_{17}H_{19}FN_4S$ | Vitamin / AOX | 3.21 | 3E-02 | 6.58 | 3E-09 | 4.17 | 8E-03 | 3.94 | 6E-03 | - | - |
| 51 | Delta-tocopherol | 528.29 | 11.36 | Roots | Neg | $C_{27}H_{46}O_2$ | Vitamin / AOX | 9.35 | 3E-06 | 4.59 | 2E-06 | 3.75 | 7E-07 | 3.29 | 5E-05 | 3.40 | 4E-02 |
| 52 | Gamma-tocopherol | 574.30 | 11.35 | Roots | Pos | $C_{28}H_{46}O_4$ | Vitamin / AOX | 9.32 | 2E-02 | 5.40 | 1E-07 | 4.21 | 8E-07 | 3.48 | 8E-06 | - | - |
| 53 | Beta-tocopherol | 542.31 | 8.07 | Roots | Neg | $C_{28}H_{48}O_2$ | Vitamin / AOX | 5.03 | 7E-07 | 3.75 | 2E-08 | 3.28 | 2E-06 | 2.83 | 3E-06 | - | - |

Abbreviations: ESI= electrospray ionisation; RT= retention time; MF= molecular formula; IST= induced systemic tolerance; MOA= mode of action; fc= fold change; *p*= probability value; Pos= positive; Neg= negative; AOX= antioxidant.

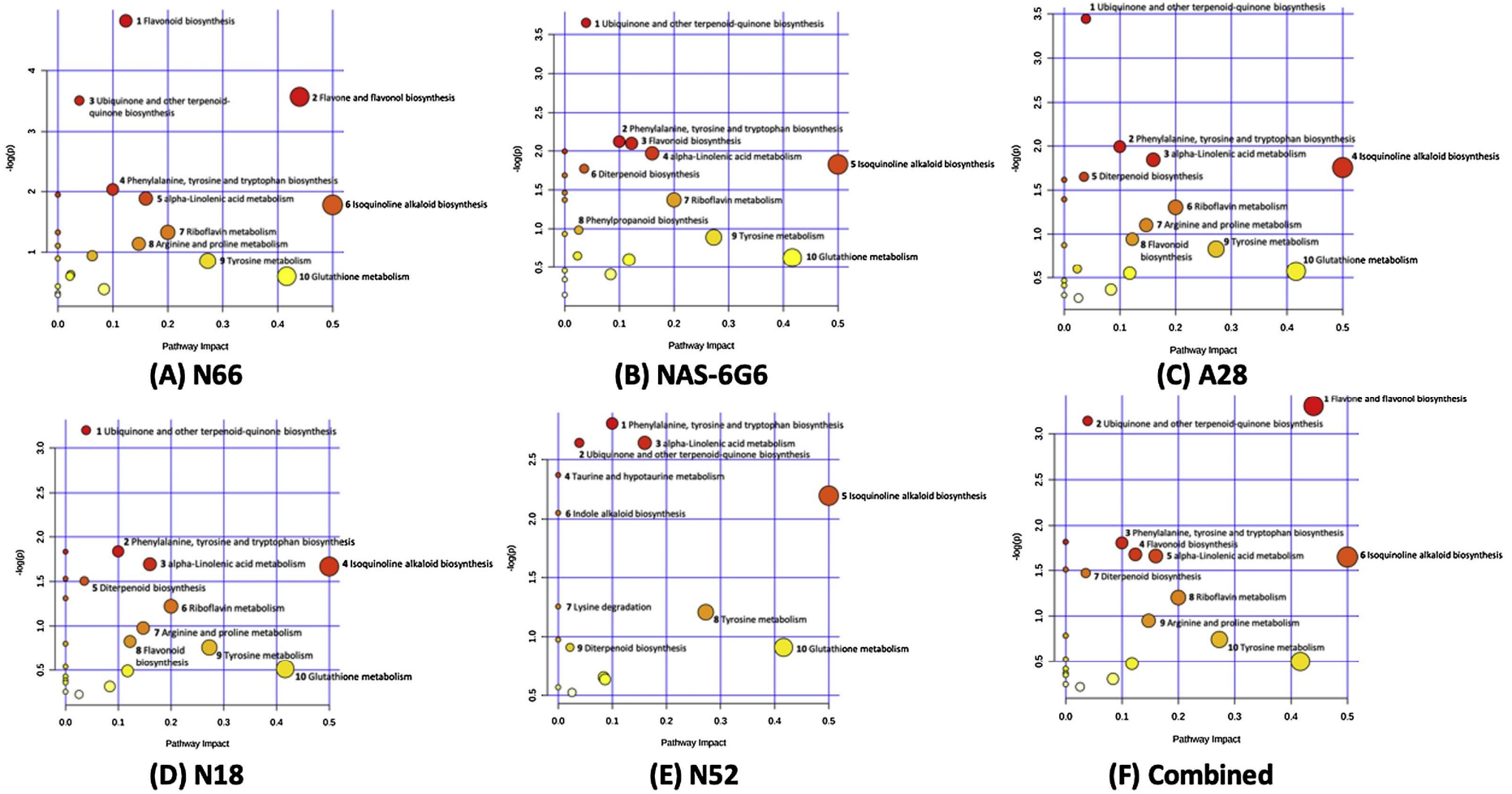



**Fig. 9** Summary of metabolic pathway analyses (MetPA) for *S. bicolor* plants treated (primed) with rhizobacterial isolates (A) N66, (B) NAS-6G6, (C) A28, (D) N18, (E) N52 and (F) combined pathways as shown in Table 1 compared to the untreated control (naïve): Representation of all MetPA-computed metabolic pathways displayed per their significance or pathway impact. The graph, "metabolome view" contains all the matched pathways (the metabolome) arranged by *p*-values (pathway enrichment analysis) on *y*-axis, and the pathway impact values (pathway topology analysis) on *x*-axis. The node colour is based on the *p*-value and the node radius is defined by the pathway impact values. The latter is the cumulative percentage from the matched metabolite nodes.

### 3.5.3. Metabolic pathway analysis

Pathway analysis with MetPa (Fig. 9 A-F) revealed that isoquinoline alkaloid biosynthesis and glutathione biosynthesis were upregulated by all five of the selected rhizobacterial isolates. Flavonoid biosynthesis, flavone biosynthesis and ubiquinone and other terpenoid quinone biosynthesis were the most significant pathways upregulated by *Pseudomonas* sp. strain N66. Ubiquinone and other terpenoid quinone biosynthesis, phenylalanine-, tyrosine- and tryptophan biosynthesis and flavonoid biosynthesis were the most significant pathways upregulated by *P. alvei* NAS-6G6. Ubiquinone and other terpenoid quinone biosynthesis, phenylalanine-, tyrosine- and tryptophan biosynthesis and alpha-linolenic acid metabolism were upregulated by both *Bacillus* sp. strain N18 and *Bacillus* sp. strain A28. Tyrosine- and tryptophan biosynthesis, alpha-linolenic acid metabolism and ubiquinone and other terpenoid quinone biosynthesis were upregulated by *Bacillus* sp. strain N52.

## 4 Discussion

Rhizobacteria belonging to the genera *Bacillus* and *Pseudomonas* are known to protect their host-plants against drought stress by eliciting induced systemic tolerance by means of either one or more of the following mechanisms: (1) growth promotion and root architecture modification as a result of the upregulation of phytohormones; (2) osmotic adjustment; (3) AOX metabolism and (4) ACC deaminase activity resulting in lowered ethylene levels (Ngumbi & Kloepper, 2016). Many rhizobacterial species employ ACC deaminase activity to lower ethylene levels under conditions of environmental stress, thus benefitting from the resultant enhanced resistance induced in their hosts (Glick, 2005). This rhizobacteria-induced drought stress tolerance in plants obtained from ACC deaminase activity has been reported for both *Bacillus* spp. [*B. circulans*, *B. firmus* and *B. globisporus* (Ghosh et al., 2003); *B. licheniformis* (Ngumbi and Kloepper, 2016); *B. mojavensis* (Pourbabaee et al., 2016)] and *Pseudomonas* spp. [*P. brassicacearum* (Belimov et al., 2007); *P. fluorescens* (Ali et al., 2014; Arshad et al., 2008), *P. taiwanensis* (Shagol et al., 2014) and *P. putida* (Arshad et al., 2008; Glick et al., 1997)]. ACC deaminase activity was confirmed for *Pseudomonas* sp. strain N66, by means of an *in vivo* lab assay and the presence of the *acdS* gene was subsequently confirmed by means of molecular techniques (Fig. 3).

Priming *S. bicolor* seedlings with each of the five selected rhizobacterial isolates had a statistically significant impact ($p < 0.05$) on *S. bicolor* growth under drought stress. This protection was the result of differential metabolic reprogramming between rhizobacteria-primed and non-primed (naïve) plants, confirming the ability of all five of the five selected rhizobacterial isolates to induce systemic tolerance in *S. bicolor* against drought stress. This reprogramming involved metabolites that are actively involved in the tolerance against drought stress in plants such as phytohormones, AOX, osmolytes, flavonoids, terpenoids, cutin, suberine and waxes.

The phytohormones upregulated in rhizobacteria-treated *S. bicolor* plants known for their role in tolerance against drought stress, by the manipulation of plant growth, included cytokinin, GA and auxins. The signalling hormones that were upregulated consisted of JA (including its lineolate-precursors) (Khan, Nazar, Iqbal, 2012), SA (Horváth et al., 2007) and brassinolide (Pieterse et al., 2009). Ethylene was down-regulated in rhizobacteria-primed *versus* non-primed *S. bicolor* plants for all five of the selected rhizobacterial isolates tested, except *Bacillus* sp. strain N52. Since ethylene is a stress-hormone involved in plant senescence, this reduction in ethylene levels supports the enhanced tolerance of drought stress observed (Glick et al.,

2007). Other signalling molecules that play a role in the induction of tolerance against drought consisted of the sphingolipids glycosylsphingosine and psychosine. A study done on *Arabidopsis* by Seo et al., (2011) provides evidence of ABA-mediated transcriptional activation of cuticular wax biosynthesis in response to drought stress. The current study provided evidence for the role of ABA as signal hormone in the activation of a drought-tolerance response through the biosynthesis of cuticular waxes such as docosanoic acid.

In addition to the upregulation of phytohormones, additional changes in primary metabolism included the amino acids. Similarly to what is observed during induced systemic resistance, primed *S. bicolor* plants showed enhanced levels of amino acids, pointing to a preconditioning for enhanced tolerance of drought stress. During abiotic stress, amino acids serve either as a source of energy, signalling hormones or as building blocks. When the tricarboxylic acid cycle is inhibited under conditions of oxidative stress for example, accumulation of amino acids such as lysine and glycine are known to promote ongoing activity of the mitochondrial electron transport chain and associated adenosine triphosphate generation (Noctor et al., 2015; Schauer et al., 2006). The accumulation of aromatic amino acids such as tryptophan and tyrosine, points to the activation of phenolic and indolic defence pathways (Noctor et al., 2015) and the production of glucosinolates, phytoalexins and auxin (Pastor et al., 2014). The signalling amino acid arginine is known to promote the production of AOX under conditions of oxidative stress (Noctor et al., 2015).

The production of ROS is an unavoidable product of aerobic metabolism and is kept in equilibrium by the production AOX. Under conditions of environmental stress, this redox equilibrium is challenged, which if left unattended, can lead to oxidative damage. The speed at which plants respond to environmental stress, through the production of AOX, is directly linked to its tolerance thereof. However, although ROS are damaging at high levels, they are important stress-signalling molecules at low levels that regulate phytohormone production and ultimately plant response to environmental stress emphasizing the importance of the maintenance of redox equilibrium (Baxter et al., 2014). PGPR possess the ability to augment the plant's capacity to maintain cellular redox-equilibrium by inducing a quicker and enhanced upregulation of AOX. This enhanced activation of the plant's AOX defence system comprises of both enzymatic and non-enzymatic components that scavenge ROS. In the current study, UHPLC-HDMS enabled measurement of the non-enzymatic AOX only. These included significant upregulation of the AOX glutathione, tyrosine, tocopherol, carotenoids and phenolic compounds (Sharma et al., 2012). Glutathione metabolism was significantly upregulated in *S. bicolor* plants under drought stress, post treatment with each of the selected rhizobacterial isolates. Glutathione is a tripeptide thiol (consisting of glycine, glutamate and cysteine) that has powerful AOX capacity and numerous MOA. It protects macromolecules such as lipids, proteins and DNA from free radical damage through a process of glutathiolation; acts as a proton donor in the presence of free radicals and plays a role in the production of other AOX such as ascorbate. Another AOX amino acid that was upregulated in *S. bicolor* plants under drought stress, post treatment with all five of the selected rhizobacterial isolates, include the monophenolic amino acid tyrosine which is known to act as free radical scavenger (Gülçin, 2007). In the current study, tyrosine was significantly upregulated in *S. bicolor* seedlings primed with each of the rhizobacterial strains tested (Table 1). In the current study tocopherol was significantly upregulated in *S. bicolor* plants under drought stress post treatment with each of the five selected rhizobacterial isolates. The carotenoid 13-apo-beta-carotenone (Pastor et al., 2014), arising from the oxidative degradation of β,β-carotene, was significantly upregulated in *S. bicolor* plants under drought stress post treatment with each of the five of the selected rhizobacterial isolates in the current study. Carotenoids are regarded as powerful AOX, mainly

because of their isoprene chains that are able to absorb and dissipate excess energy as heat (Sharma et al., 2012). Furthermore, other pathways that were involved were those of the phenolic compounds. The latter play important roles as AOX include the hydroxycinnamic acids, flavonoids, tannins and lignins. The phenolic compounds that were upregulated in *S. bicolor* plants under drought stress post treatment with each of the five selected rhizobacterial isolates included the hydroxycinnamic acids and -conjugates: caffeoylshikimate, caffeoylquinate, coumaroylquinate, hesperetin, hydroxyflavone, luteolin, naringenin, 1-O-sinapoyl-beta-D-glucose and shikimic acid; the flavonoids quercetin, hesperetin, hydroxyflavone, luteolin and naringenin; the terpenoids loganin and secologanin; and the terpenoid quinones menaquinol, benzoquinol and ubiquinone (Karuppanapandian et al., 2011; Martinez et al., 2016). The group of osmolytes that were upregulated in primed *S. bicolor* plants included the amino acids proline (Szabados and Savouré, 2010), glutamic acid (Tari et al., 2013); and the amine choline (Dawood, 2016). Choline plays a critical role in plant stress resistance, mainly for enhancing glycine betaine synthesis and accumulation and improved leaf RWC.

Several members of *Bacillus* spp. are known to elicit systemic tolerance against drought stress in their host plants. The mechanisms involved comprise mainly of (1) augmented AOX capacity (Kasim et al., 2013; Merkl et al., 2010; Vardharajula et al., 2011), (2) growth promotion (Dimkpa et al., 2009; Grover et al., 2014) and root architecture modification (Sharma et al., 2015; Timmusk et al., 2014) by the production of phytohormones, (3) the early activation of induced systemic tolerance by signalling hormones (Lim and Kim, 2013; Kang et al., 2015), (4) enhanced osmoregulation through the augmented osmolyte levels (Ortiz et al., 2015; Sharma et al., 2015; Vardharajula et al., 2011) and (5) lowered ethylene levels as a result of ACC deaminase activity (Lim and Kim, 2013; Saikia et al., 2018). In the current study *Bacillus* sp. strain N18, *Bacillus* sp. strain N52 and *Bacillus* sp. strain A28 attributed to similar changes in the *S. bicolor* metabolome that attributed to an enhanced drought stress tolerance. In addition to the MOA mentioned above, the *Bacillus* spp. tested were able to elicit unique changes in the *S. bicolor* metabolome. Interestingly, *Bacillus* sp. strain N18 and *Bacillus* sp. strain A28 elicited a similar response in *S. bicolor* which comprised of augmented AOX capacity, growth promotion through the upregulation of IAA, cytokinin and GA; enhanced induced systemic tolerance signalling through upregulation of SA, brassinolide, JA, sphingosine and psychosine; enhanced osmoregulation through the osmolytes proline, glutamic acid and choline; the production of epicuticular wax; and lowered ethylene levels. Although *B. pumilus* is known to enhance osmotic stress tolerance by means of augmented antioxidant- and osmolyte levels (Jha et al., 2011), in the current study the capacity of *Bacillus* sp. strain N52 to protect *S. bicolor* against drought stress was inferior to that of the other two *Bacillus* strains. Similar changes were observed in the *S. bicolor* metabolome in response to treatment with *Bacillus* sp. strain N52. However, when compared to the other two *Bacillus* strains, *Bacillus* sp. strain N52 lacked the ability to augment levels of IAA, cytokinin, JA, sphingosine, psychosine, choline, epicuticular wax and lowering of ethylene levels.

*Paenibacillus* spp. are also reported to elicit drought stress tolerance in host plants. Reported mechanisms include the augmentation of both AOX (Rodrigues et al., 2013; Timmusk et al., 2014; Vardharajula et al., 2011) and osmolytes (Vardharajula et al., 2011). Similar changes in the *S. bicolor* metabolome were elicited by *P. alvei* NAS-6G6, although it lacked the ability to augment levels of SA, proline and glutamic acid when compared to that of *Bacillus* sp. strain N18 and *Bacillus* sp. strain A28. The metabolomic changes in *S. bicolor* elicited by *Pseudomonas* sp. strain N66 were similar to that of the two selected *Bacillus* strains, but it lacked in the ability to upregulate brassinolide- and IAA levels. Regardless of this,

*Pseudomonas* sp. strain N66 were able to elicit superior protection against drought in *S. bicolor* plants. This was not only evident in the plant growth promotion- and induction of drought stress data (Fig. 4 and Table S2), but also visually discernible (Fig. 5).

Thus, the present study unraveled a molecular landscape describing metabolic reconfigurations involved in rhizobacteria-induced systemic tolerance against drought stress. This study specifically elucidated the metabolic reprogramming related to responses of rhizobacteria-primed and non-primed *S. bicolor* seedlings to drought condition, pointing out differential metabolic profiles described by key changes in different metabolic pathways. This provides insightful characterization of molecular events induced by the rhizobacteria for drought stress tolerance in sorghum plants. However, further studies are still needed to unpack regulatory details of this rhizobacteria-induced systemic tolerance such as early signaling cascade events and genome repgromming. For instance, the use of transcriptomics to determine the transcript factors involved in the early stages of rhizobacteria-induced systemic tolerance against drought stress might shed light on the molecular mechanism associated with signal transduction (Vinocur and Altman, 2005) and is proposed for future research.

## 5 Conclusion

PGPR are able to induce systemic tolerance in plants against drought stress. This systemic acquired tolerance is the result of physical, biochemical and molecular modifications that occur *in planta*. The MOA involved in rhizobacteria-induced drought stress tolerance consist of (1) osmotic adjustment, (2) augmented AOX metabolism and (3) changes in plant growth and root architecture. All five of the selected rhizobacterial isolates were able to protect *S. bicolor* against drought stress. Putative MOA, involved in rhizobacteria-induced drought stress tolerance, identified as key elements in the observed induced systemic tolerance were (1) AOX capacity; (2) growth promotion and root architecture modification as a result of the upregulation of the hormones GA, IAA and cytokinin; (3) the early activation of induced systemic tolerance through the signalling hormones brassinolides, SA and JA and signalling molecules sphingosine and psychosine; (4) the production of the osmolytes proline, glutamic acid and choline; (5) the production of the epicuticular wax docosanoic acid and (6) ACC-deaminase activity and lowered ethylene levels. The superior drought stress tolerance elicited by *Pseudomonas* sp. strain N66 in *S. bicolor* plants points to an important role of ACC deaminase activity in rhizobacteria-induced drought stress tolerance. Although significant overlap existed in the metabolomic responses of *S. bicolor* upon treatment with each of the five selected rhizobacterial isolates tested, each isolate induced a unique set of biochemical pathway responses in *S. bicolor*. Each isolate thus had a unique effect on the *S. bicolor* metabolome, pointing to unique MOA involved in the rhizobacteria-induced drought stress tolerance observed for each of the isolates tested. This points to an important relationship between the plant's rhizobiome diversity, its phenotype and ultimately resilience against environmental stress.

## 6 Acknowledgement of financial and other support

The National Research Foundation (NRF) of South Africa is duly acknowledged for partially supporting this work through grant holder's bursary to René Carlson, grant number 98863 (ref. no. CSUR150624120744) and for funding the Bruker Daltonik MALDI Biotyper under grant number 74426. Due acknowledgement also goes to the University of Pretoria who rendered the fellowship towards her PhD study.

## 7 Author contributions

Nico Labuschagne and René Carlson conceived and guided the project. Nico Labuschagne, Ahmed Idris Hassen, Ian A. Dubery and Fidele Tugizimana guided and coordinated the research. René Carlson performed the experimental work. Paul A. Steenkamp did the instrumental analyses of samples. René Carlson performed the molecular work and was guided by Ahmed Idris Hassen. René Carlson and Fidele Tugizimana analysed the data and performed the chemometric analyses. All authors contributed to writing and editing of the manuscript. All authors have read and approved the final version of the manuscript.

## 8 Declarations of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## 9 Supplementary material

Materials and methods for the ultra-high performance liquid chromatography-high definition mass spectrometry. **Table S1** Screening of the rhizobacterial isolates for their potential to induce drought stress tolerance in *Sorghum bicolor*. **Table S2** Plant growth promotion and induction of drought stress tolerance in *S. bicolor* after treatment with the selected rhizobacterial isolates for induced systemic tolerance. **Fig. S1** UHPLC-HDMS BPI chromatograms of ESI-positive data obtained from *S. bicolor* roots. **Fig. S2** UHPLC-HDMS BPI chromatograms of ESI-negative data obtained from *S. bicolor* shoots. **Fig. S3** UHPLC-HDMS BPI chromatograms of ESI-negative data obtained from *S. bicolor* roots. **Fig. S4** PCA score / scatter plot of *S. bicolor* root samples computed from ESI-positive data representing the first two PCs of a 15-component PCA model. **Fig. S5** PCA score scatter plot of *S. bicolor* shoot samples computed from ESI-negative data representing the first two PCs of a 16-component PCA model. **Fig. S6** PCA score scatter plot of *S. bicolor* root samples computed from ESI-negative data representing the first two PCs of a 15-component PCA model. **Fig. S7** OPLS-DA modelling of *S. bicolor* root samples and variable/feature selection ESI-positive data. **Fig. S8** OPLS-DA modelling of *S. bicolor* shoots samples and variable/feature selection ESI-negative data. **Fig. S9** OPLS-DA modelling of *S. bicolor* roots samples and variable/feature selection ESI-negative data.

## 10 Data availability

The metabolomics data and the raw data from the screening- and greenhouse trials have been deposited to the EMBL-EBI MetaboLights database (DOI: 10.1093/nar/gks1004. PubMed PMID: 23109552) with the identifier MTBLS1368 (Haug *et al*., 2013). The complete dataset can be accessed here https://www.ebi.ac.uk/metabolights/MTBLS1368.

## 11 Reference list

Ali, S., Charles, T.C., Glick, B.R., 2014. Amelioration of high salinity stress damage by plant growth-promoting bacterial endophytes that contain ACC deaminase. Plant Physiol. Biochem. 80, 160–7. https://doi.org/10.1016/j.plaphy.2014.04.003

Arshad, M., Shaharoona, B., Mahmood, T., 2008. Inoculation with *Pseudomonas* spp. containing ACC-deaminase partially eliminates the effects of drought stress on growth, yield, and ripening of pea (*Pisum sativum* L.). Pedosphere 18, 611–620. https://doi.org/10.1016/S1002-0160(08)60055-7

Baker, R., 1991. Diversity in biological control. Crop Prot. 10, 85–94. https://doi.org/10.1016/0261-2194(91)90054-U

Bashan, Y., De-Bashan, L.E., 2010. Chapter two: How the plant growth-promoting bacterium *Azospirillum* promotes plant growth - a critical assessment, in: Sparks, D. (Ed.), Advances in Agronomy. Elsevier Inc., Amsterdam, Netherlands, pp. 77–136. https://doi.org/10.1016/S0065-2113(10)08002-8

Baxter, A., Mittler, R., Suzuki, N., 2014. ROS as key players in plant stress signalling. J. Exp. Bot. 65, 1229–1240. https://doi.org/10.1093/jxb/ert375

Belimov, A.A., Dodd, I.C., Safronova, V.I., Hontzeas, N., Davies, W.J., 2007. *Pseudomonas brassicacearum* strain Am3 containing 1-aminocyclopropane-1-carboxylate deaminase can show both pathogenic and growth-promoting properties in its interaction with tomato. J. Exp. Bot. 58, 1485–1495. https://doi.org/10.1093/jxb/erm010

Birben, E., Sahiner, U.M., Sackesen, C., Erzurum, S., Kalayci, O., 2012. Oxidative stress and antioxidant defense. World Allergy Organ. J. 5, 9–19. https://doi.org/10.1097/WOX.0b013e3182439613

Blaha, D., Prigent-combaret, C., Mirza, M.S., Moe, Y., 2006. Phylogeny of the 1-aminocyclopropane-1-carboxylic acid deaminase-encoding gene *acdS* in phytobeneficial and pathogenic *Proteobacteria* and relation with strain biogeography. FEMS Microbiol. Ecol. 56, 455–470. https://doi.org/10.1111/j.1574-6941.2006.00082.x

Carlson R., Tugizimana F., Steenkamp P.A., Dubery I.A., Labuschagne N., 2019. Differential metabolic repogramming in *Paenibacillus alvei*-primed *Sorghum bicolor* seedlings in response to *Fusarium pseudograminearum* infection. Metabolites 9, 150. https://doi.org/10.3390/metabo9070150

Carvalho, F.P., 2006. Agriculture, pesticides, food security and food safety. Environ. Sci. Policy 9, 685–692. https://doi.org/10.1016/j.envsci.2006.08.002

Dawood, M.G., 2016. Influence of osmoregulators on plant tolerance to water stress. Sci. Agric. 13, 42–58. https://doi.org/10.15192/PSCP.SA.2016.13.1.4258

DeBrito Alvarez, M., Gagne, S., Antoun, H., 1995. Effect of compost on rhizosphere microflora of the tomato and on the incidence of plant growth-promoting rhizobacteria. Appl. Environ. Microbiol. 61, 194–199. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1388325/

Dimkpa, C., Weinand, T., Asch, F., 2009. Plant-rhizobacteria interactions alleviate abiotic stress conditions. Plant, Cell Environ. 32, 1682–1694. https://doi.org/10.1111/j.1365-3040.2009.02028.x

Dugas, D. V., Monaco, M.K., Olsen, A., Klein, R.R., Kumari, S., Ware, D., Klein, P.E., 2011. Functional annotation of the transcriptome of *Sorghum bicolor* in response to osmotic stress and abscisic acid. BMC Genomics 12, 1–21. https://doi.org/10.1186/1471-2164-12-514

Dworkin, M., Foster, J.W., 1958. Experiments with some microorganisms which utilize ethane and hydrogen. Am. Soc. Microbiol. 75, 1916–2017. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC290115/

Embley, T.M., Smida, J., Stackebrandt, E., 1988. Reverse transcriptase sequencing of 16s ribosomal RNA from *Faenia rectivirgula, Pseudonocardia themtophila* and *Sacchuropolysporu hivsuta,* three wall type IV Actinomycetes which lack mycolic acids. J. Gen. Microbiol. 134, 961–966. https://www.ncbi.nlm.nih.gov/pubmed/2460582

Farooq, M., Wahid, A., Kobayashi, D., 2009. Plant drought stress: effects, mechanisms and management. Agron. Sustain. Dev. Springer Verlag 29, 185–212. https://doi.org/10.1051/agro:2008021

Finkel, O.M., Castrillo, G., Herrera Paredes, S., Salas González, I., Dangl, J.L., 2017. Understanding and exploiting plant beneficial microbes. Curr. Opin. Plant Biol. 38, 155–163. https://doi.org/10.1016/j.pbi.2017.04.018

Ghosh, S., Penterman, J.N., Little, R.D., Chavez, R., Glick, B.R., 2003. Three newly isolated plant growth-promoting bacilli facilitate the seedling growth of canola, *Brassica campestris*. Plant Physiol. Biochem. 41, 277–281. https://doi.org/10.1016/S0981-9428(03)00019-6

Ghyselinck, J., Van Hoorde, K., Hoste, B., Heylen, K., De Vos, P., 2011. Evaluation of MALDI-TOF MS as a tool for high-throughput dereplication. J. Microbiol. Methods 86, 327–336. https://doi.org/10.1016/j.mimet.2011.06.004

Ghyselinck, J., Velivelli, S.L.S., Heylen, K., O'Herlihy, E., Franco, J., Rojas, M., De Vos, P., Prestwich, B.D., 2013. Bioprospecting in potato fields in the Central Andean Highlands: Screening of rhizobacteria for plant growth-promoting properties. Syst. Appl. Microbiol. 36, 116–127. https://doi.org/10.1016/j.syapm.2012.11.007

Gill, S.S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909–930. https://doi.org/10.1016/j.plaphy.2010.08.016

Glick, B.R., 2005. Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. FEMS Microbiol. Lett. 251, 1–7. https://doi.org/10.1016/j.femsle.2005.07.030

Glick, B.R., Bashan, Y., 1997. Genetic manipulation of plant growth-promoting bacteria to enhance biocontrol of phytopathogens. Biotechnol. Adv. 15, 353–378. https://doi.org/10.1016/S0734-9750(97)00004-9

Glick, B.R., Cheng, Z., Czarny, J., Duan, J., Cheng, Z., Czarny, J., 2007. Promotion of plant growth by ACC deaminase-producing soil bacteria. New Perspect. Approaches Plant Growth-Promoting Rhizobacteria Res. 119, 329–339. https://doi.org/10.1007/978-1-4020-6776-1_8

Glick, B.R., Liu, C., Ghosh, S., Dumbroff, E.B., 1997. Early development of canola seedlings in the presence of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2. Soil Biol. Biochem. 29, 1233–1239. https://doi.org/10.1016/S0038-0717(97)00026-6

Gliessman, S.R., 1995. 3 Sustainable agriculture: An agroecological perspective. Adv. Plant Pathol. 11, 45–57. https://doi.org/10.1016/S0736-4539(06)80005-X

Grover, M., Ali, S.Z., Sandhya, V., Rasul, A., Venkateswarlu, B., 2011. Role of microorganisms in adaptation of agriculture crops to abiotic stresses. World J. Microbiol. Biotechnol. 27, 1231–1240. https://doi.org/10.1007/s11274-010-0572-7

Grover, M., Madhubala, R., Ali, S.Z., Yadav, S.K., Venkateswarlu, B., 2014. Influence of *Bacillus* spp. strains on seedling growth and physiological parameters of sorghum under moisture stress conditions. J. Basic Microbiol. 54, 951–961. https://doi.org/10.1002/jobm.201300250

Gülçin, I., 2007. Comparison of in vitro antioxidant and antiradical activities of L-tyrosine and L-Dopa. Amino Acids 32, 431–438. https://doi.org/10.1007/s00726-006-0379-x

Harish, S., Kavino, M., Kumar, N., Saravanakumar, D., Soorianathasundaram, K., Samiyappan, R., 2008. Biohardening with plant growth promoting rhizosphere and endophytic bacteria induces systemic resistance against *Banana bunchy top virus*. Appl. Soil Ecol. 39, 187–200. https://doi.org/10.1016/j.apsoil.2007.12.006

Hassen, A.I., 2007. Efficacy of rhizobacteria for growth promotion and biocontrol of *Pythium ultimum* and *Fusarium oxysporum* on sorghum in Ethiopia and South Africa. PhD dissertation, University of Pretoria, Pretoria. https://repository.up.ac.za/handle/2263/26300

Haug K., Salek R.M., Conesa P., Hastings J., de Matos P., Rijnbeek M., Mahendraker T., Williams M., Neumann S., Rocca-Serra P., et al., 2013. MetaboLights - an open-access general-purpose repository for metabolomics studies and associated meta-data. Nucleic Acids Res. 41:D781-D786. doi:10.1093/nar/gks1004 PubMed

Horrigan, L., Lawrence, R.S., Walker, P., 2002. How sustainable agriculture can address the environmental and human health harms of industrial agriculture. Environ. Health Perspect. 110, 445–456. https://doi.org/10.1289/ehp.02110445

Horváth, E., Szalai, G., Janda, T., 2007. Induction of abiotic stress tolerance by salicylic acid signaling. Plant Growth Regul. 26, 290–300. https://doi.org/10.1007/s00344-007-9017-4

Jha, Y., Subramanian, R.B., Patel, S., 2011. Combination of endophytic and rhizospheric plant growth promoting rhizobacteria in *Oryza sativa* shows higher accumulation of osmoprotectant against saline stress. Acta Physiol. Plant. 33, 797–802. https://doi.org/10.1007/s11738-010-0604-9

Jones, H.G., Serraj, R., Loveys, B.R., Xiong, L., Wheaton, A., Price, A.H., 2009. Thermal infrared imaging of crop canopies for the remote diagnosis and quantification of plant responses to water stress in the field. Funct. Plant Biol. 36, 978–989. https://doi.org/10.1071/FP09123

Kang, S-M., Khan A.L., Waqas, M., You Y-H., Hamayun, M., Joo G-J., Shahzad, R., Choi K-S., Lee, I.-J., 2015. European Journal of Soil Biology Gibberellin-producing *Serratia nematodiphila* PEJ1011 ameliorates low temperature stress in *Capsicum annuum* L . Eur. J. Soil Biol. 1–9. https://doi.org/10.1016/j.ejsobi.2015.02.005

Kang, Y., DiLeo, M. V., Parnell, J.J., Barnhart, D.M., Berka, R., Sturino, J.M., Young, H.A., 2016. From the lab to the farm: An industrial perspective of plant beneficial microorganisms. Front. Plant Sci. 7, 1–12. https://doi.org/10.3389/fpls.2016.01110

Karuppanapandian, T., Moon, J.C., Kim, C., Manoharan, K., Kim, W., 2011. Reactive oxygen species in plants: Their generation, signal transduction, and scavenging mechanisms. Aust. J. Crop Sci. 5, 709–725. https://search.informit.com.au/documentSummary;dn=282079847301776;res=IELHSS

Kasim, W.A., Osman, M.E., Omar, M.N., Abd El-Daim, I.A., Bejai, S., Meijer, J., 2013. Control of drought stress in wheat using plant-growth-promoting bacteria. J. Plant Growth Regul. 32, 122–130. https://doi.org/10.1007/s00344-012-9283-7

Kaushal, M., Wani, S.P., 2016. Rhizobacterial-plant interactions : Strategies ensuring plant growth promotion under drought and salinity stress. Agric. Ecosyst. Environ. 231, 68–78. https://doi.org/10.1016/j.agee.2016.06.031

Khan, N.A., Nazar, R., Iqbal, N., Anjum N.A., 2012. Phytohormones and abiotic stress tolerance in plants. Springer-Verlag Berlin Heidelberg, 1-306. https://doi.org/10.1007/978-3-642-25829-9

Kloepper, J.W., 1994. Plant-Growth Promoting Rhizobacteria, in: Azospirillum / Plant Associations. Springer, pp. 147–166. https://doi.org/10.1016/j.apsoil.2005.03.007

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549. https://doi.org/10.1093/molbev/msy096

Lane, D.J., 1991. 16S/23S rRNA sequencing, in: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley and Sons Ltd., New York, pp. 115–175.

Lim, J.H., Kim, S.D., 2013. Induction of drought stress resistance by multi-functional PGPR *Bacillus licheniformis* K11 in pepper. Plant Pathol. J. 29, 201–208. https://doi.org/10.5423/PPJ.SI.02.2013.0021

Lucas, J.A., García-Cristobal, J., Bonilla, A., Ramos, B., Gutierrez-Mañero, J., 2014. Beneficial rhizobacteria from rice rhizosphere confers high protection against biotic and abiotic stress inducing systemic resistance in rice seedlings. Plant Physiol. Biochem. 82, 44–53. https://doi.org/10.1016/j.plaphy.2014.05.007

Mareya, C., Tugizimana, F., Piater, L., Madala, N., Steenkamp, P., Dubery, I., 2019. Untargeted metabolomics reveal defensome-related metabolic reprogramming in *Sorghum bicolor* against infection by *Burkholderia andropogonis*. Metabolites 9, 1–23. https://doi.org/10.3390/metabo9010008

Martinez, V., Mestre, T.C., Rubio, F., Girones-vilaplana, A., Moreno, D.A., Mittler, R., Rivero, R.M., 2016. Accumulation of flavonols over hydroxycinnamic acids favors oxidative damage protection under abiotic stress. Front. Plant Sci. 7, 1–28. https://doi.org/10.3389/fpls.2016.00838

Merkl, R., Hrádková, I., Filip, V., Šmidrkal, J., 2010. Antimicrobial and antioxidant properties of phenolic acids alkyl esters. Czech J. Food Sci. 28, 275–279. https://doi.org/10.17221/132/2010-CJFS

National Center for Biotechnology Information. Available online: https://www.ncbi.nlm.nih.gov/WebSub/ (accessed on 30 April 2019).

Naseem, H., Bano, A., 2014. Role of plant growth-promoting rhizobacteria and their exopolysaccharide in drought tolerance of maize. J. Plant Interact. 9, 689–701. https://doi.org/10.1080/17429145.2014.902125

Ngumbi, E., Kloepper, J., 2016. Bacterial-mediated drought tolerance: Current and future prospects. Appl. Soil Ecol. 105, 109–125. https://doi.org/10.1016/j.apsoil.2016.04.009

Noctor, G., Lelarge-trouverie, C., Mhamdi, A., 2015. The metabolomics of oxidative stress. Phytochemistry 112, 33–53. https://doi.org/10.1016/j.phytochem.2014.09.002

Ortiz, N., Armada, E., Duque, E., Roldán, a, Azcón, R., 2015. Contribution of arbuscular mycorrhizal fungi and/or bacteria to enhancing plant drought tolerance under natural soil conditions: Effectiveness of autochthonous or allochthonous strains. J. Plant Physiol. 174, 87–96. https://doi.org/10.1016/j.jplph.2014.08.019

Pastor, V., Balmer, A., Gamir, J., Flors, V., Mauch-Mani, B., 2014. Preparing to fight back: generation and storage of priming compounds. Front. Plant Sci. 5, 1–12. https://doi.org/10.3389/fpls.2014.00295

Penrose, D.M., Glick, B.R., 2003. Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiol Plant 118, 10–15. https://doi.org/10.1034/j.1399-3054.2003.00086.x

Pieterse, C.M.J., Leon-Reyes, A., Van der Ent, S., Van Wees, S.C.M., 2009. Networking by small-molecule hormones in plant immunity. Nat. Chem. Biol. 5, 308–316. https://doi.org/10.1038/nchembio.164

Pieterse, C.M.J., Zamioudis, C., Berendsen, R.L., Weller, D.M., Van Wees, S.C.M., Bakker, P.A.H.M., 2014. Induced systemic resistance by beneficial microbes. Annu. Rev. Phytopathol. 52, 347–75. https://doi.org/10.1146/annurev-phyto-082712-102340

Rooney, W.L., Blumenthal, J., Bean, B., Mullet, J.E., 2007. Designing sorghum as a dedicated bioenergy feedstock. Biofuel Bioprod Bior. 1, 147-157. https://doi.org/10.1002/bbb.15

Pourbabaee, A.A., Bahmani, E., Alikhani, H.A., Emami, S., 2016. Promotion of wheat growth under salt stress by halotolerant bacteria containing ACC deaminase. JASTA. 18, 855–864. http://hdl.handle.net/123456789/3974

Reganold, J.P., Papendick, R.I., Parr, J.F., 1990. Sustainable agriculture. Sci. Am. 262, 112–120. https://doi.org/10.1038/scientificamerican0690-112

Rodrigues, A.C., Bonifacio, A., Antunes, J.E.L., da Silveira, J.A.G., Figueiredo, M.D.V.B., 2013. Minimization of oxidative stress in cowpea nodules by the interrelationship between *Bradyrhizobium* sp. and plant growth-promoting bacteria. Appl. Soil Ecol. 64, 245–251. https://doi.org/10.1016/j.apsoil.2012.12.018

Saikia, J., Sarma, R.K., Dhandia, R., Yadav, A., Bharali, R., Gupta, V.K., Saikia, R., 2018. Alleviation of drought stress in pulse crops with ACC deaminase producing rhizobacteria isolated from acidic soil of Northeast India. Sci. Rep. 8, 3560. https://doi.org/10.1038/s41598-018-21921-w

Sandhya, V., Z, A.S.K., Grover, M., Reddy, G., Venkateswarlu, B., Z., A.S., Grover, M., Reddy, G., Venkateswarlu, B., 2009. Alleviation of drought stress effects in sunflower seedlings by the exopolysaccharides producing *Pseudomonas putida* strain GAP-p45. Biol. Fertil. Soils 46, 17–26. https://doi.org/10.1007/s00374-009-0401-z

Schauer, N., Baxter, C.J., Redestig, H., Fernie, A.R., Liu, J., Patil, K.R., Repsilber, D., Nielsen, J., Sweetlove, L.J., Selbig, J., 2006. The metabolic response of heterotrophic Arabidopsis cells to oxidative stress. Plant Physiol. 143, 312–325. https://doi.org/10.1104/pp.106.090431

Schisler, D. a, Slininger, P.J., Behle, R.W., Jackson, M. a, 2004. Formulation of *Bacillus* spp. for biological control of plant diseases. Phytopathology 94, 1267–1271. https://doi.org/10.1094/PHYTO.2004.94.11.1267

Seelig, H. -D., Hoehn, A., Stodieck, L.S., Klaus, D.M., Adams III, W.W., Emery, W.J., 2008. The assessment of leaf water content using leaf reflectance ratios in the visible, near-, and short-wave-infrared. Int. J. Remote Sens. 29, 3701–3713. https://doi.org/10.1080/01431160701772500

Seo, P.J., Lee, S.B., Suh, M.C., Park, M.-J., Go, Y.S., Park, C.-M., 2011. The MYB96 transcription factor regulates cuticular wax biosynthesis under drought conditions in *Arabidopsis*. Plant Cell 23, 1138–1152. https://doi.org/10.1105/tpc.111.083485

Shagol, C.C., Krishnamoorthy, R., Kim, K., Sundaram, S., Sa, T., 2014. Arsenic-tolerant plant-growth-promoting bacteria isolated from arsenic-polluted soils in South Korea. Environ. Sci. Pollut. Res. 21, 9356–9365. https://doi.org/10.1007/s11356-014-2852-5

Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M., 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J. Bot. 2012, 1–26. https://doi.org/10.1155/2012/217037

Sharma, P., Khanna, V., Kumari, S., 2015. Potential of ACC-deaminase producing plant growth promoting rhizobacteria on water stress mitigation in lentil (*Lens culinaris* L. Medikus) under axenic conditions 3, 59–67. https://doi.org/10.1139/cjm-2018-0636

Singh, D., Singh, H., Prabha, R., 2017. Plant-microbe interactions in agro-ecological perspectives. Springer. https://doi.org/10.1007/978-981-10-5813-4

Stewart, A., 2001. Commercial biocontrol - Reality or fantasy? Australas. Plant Pathol. 30, 127–131. https://doi.org/10.1071/AP01011

Sturz, A., Christie, B., 2003. Beneficial microbial allelopathies in the root zone: The management of soil quality and plant disease with rhizobacteria. Soil Tillage Res. 72, 107–123. https://doi.org/10.1016/S0167-1987(03)00082-5

Sukumar, P., Legué, V., Vayssières, A., Martin, F., Tuskan, G.A., Kalluri, U.C., 2013. Involvement of auxin pathways in modulating root architecture during beneficial plant – microorganism interactions. Plant Cell Physiol. 36, 909–919. https://doi.org/10.1111/pce.12036

Szabados, L., Savouré, A., 2010. Proline: a multifunctional amino acid. Trends Plant Sci. 15, 89–97. https://doi.org/10.1016/j.tplants.2009.11.009

Tari, I., Laskay, G., Poor, P., 2013. Response of sorghum to abiotic stresses: A review. J. Agron. Crop Sci. 199, 264–274. https://doi.org/10.1111/jac.12017

Timmusk, S., Abd El-Daim, I.A., Copolovici, L., Tanilas, T., Kannaste, A., Behers, L., Nevo, E., Seisenbaeva, G., Stenstrom, E., Niinemets, U., 2014. Drought-tolerance of wheat improved by rhizosphere bacteria from harsh environments: Enhanced biomass production and reduced emissions of stress volatiles. PLoS One 9, 1–13. https://doi.org/10.1371/journal.pone.0096086

Tugizimana, F., Piater, L., Dubery, I., 2013. Plant metabolomics: A new frontier in phytochemical analysis. S. Afr. J. Sci. 109, 1–11. https://doi.org/10.1590/sajs.2013/20120005

Vardharajula, S., Ali, S.Z., Grover, M., Reddy, G., Bandi, V., 2011. Drought-tolerant plant growth promoting *Bacillus* spp.: Effect on growth, osmolytes, and antioxidant status of maize under drought stress. J. Plant Interact. 6, 1–14. https://doi.org/10.1080/17429145.2010.535178

Vinocur, B., Altman, A., 2005. Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Curr. Opin. Biotech. 16, 123-132. https://doi.org/10.1016/j.copbio.2005.02.001

Walters, D.R., Fountaine, J.M., 2009. Practical application of induced resistance to plant diseases: An appraisal of effectiveness under field conditions. J. Agric. Sci. 147, 523. https://doi.org/10.1017/S0021859609008806

Welbaum, G.E., Sturz, A. V., Dong, Z., Nowak, J., 2004. Managing soil microorganisms to improve productivity of agro-ecosystems. CRC. Crit. Rev. Plant Sci. 23, 175–193. https://doi.org/10.1080/07352680490433295

Yang, J., Kloepper, J.W., Ryu, C.M., 2009. Rhizosphere bacteria help plants tolerate abiotic stress. Trends Plant Sci. 14, 1–4. https://doi.org/10.1016/j.tplants.2008.10.004

Zhang, Z., Schwartz, S., Wagner, L., Miller, W., 2000. A greedy algorithm for aligning DNA sequences. J. Comput. Biol. 7, 203–214. https://doi.org/10.1089/10665270050081478