Phytopathologia Mediterranea
The international journal of the
Mediterranean Phytopathological Union

Firenze University Press
www.fupress.com/pm

Research Papers

Combined interaction between the diazotrophic *Niallia circulans* strain YRNF1 and arbuscular mycorrhizal fungi in promoting growth of eggplant and mitigating root rot stress caused by *Rhizoctonia solani*

Younes M. RASHAD[1], Nahla Alsayd BOUQELLAH[2,*], Mohamed HAFEZ[3], Sara A. ABDALLA[1], Mohamed M. SLEEM[1], Adel K. MADBOULY[4]

[1] *Plant Protection and Biomolecular Diagnosis Department, Arid Lands Cultivation Research Institute (ALCRI), City of Scientific Research and Technological Applications (SRTA-City), New Borg El-Arab, Alexandria, 21934, Egypt*
[2] *Biology Department, College of Science, Taibah University, 42317-8599, Al Madinah Al Munawwarah, Saudi Arabia*
[3] *Land and Water Technologies Department, Arid Lands Cultivation Research Institute (ALCRI), City of Scientific Research and Technological Applications (SRTA-City), New Borg El-Arab, 21934, Egypt*
[4] *Microbiology Department, Faculty of Science, University of Ain Shams, Abbassia, Cairo, Egypt*
*Corresponding author. E-mail: nahlaalsaydbouqellah1992@gmail.com

Summary. Rhizoctonia root rot of eggplant, caused by *Rhizoctonia solani*, is an economically important disease. *Niallia circulans* YRNF1 and arbuscular mycorrhizal fungi (AMF) were assessed for their biocontrol and biofertilizing effects against *R. solani*, as potential replacements for synthetic fungicides and fertilizers. The diazotrophic *N. circulans* YRNF1, isolated from soil, reduced *in vitro* growth of *R. solani* by 42%. GC-MS analysis of culture filtrate of *N. circulans* YRNF1 detected bioactive compounds, including butyric acid (85%) and ethylene glycol (8%). In greenhouse experiments, combined application of *N. circulans* YRNF1 and AMF reduced the severity of eggplant root rot by 26%. This combined treatment triggered the transcriptional expression of five resistance genes (*JERF3, PAL1, C3H*, *CHI2*, and *HQT*) in the treated eggplants. Biochemical analyses of the infected eggplant roots treated with the combined bio-inoculants showed enhancement of the phenol content (+188%), and increased antioxidant enzyme activity, mainly of POD (+104%) and PPO (+72%). Combined application of *N. circulans* YRNF1 and AMF also promoted eggplant growth and improved the total NPK concentrations in treated plant leaves. Inoculation of eggplant with *N. circulans* YRNF1 in the presence of AMR increased the mycorrhization level. This is the first report of *N. circulans* and AMF as potential agents for biological control of Rhizoctonia root rot and growth promotion of eggplant.

Keywords. Biocontrol, mycorrhization, antifungal activity, biofertilizer, biofungicide.

OPEN ACCESS

Citation: Y.M. Rashad, N.A. Bouqellah, M. Hafez, S.A. Abdalla, M.M. Sleem, A.K. Madbouly (2024) Combined interaction between the diazotrophic *Niallia circulans* strain YRNF1 and arbuscular mycorrhizal fungi in promoting growth of eggplant and mitigating root rot stress caused by *Rhizoctonia solani*. *Phytopathologia Mediterranea* 63(1): 25-43. doi: 10.36253/phyto-14896

Accepted: February 19, 2024

Published: April 9, 2024

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Editor: Maurizio Vurro, National Research Council, (CNR), Bari, Italy.

ORCID:
YMR: 0000-0002-7702-8023
NAB: 0000-0002-2379-3475
MH: 0000-0001-9269-2128
SAA: 0000-0002-1050-2894
MMS: 0000-0002-2748-2615
AKM: 0000-0002-6261-9779

INTRODUCTION

Solanum melongena L. (eggplant) is the most important member of *Solanaceae* (Kaniyassery *et al*., 2022). China is the greatest eggplant producer and Egypt is the 3rd largest producer, recording annual production of 1,396,725 tons (FAOSTAT, 2024). Rhizoctonia root rot of eggplant is caused by *Rhizoctonia solani* Kühn (Almammory and Matloob, 2019). The soil-borne pathogen causes diseases of several hosts, including root rots, damping-off, leaf spots, and leaf blights, which result in a significant reductions in crop yields (Rashad *et al*., 2018).

Effective control of *R. solani* is difficult, due to its wide host range (Cook *et al*., 2002). The most widely used control method is application of synthetic fungicides, but these can have deleterious effects on human and animal health, may induce fungicide resistance in pathogens (Hollomon, 2015), and lead to environmental pollution (Baite *et al*., 2021). Biological control of plant diseases has received increasing attention as a possible effective, eco-friendly and safe control strategy against various plant diseases (Al-Askar *et al*., 2014).

Arbuscular mycorrhizal fungi (AMF: *Glomeromycotina*) live in symbiotic associations with more than 85% of the terrestrial plants (Spatafora *et al*., 2016; Mathur *et al*., 2018). AMF have been extensively studied as potential bio-protectants against several fungal pathogens, including *R. solani, Colletotrichum* spp., *Alternaria* spp., *Phytophthora* spp., *Fusarium* spp., and *Puccinia* spp. (Devi *et al*., 2022). AMF can also promote plant growth (de Oliveiraa *et al*., 2022). For example, mycorrhization of pea roots gave effective biocontrol of Rhizoctonia root rot (Rashad *et al*., 2022a). However, improvement of AMF biocontrol efficiency by application with other compatible antifungal microorganisms may also be worthwhile.

Nitrogen-fixing bacteria convert atmospheric nitrogen to ammonia, which can be absorbed by plants. These bacteria can promote plant growth, facilitate nutrient uptake and phosphorous solubilization, and produce siderophores and phytohormones (Shameem *et al*., 2023). In addition, *Bacillus circulans* CB7 Jordan, 1890 (now known as *Niallia circulans*), a non-symbiotic nitrogen-fixing bacterium, with plant growth-promoting properties due to auxin production, P-solubilization and siderophore production, has also shown antagonistic effects against *Dematophora necatrix* (Mehta *et al*., 2015).

Triggering immunity-related genes in plants is an important mode of action of biocontrol agents. Jasmonic acid and ethylene-response factor 3 gene (*JERF3*), the responsive gene that manages several defence-response genes through the jasmonate/ethylene signalling pathway, is elicited by abiotic and biotic plant stresses (Rashad *et al*., 2022b).

Polyphenols have potential as natural antioxidants, which can protect the living organisms from deleterious effects of the reactive oxygen species (ROS) (Elshafie *et al*., 2023). These compounds have antioxidant activity, as well as antihypertensive, antimicrobial, and antiviral activity (Losada-Barreiro *et al*., 2022). Excessive production of ROS causes oxidative stress in humans, leading to the development of several ailments (Forman, and Zhang, 2021). Antioxidant polyphenols have several modes of action, including reductive ability to neutralize ROS, chelation of metal ions that elicit the oxidative stress, inhibition of enzymes involved in the formation of ROS, and activation of the antioxidant enzymes (Dias *et al*., 2021). Upon infection of plants by the microbial pathogens, their cell walls accumulate large amounts of lignin (Rashad *et al*., 2020a). Increased lignification is a main barrier against the pathogen spread, and reduces the infiltration of toxins and fungal enzymes into plant cell walls. Lignin compounds also cause fungal pathogens to lose abilities to infect host plants, and prevent pathogen movement and multiplication (Ma *et al*., 2017). Flavonoids are plant responsive metabolites that contribute to host resistance in response to various abiotic and biotic stresses. These compounds act as physical or chemical barriers to prevent the microbial invasion, and are toxic defences against the microbial pathogens and/or insects. They interfere with pathogen cellular processes and structures (Ramaroson *et al*., 2022). The phenylalanine ammonia lyase 1 gene (*PAL1*) encodes phenylalanine ammonia lyase, which is involved in biosynthesis of polyphenolic compounds that have roles in host plant systemic resistance (Rashad *et al*., 2020a). During the early stages of lignin biosynthesis, the 4-coumarate 3-hydroxylase gene (*C3H*) catalyzes conversion of 4-coumarate to caffeate (Shrestha *et al*., 2022). The Chalcone isomerase 2 gene (*CHI2*) encodes for chalcone flavonone isomerase that catalyses the first two steps of flavonoid biosynthesis (Chao *et al*., 2021). Niggeweg *et al*. (2004) reported that the Hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase gene (*HQT*) catalyzes biosynthesis of chlorogenic acid from caffeoyl-CoA and quinic acid.

The present study had the following objectives: 1) to investigate *in vitro* suppressive potential of the diazotrophic bacterium *N. circulans* YRNF1 against *R. solani*; 2) to assess biocontrol effects of a combined treatment with *N. circulans* YRNF1 and AMF on Rhizoctonia root rot of eggplant, 3) elucidate the host defensive mechanisms elicited by these combined bioagents, based on the transcription of some responsive genes, phenolic com-

pound content, and antioxidant activity; and 4) assess effects of combined bioagents on eggplant development.

MATERIALS AND METHODS

Fungi and eggplant cultivar

The pathogenic fungus *R. solani* (AG-2-2 IIIB) was provided from Mansoura University, Egypt. To prepare the inoculum, a 500 ml glass flask containing 100 g of sterilized oat grains mixed with sand (1:2 v:v) was aseptically inoculated with five discs (6 mm diam.) cut from a 5-d-old culture of *R. solani*, incubated at 28±2°C, and was shaken daily for two weeks to ensure uniform growth of the fungus (Youssef *et al.*, 2016). AMF inoculum (73% colonization index) was provided by the Agricultural Research Center (ARC), Giza, Egypt. The mixed inoculum of potential biocontrol AMF contained spores of *Claroideoglomus etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schüsler and *Rhizoglomus intraradices* (N.C. Schenck & G.S. Sm.) Sieverd, G.A. Silva & Oehl (in an equal ratio). AMF were propagated by inoculation of sterilized sandy-clay soil with 10 g of grain of the AMF inoculum (approx. 50 AM spores and root pieces g^{-1} soil). The inoculum was applied as a grain bed before planting grains of maize (potential host) in a sterilized plastic pot under the greenhouse conditions (27/22°C, 75% relative humidity, and 16 h daily light period). No fertilizers were added and irrigation was regularly applied to 50% field capacity. After 3 months, the AMF colonized roots were cut using a sterile scalpel into small segments, and were mixed with the AMF spores in the maize rhizosphere soil to be used as AMF inoculum (approx. 45 AM spores and mycorrhizal root pieces g^{-1} soil) (Nafady *et al.*, 2019). Seeds of the eggplant cultivar 'Rondona' were used, which were provided by the ARC, Giza, Egypt.

Collection of soil samples

Twenty-one soil samples were collected from several cultivated fields in Alexandria and El- Beheira governorates, Egypt, and were immediately transported to the laboratory. These samples were stored for subsequent studies at 4°C.

Isolation of the diazotrophic bacteria

Approximately 10 g of each soil sample were added aseptically to 90 mL of sterilized water in an Erlenmeyer flask, vigorously mixed for 45 min at 150 rpm, and then serial dilutions were prepared (10^{-1} to 10^{-4}). Diazotrophic bacteria were isolated from the soil according to Döbereiner (1988) with slight modifications. Plates of nitrogen free (NF) agar medium, containing glucose (20 g L^{-1}); K_2HPO_4 (0.2 g L^{-1}), NaCl (0.2 g L^{-1}), $MgSO_4.7H_2O$ (0.2 g L^{-1}), K_2SO_4 (0.1 g L^{-1}), $CaCO_3$ (5 g L^{-1}), and agar (20 g L^{-1}), were individually inoculated with 0.1 mL of each serial dilution of soil suspension; Three replicated plates were used for each dilution. The plates were then incubated for 5 d at 28±2°C. Growing bacterial colonies were then singly transferred onto fresh NF plates and incubated at the same conditions. The resulting cultures were stored in 15% glycerol at -80°C until used (Kifle and Laing, 2016).

Screening of the diazotrophic bacteria for antifungal potential against R. solani

The ability of the isolated diazotrophic bacteria to antagonize *R. solani* was evaluated using the *in vitro* dual plate method (Jasim *et al.*, 2016) on potato dextrose agar plates (PDA, Difco). In each plate, 10 µL of each bacterial isolate (10^6 cells mL^{-1}) were streaked aseptically as a longitudinal line 2 cm away from the border of the plate. A disc (6 mm diam.) cut from a freshly grown *R. solani* culture was placed 20 mm from the opposite side of the plate. Plates inoculated only with pathogen discs served as experimental controls. Each treatment was applied in six replicates, and the assay was repeated twice. After incubating the plates for 4 d at 25°C, and upon complete coverage of control plates with fungal growth, inhibition of fungal growth in the dual plates was measured using a calibrated ruler, and compared with the fungal diameter in the corresponding control plate. Suppression of the fungal growth (S %) was determined using the equation of Ferreira *et al.* (1991):

$$S\,\% = \frac{C - T}{C} \times 100$$

where C = radial growth in the control plate, and T = radial growth in the treated plate.

Biochemical analyses of bacterial culture filtrate using gas chromatography-mass spectrometry (GC-MS)

Secondary metabolites in cell free culture filtrate of *N. circulans* YRNF1 were identified qualitatively using GC-MS. The culture filtrate of *N. circulans* YRNF1 was obtained by inoculating 200 µL of the bacterial suspension in sterile distilled water (6×10^6 cfu mL^{-1}) into a 500 mL capacity Erlenmeyer flask containing 250 mL of

nutrient broth (NB) [1% (w/v) each of beef extract and peptone, and 5% (w/v) NaCl]. The culture was incubated under shaking at 120 rpm at 28°C (Nisa *et al.*, 2019). After 3 d, the NB was centrifuged for 10 min. at 4°C at 11,200 g (Thermo Fisher Scientific). The collected cell-free supernatant was filter sterilized using 0.22 µm filters (Millex-GS, Millipore). The cell-free culture filtrate was lyophilized to complete dryness, re-suspended in methoxyamine hydrochloride (50 µL) dissolved in pyridine, followed by incubation for 90 min. For derivatization of the sample, 50 µL of silylation reagent [bis(trimethylsilyl) trifluoroacetamide + trimethylchloro-silane, 99:1 v:v] were added to the sample. The GC-MS system (GCMS-QP2010 Plus, Shimadzu), equipped with a gas chromatograph (7890B) and a mass spectrometer detector (5977A), was used to analyze the sample, at the Central Laboratories Network, NRC, Giza, Egypt. This system involved an HP-5MS column (30 m × 0.25 mm × 0.25 µm). Hydrogen was used as the carrier gas at the flow rate of 2.0 mL min^{-1}. In this assay, 1 µL was used as an injection volume according to several processing conditions, including 50°C for 5 min, which rose at 10°C min^{-1} to 100°C, and then at 20°C min^{-1} to 320°C. Mass spectra were obtained by electron ionization (EI) at 70 eV, involving a spectral range of m/z 50–700, and a solvent delay of 4 min, where 230°C was the mass temperature used and the Quad was at 150°C. Biochemical characterization of the different bacterial filtrate constituents was obtained by comparing the spectrum fragmentation pattern with that stored in the data of Wiley and the National Institute of Standards and Technology (NIST) Mass Spectral Library.

*Detection of the nitrogenase gene (*nifH*)*

Presence of the nitrogenase (*nifH*) gene was detected in the isolated diazotrophic bacteria according to Tan *et al.* (2009), as follows:

DNA extraction. A 2-d culture of each bacterial isolate grown on NF medium was centrifuged for 2 min at 1792 g. The resulting pellet was re-suspended in sterilized water (100 mL) and re-centrifuged for an additional 2 min at 1792 g, followed by heating in a water bath (90°C) for 10 min. After re-centrifugation, the supernatant was added to a sterile tube (0.5 mL capacity) and used as a DNA template. The resulting crude DNA concentration was estimated at using a UV spectrophotometer at OD_{260} and OD_{280} (PERSEE).

Polymerase chain reaction (PCR). The reaction mixture (25 mL) of PCR reaction involved a template DNA (≈ 100 ng); primer (1 mM of each primer), DNA polymerase (25 μL^{-1}), 5 × buffer, $MgCl_2$ (1 mM), dNTPs (0.2 mM), and sterile H_2O. Sequences of the *nifH* primers used were as follows: *nifH*-F (5'AAAGGYGGWATCG-GYAARTCCACCAC3') and *nif*H-R (5'TTGTTSGCS-GCRTACATSGCCATCAT3') (Turk-Kubo *et al.*, 2012). The processing reaction was conducted on a thermocycler (Eppendorf) under processing conditions of one cycle (95°C for 3 min), then 30 cycles (each of 95°C for 1 min, 52°C for 1 min, 72°C for 1 min, and 72°C for 5 min).

Molecular identification of the selected bacterium using 16S rRNA gene

DNA of the promising nitrogen-fixing bacterial isolate was subjected to amplification of the 16S-rRNA region, using the primer 16S-27F: 5'-AGAGTTTGATC-MTGGCTCAG-3' and 16S-1492R: 5'-CGGTTACCTT-GTTACGACTT-3' (dos Santos *et al.*, 2019). The PCR mixture was subjected to the Exosap-IT (GE Healthcare) PCR clean up protocol. The 16S rRNA gene nucleotide sequence was determined through Sanger sequencing using the DNA Analyzer of Applied Biosystems 3730 × l. Using the Big Dye Terminator from ABI; the two primers 27b F and 1492uR were used for setting up the PCR reactions (dos Santos *et al.*, 2019). The Vector NTI software (Invitrogen) was used to align the sequences from the forward and reverse primers, while the contigs were subjected to BLAST to search for nucleotide similarity (Zhang *et al.*, 2000). The maximum likelihood method through MEGA X software (10.2.4) was used to generate a phylogenetic tree of the selected isolate.

Greenhouse experiment

Eggplant seeds were surface sterilized (using 0.05% sodium hypochlorite) and planted (one per pot) into 20 cm diam. plastic pots containing sterilized clay soil. The soil physical composition was: silt (35 ± 0.13 g kg^{-1}), sand (110.2 ± 0.21 g kg^{-1}), and clay (453 ± 0.18 g kg^{-1}). The soil chemical properties were: pH = 7.58, EC, 1.32 dS m^{-1}, available P (26 mg kg^{-1}), total N (2.84 g kg^{-1}), available K (310 mg kg^{-1}), organic matter (1.72 g kg^{-1}), total organic carbon (0.99 g kg^{-1}), and total $CaCO_3$ (5.9%). For AMF colonization, an AMF inoculum was added to each pot under the seeds (10 g $seed^{-1}$ of the AMF inoculum). Infestation of the soil was carried out by mixing the upper layer with the *R. solani* inoculum (3% w/w), and the pots were watered daily for 10 d before planting. For preparation of bacterial inoculum, the diazotrophic bacterium *N. circulans* strain YRNF1 was grown in NF broth on a rotary shaker for 3 d. The resulting bacterial suspension (adjusted at 10^8 cell mL^{-1}) was mixed with 1% Arabic gum. For application of *N. circulans* YRNF1,

eggplant seeds were soaked for 30 min before planting in freshly prepared bacterial inoculum. Three hours before planting, the seeds in control pots were treated with the fludioxonil (50.0%) fungicide at 3.5 mL kg^{-1} as a positive fungicide control. The negative control involved a set of pots that was untreated. Nine treatments were applied in this experiment. These were:

- non-mycorrhizal, untreated, uninfected plants (designated C);
- non-mycorrhizal, untreated and infected plants (P);
- non-mycorrhizal, treated with *N. circulans* YRNF1 and uninfected plants (B);
- mycorrhizal, untreated and uninfected plants (M);
- mycorrhizal, treated with *N. circulans* YRNF1 and uninfected plants (B+M);
- non-mycorrhizal, treated with *N. circulans* YRNF1 and infected plants (B+P);
- mycorrhizal, untreated and infected plants (M+P);
- non-mycorrhizal, treated with the fungicide and infected plants (F+P); and
- mycorrhizal, treated with *N. circulans* YRNF1 and infected plants (B+M+P).

No fertilization was applied in this experiment. Five replicates were used, and the pots were arranged in a randomized complete block design. They were maintained at 70% relative humidity, in a 27°C day 17°C night temperature regime in a greenhouse, and were irrigated with tap water when necessary. This experiment was repeated twice

Assessment of the disease severity

Five plants from each treatment were carefully uprooted, and the adhering soil was removed with tap water. The disease severity was evaluated according to Wen *et al.* (2005). This scale included six severity categories: 0 = no necrosis; 1 = small root necroses (2.5 mm length); 2 = necrosis (2.5–5 mm); 3 = necrosis ≥ 5 mm; 4 = crown and shoots covered with necrotic lesions; or 5 = seedlings damped-off. The disease severity (DS%) was estimated according to Taheri and Tarighi (2010):

$$\mathrm{DS}\ (\%) = \frac{1n1 + 2n2 + 3n3 + 4n4 + 5n5}{5N} \times 100$$

where n1 was the number of plants that had severity category 1; n2 was number of plants that had level 2; *etc*., and N was the total number of evaluated plants.

Quantifying the expression of the defense-related genes using qPCR

Transcription of some responsive genes in eggplant roots was quantified at 14 d post planting (dpp). The studied genes included *JERF3*, *PAL1*, *C3H*, *CHI2*, and *HQT*. *α*-Tubulin and *β*-actin were used as reference genes, based on their stability in the mycorrhizal plants (Fuentes *et al*., 2016). The primer sequences used are shown in Table 1. The RNeasy Kit (Qiagen) was used for extraction of total RNA, in accordance with the manufacturer's instructions. A SureCycler 8800 (Agilent, USA) was used to synthesize the cDNA. The total volume (20 µL) of the reaction mixture was composed of 3.5 µL RNase-free H_2O; 3 µL 5×-buffer; 3 µL RNA (30 ng), 3 µL of dNTPs (10 mM), 7 µL of dT primer (5 pmol μL^{-1}), and 0.5 µL of the RT enzyme.

The reaction was carried out using the RotorGen 6000 (Qiagen) real-time system The qPCR was run for 1 h at 43°C, then 10 min at 71°C. The qPCR mixture consisted of 3 µL cDNA, 1.6 µL sterile water, 1.5 µL of each

Table 1. Primer sequences of the defense-related genes used in this study (Rashad *et al*., 2020b).

Primer name	Abbreviation		(5'-3')
Jasmonate and ethylene-responsive factor 3	*JERF3*	F	GCCATTTGCCTTCTCTGCTTC
		R	GCAGCAGCATCCTTGTCTGA
Phenylalanine ammonia lyase 1	*PAL1*	F	ACGGGTTGCCATCTAATCTGACA
		R	CGAGCAATAAGAAGCCATCGCAAT
4-coumarate 3-hydroxylase	*C3H*	F	TTGGTGGCTACGACATTCCTAAGG
		R	GGTCTGAACTCCAATGGGTTATTCC
Chalcone isomerase 2	*CHI2*	F	GGCAGGCCATTGAAAAGTTCC
		R	CTAATCGTCAATGATCCAAGCGG
Hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase	*HQT*	F	CCCAATGGCTGGAAGATTAGCTA
		R	CATGAATCACTTTCAGCCTCAACAA
α-tubulin	*α*-tubulin	F	TATCTGCTACCAGGCTCCCGAGAA
		R	TGGTGTTGGACAGCATGCAGACAG
β-actin	*β*-actin	F	GTGGGCCGCTCTAGGCACCAA
		R	CTCTTTGATGTCACGCACGATTTC

primer, and 12.4 µL 2× SYBR® Green Mix. The qPCR reaction was run with one cycle at 95°C (for 3 min), 45 cycles each of 95°C for 15 s and 56°C for 35 s, then 75°C for 250 s. The comparative CT method ($2^{-\Delta\Delta CT}$) was used to estimate the transcriptional expression of the tested genes, in accordance with Schmittgen and Livak (2008). Triplicates of each treatment (biological and technical) were used, and the assay was conducted twice.

Determination of the biochemical plant defense responses

At 30 dpp, samples from the eggplant roots were collected from each treatment. Estimation of the total phenolics, and activities of peroxidase (POD) and polyphenol oxidase (PPO) were assessed (five replicates). The Folin-Ciocalteu test was used to determine the total phenolic content (Singleton *et al.*, 1999). Approximately 1 g of roots was ground in 5 mL of 80% methanol, at 5°C overnight. This homogenate was then centrifuged at 1344 g for 10 min. The supernatant (100 µL) was then mixed with 20% Na_2CO_3 (50 µL), 1750 µL of dH_2O, and 250 µL of Folin-Ciocalteu reagent (Sigma-Aldrich), and the resulting mixture was left at 40°C for 35 min. Caffeic acid was used as a reference. Phenolic contents were estimated using spectrophotometry at 760 nm. For POD activity determination, approx. 1 g of the root was ground in 3 mL of 0.1 M Na_2HPO_4 buffer, and the homogenate was centrifuged for 10 min at 1792 g. POD enzyme activity was estimated spectrophotometrically at 470 nm, which was represented as ΔA_{470} min^{-1} g^{-1} f wt (Gong *et al.*, 2001). Estimation of the PPO activity was carried out according to Singh and Ravindranath (1994). Five g of root tissue were homogenized and kept for 35 min in acetone at 4°C. PPO enzyme potential was evaluated spectrophotometrically at 420 nm using catechin as the substrate, and was recorded as ΔA_{420} min^{-1} g^{-1} f wt.

Estimation of levels of plant mycorrhization

Mycorrhizal colonization in eggplant roots was estimated 45 dpp according to Trouvelot *et al.* (1986). Plant roots were fragmented (10 mm diam.) using a scalpel and were boiled in 10% potassium hydroxide solution. The treated segments were stained using 0.05% trypan-blue, as described by Phillips and Hayman (1970). For each root treatment, approx. 50 stained fragments were checked under a light microscope. Three mycorrhization parameters were assessed, including frequency of mycorrhizal colonization, colonization intensity, and frequency of formation of arbuscules (Trouvelot *et al.*, 1986). Estimation of mycorrhization was repeated twice, each time on 50 stained fragments.

Evaluation of the eggplant growth parameters

At 45 dpp, approx. five replicate eggplant were carefully uprooted. Adhering soil particles were removed and plant growth was estimated using the following parameters: shoot height, root length, shoot and root dry weights, and leaf area. For the evaluation of the dry weights, the plants were dried at 80°C in an oven (3 d). This assay was conducted twice.

Influence of bioagents on macronutrient contents of eggplant leaves

For quantitative analysis of the nutrients content in eggplant leaves, approx. ten leaves from each treatment were collected, and then air dried. The leaves were fragmented using a grinding machine, and then used for estimation of the total contents of nitrogen (N), phosphorus (P), and potassium (K). Using Kjeldhal assays, N was estimated by titration following distillation (Goyal *et al.*, 2022). Total P was evaluated as per Singh *et al.* (2022). Total K was determined based on Goyal *et al.* (2022). Determinations were each carried out twice.

Statistical analyses

The results were statistically analyzed using CoStat software 6.4. Data were checked for normality before applying the analyses of variance. Treatment means were compared using Tukey's HSD test ($P \leq 0.05$).

RESULTS

Antagonistic potential of the isolated diazotrophic bacteria against R. solani

Eight isolates of diazotrophic bacteria were recovered from the collected soil samples. Results of the *in vitro* antifungal assays against *R. solani* showed that inhibition of *R. solani* mycelial growth varied among the eight isolates ranging from no inhibition to medium inhibition. The greatest level of growth inhibition (42% growth, compared to control plates) was observed with the isolate designated as YRNF (Figure 1, a and b). Growth of the *R. solani* colony was suppressed and the colony had an arc-shaped border with the diazotrophic bacterium YRNF1, with a clear no-growth zone in

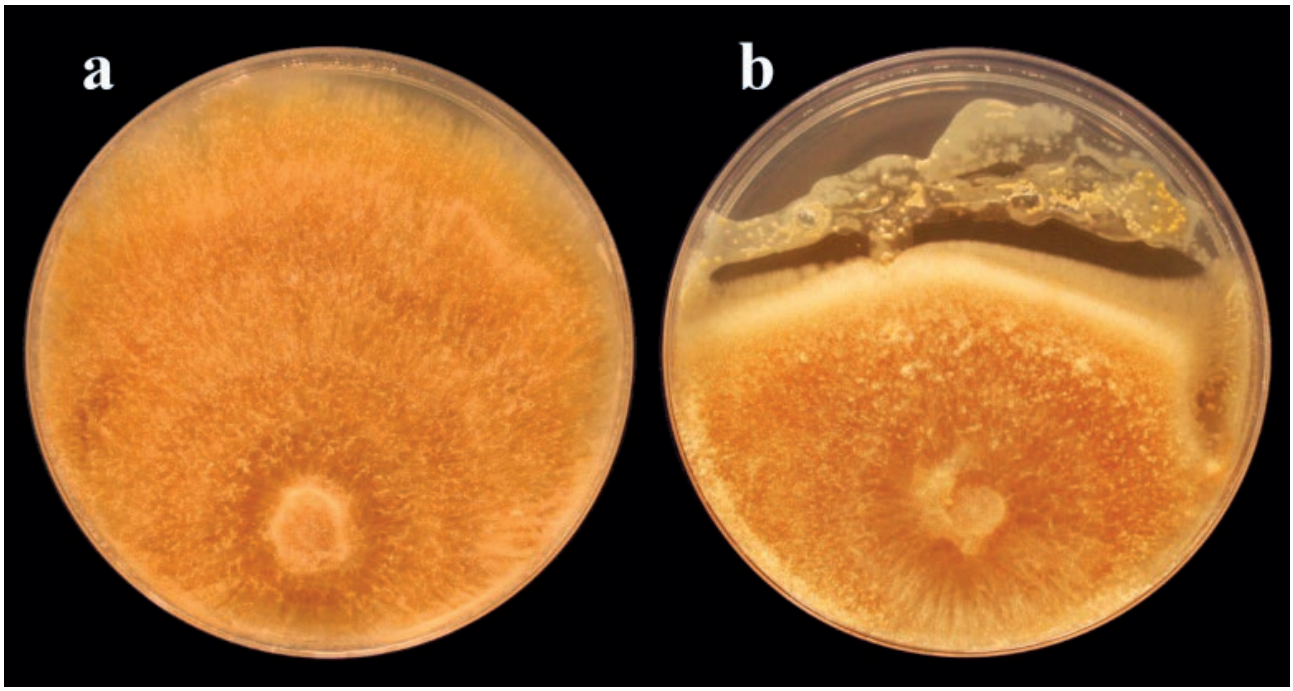

Figure 1. *In vitro* antagonism of the diazotrophic bacterium *Niallia circulans* strain YRNF1 against *Rhizoctonia solani*. Confrontation test in dual culture; a) Unchallenged *R. solani* (control); b) *R. solani* challenged by *N. circulans* strain YRNF1.

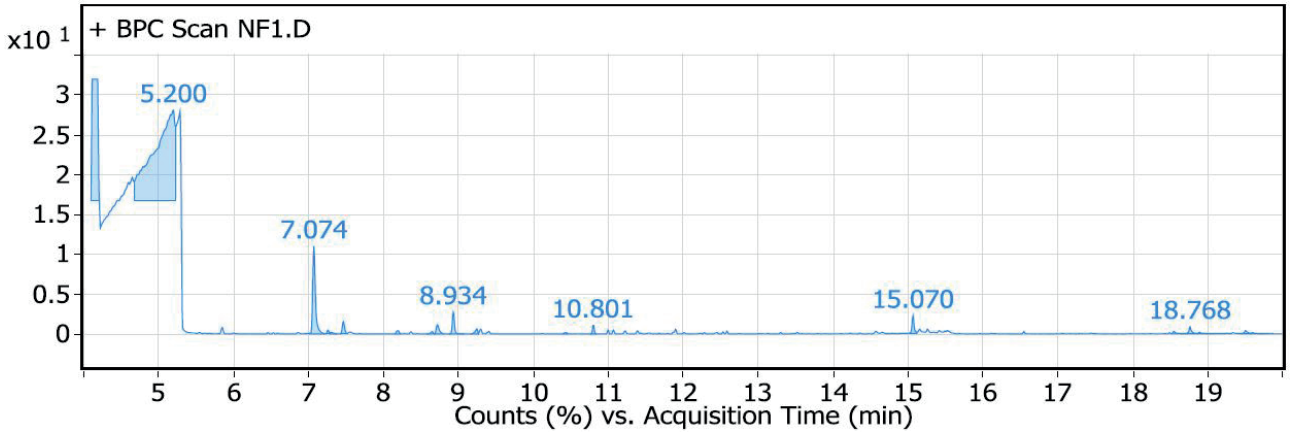

Figure 2. GC-MS chromatogram of the secondary metabolites detected in culture filtrates of *Niallia circulans* YRNF1.

between the fungus and bacterium, indicating release of antifungal metabolites by the bacterium, and an antibiosis-based mechanism of antifungal activity (Figure 1).

GC-MS analysis of the bacterial culture filtrate

Nineteen chemical constituents were detected in the culture filtrate of *N. circulans* YRNF1, which were identified by the GC-MS analysis (Figure 2). As presented in Table 2, the major biochemical constituents were butyric acid (85%) and ethylene glycol (8%). Some metabolites were recorded at intermediate proportions, including lactic acid, propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester (2TMS) derivative (1.5%) and α-D-mannopyranoside, methyl, cyclic 2,3:4,6-bis (butylboronate) (1.2%). The least detected components were β-hydroxyquebrachamine; 1,3-Dipalmitin trimethylsilyl ester (TMS) derivative; octadecanoic acid, 2,3-bis[(trimethylsilyl) oxylpropyl ester (Glycerol mon-

Table 2. Secondary metabolites identified in culture filtrates of *Niallia circulans* YRNF1.

Peak no.	Retention time (min)	Peak area (%)	Compound name	Chemical formula
1	4.153	23.11	Butyric Acid, TMS derivative	$C_7H_{16}O_2Si$
2	5.2	61.53	Butyric acid	$C_4H_8O_2$
3	7.074	7.75	Ethylene glycol, 2TMS derivative	$C_8H_{22}O_2Si_2$
4	7.265	0.22	Cyclobarbital	$C_{12}H_{16}N_2O_3$
5	7.309	0.16	2,4,6(1H,3H,5H)-Pyrimidinetrione, 5-(1-cyclohexen-1-yl)-5-ethyl-	$C_{12}H_{16}N_2O_3$
6	7.47	0.91	Propylene glycol, 2TMS derivative	$C_9H_{24}O_2Si_2$
7	8.195	0.28	2-Ethoxyethanol, TMS derivative	$C_7H_{18}O_2Si$
8	8.649	0.17	Cedran-diol, 8S,13-	$C_{15}H_{26}O_2$
9	8.722	0.85	1-Ethyl-1-(2-phenylethoxy)-1-silacyclohexane	$C_{15}H_{24}OSi$
10	8.934	1.48	Lactic Acid, 2TMS derivative	$C_9H_{22}O_3Si_2$
11	9.249	0.4	β-D-Galactopyranoside, methyl 2,3-bis-O-(trimethylsilyl)-, cyclic butylboronate	$C_{17}H_{37}BO_6Si_2$
12	10.443	0.11	β-Hydroxyquebrachamine	$C_{19}H_{26}N_2O$
13	10.801	0.46	Acetin, bis-1,3-trimethylsilyl ether	$C_{11}H_{26}O_4Si_2$
14	15.07	1.19	α-D-Mannopyranoside, methyl, cyclic 2,3:4,6-bis (butylboronate)	$C_{15}H_{28}B_2O_6$
15	18.555	0.23	1-Heptatriacotanol	$C_{37}H_{76}O$
16	18.768	0.57	1-Monopalmitin, 2TMS derivative	$C_{25}H_{54}O_4Si_2$
17	18.892	0.13	1,3-Dipalmitin trimethylsilyl ester (TMS) derivative	$C_{38}H_{76}O_5Si$
18	19.507	0.31	2-Oleoylglycerol, 2TMS derivative	$C_{27}H_{56}O_4Si_2$
19	19.603	0.14	Glycerol monostearate, 2TMS derivative	$C_{27}H_{58}O_4Si_2$

ostearate, 2TMS derivative); 2,4,6(1H,3H,5H)-pyrimidine-trione, 5-(1-cyclohexen-1-yl)-5-ethyl- (cyclobarbital), and cedrane-8,13-diol (cedran-diol, 8S,13-).

Detection of the nitrogenase gene (nifH) in the diazotrophic bacteria

Results obtained from PCR analyses showed that the selected isolate YRNF1 possessed the *nifH* nitrogenase gene, which was observed as a single band at 450 bp (Figure 3). This indicated that the isolate was a nitrogen fixing bacterium.

Molecular identification of the diazotrophic bacterium YRNF1

Results of BLAST analysis of the 16S rRNA sequence showed that the diazotrophic bacterial strain YRNF1 had 99.71% similarity with *N. circulans* (reference strain MH130347). The nucleotide sequence of *N. circulans* YRNF1 was deposited in the GenBank under accession number OP703372. The phylogenetic analysis of *N. circulans* YRNF1 in comparison to ten species of the genus *Bacillus* (Figure 4) showed that the strain grouped with *B. circulans* (MW547978) in a single distinct clade, with 66% bootstrap support. The *Bacillus* spp. strains clustered in two major groups. The first contained *B. altitudenis* (MK424248), *B. velezensis* (MG651075), and *B. subtilis* (HE610894), with 70% bootstrap support. *Bacillus mycoides* (ON464184) clustered with 64% bootstrap support in the other clade, while *B. toyonensis* (MZ773910) represented an outgroup. The second major group also included *B. licheniformis* (MK280728) and *B. amyloliquefaciens* (MF423459), with 64% bootstrap support, and clustered in a separate clade. The other clade involved *B. cereus* (OQ152624) and *B. thuringiensis* (MY912020), with 62% bootstrap support.

Disease severity

Mean severity of Rhizoctonia root rot in the infected, non-treated eggplant plants was 69%, which was the greatest of the nine treatments. Control plants with either one of the two bio-inoculants had no disease. Moderate disease severity was observed for the infected plants inoculated with *N. circulans* YRNF1 (45%), and 32% for that inoculated with AMF. Application of both bio-inoculants reduced disease severity by 26% compared to the non-treated infected plants. This indicated additive activity of these two bio-inoculants. Treatment with fludioxonil decreased the disease severity by 25% (Figure 5).

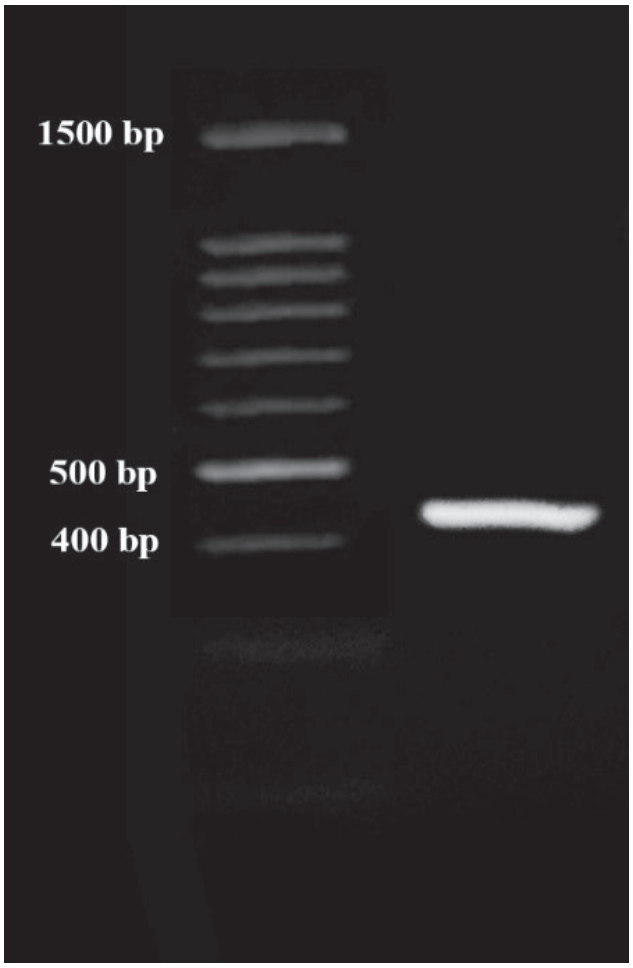

Figure 3. Agarose gel showing the amplified DNA product of the nitrogenase gene (*nifH*) of the diazotrophic bacterium YRNF1 as a single band (450 bp).

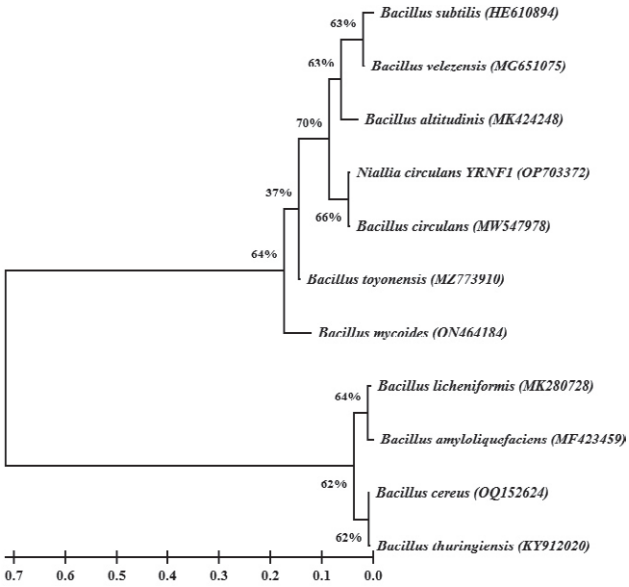

Figure 4. Phylogenetic tree of the diazotrophic bacterium *Niallia circulans* YRNF1.

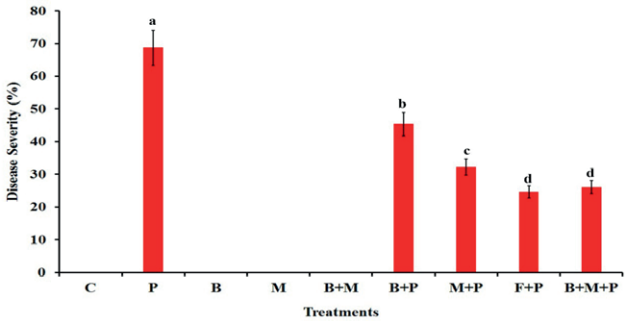

Figure 5. Mean root rot disease severities of eggplant plants treated with the diazotrophic bacterium *Niallia circulans* YRNF1 and/or AMF. Columns accompanied by the same superscript letters are not significantly different (Tukey's HSD test, $P \leq 0.05$). Error bars are Standard deviations (± SD). Treatments were: C, non-mycorrhizal, untreated and uninfected; P, non-mycorrhizal, untreated and infected; B, non-mycorrhizal, treated with *N. circulans* YRNF1 and uninfected; M, mycorrhizal, untreated and uninfected; B+M, mycorrhizal, treated with *N. circulans* YRNF1 and uninfected; B+P, non-mycorrhizal, treated with *N. circulans* YRNF1 and infected; M+P, mycorrhizal, untreated and infected; F+P, non-mycorrhizal, treated with the fungicide and infected; and B+M+P, mycorrhizal, treated with *N. circulans* YRNF1 and infected.

Triggering of the resistance-related genes in eggplant roots

Transcriptional expression of the resistance genes *JERF3, PAL1, C3H, CHI2,* and *HQT* in eggplant roots inoculated with *N. circulans* YRNF1 and colonized with AMF is shown in Figure 6. Compared to the control treatment, all of the experimental treatments elicited expression of *JERF3*. Combined application of *N. circulans* YRNF1 and AMF up-regulated the *JERF3* gene more than the single treatments. The greatest gene expression (29.4-fold) was for infected eggplant roots after inoculation with *N. circulans* YRNF1 and colonization by the AMF. Both applied bioagents increased the expression of *PAL1,* compared to the control plants. Greatest gene expression (17-fold) was for infected eggplant roots inoculated with *N. circulans* YRNF1 and colonized with the AMF. This was the same for *C3H,* where all the treatments induced expression of this gene, compared to the control treatment. Overexpression (8.6-fold) was detected for infected eggplant roots inoculated with *N. circulans* YRNF1 and colonized with the AMF. The applied treatments up-regulated expression of *CHI2*

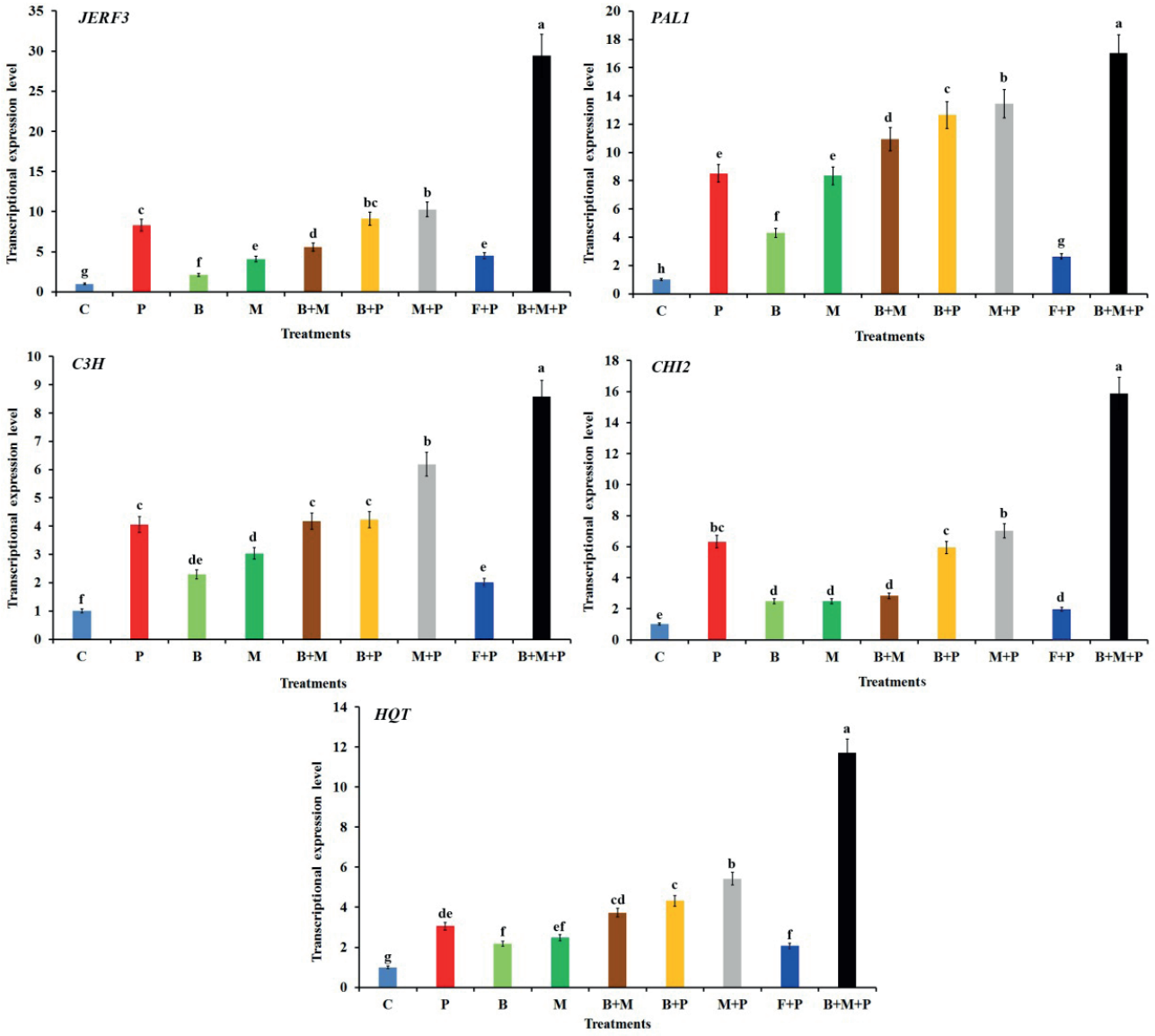

Figure 6. Mean transcriptional expression of resistance genes (*JERF3*, *PAL1*, *C3H*, *CHI2*, and *HQT*) in eggplant roots treated with *Niallia circulans* YRNF1 and colonized with AMF and inoculated with *Rhizoctonia solani*. For each gene, columns accompanied by the same superscript letter are not different ($P \leq 0.05$), according to Tukey's HSD test. The error bars are the standard deviations of the means. The experimental treatments were: C, non-mycorrhizal, untreated and uninfected; P, non-mycorrhizal, untreated and infected; B, non-mycorrhizal, treated with *N. circulans* YRNF1 and uninfected; M, mycorrhizal, untreated and uninfected; B+M, mycorrhizal, treated with *N. circulans* YRNF1 and uninfected; B+P, non-mycorrhizal, treated with *N. circulans* YRNF1 and infected; M+P, mycorrhizal, untreated and infected; F+P, non-mycorrhizal, treated with the fungicide and infected; and B+M+P, mycorrhizal, treated with *N. circulans* YRNF1 and infected.

at varying levels compared to the untreated non-infected eggplants, with greatest expression of this gene recorded value showing a 15.9-fold increase. Application of all the treatments led to overexpression of *HQT*, compared to the control plants. The infected eggplant roots inoculated with *N. circulans* YRNF1 and colonized with the AMF had maximum expression of 11.7-fold.

Effects of bioagents on phenol levels and antioxidant enzyme activities in treated eggplant roots

Eggplants challenged with *N. circulans* YRNF1 and AMF and inoculated with *R. solani* had increased level in total phenol contents, and increased POD and PPO enzyme potential (Table 3). At 30 dpp, mean phenolic content increased in the infected, non-treated eggplant

Table 3. Mean phenolic contents and antioxidant enzyme (POD or PPO) activities in eggplant roots 30 d after inoculation with *Rhizoctonia solani* and treatments with *Niallia circulans* YRNF1 and/or mycorrhizae.

Treatment	Phenolic content ($mg^{-1}g^{-1}$ f wt)*	POD (ΔA_{470} min^{-1} g^{-1} f wt)*	PPO (ΔA_{420} min^{-1} g^{-1} f wt)*
C	415.7 ± 8.19^{f}	1.14 ± 0.07^{h}	1.08 ± 0.05^{g}
P	673.2 ± 11.20^{d}	1.53 ± 0.05^{e}	1.34 ± 0.04^{e}
B	579.0 ± 7.32^{e}	1.38 ± 0.05^{f}	1.24 ± 0.05^{f}
M	594.4 ± 8.17^{e}	1.76 ± 0.09^{d}	1.56 ± 0.07^{c}
B+M	763.3 ± 8.41^{c}	1.90 ± 0.07^{c}	1.47 ± 0.09^{d}
B+P	772.5 ± 10.50^{c}	1.68 ± 0.05^{d}	1.57 ± 0.07^{c}
M+P	832.4 ± 9.13^{b}	2.12 ± 0.08^{b}	1.69 ± 0.05^{b}
F+P	668.2 ± 4.88^{d}	1.25 ± 0.04^{g}	1.29 ± 0.07^{ef}
B+M+P	1197.4 ± 12.02^{a}	2.32 ± 0.08^{a}	1.86 ± 0.09^{a}

*Means followed by different superscript letters are significantly different ($P \leq 0.05$), according to Tukey's HSD tests. The data are means of five replicates ± SD. Treatments applied were: C, non-mycorrhizal, untreated and uninfected; P, non-mycorrhizal, untreated and infected; B, non-mycorrhizal, treated with *N. circulans* YRNF1 and uninfected; M, mycorrhizal, untreated and uninfected; B+M, mycorrhizal, treated with *N. circulans* YRNF1 and uninfected; B+P, non-mycorrhizal, treated with *N. circulans* YRNF1 and infected; M+P, mycorrhizal, untreated and infected; F+P, non-mycorrhizal, treated with the fungicide and infected; B+M+P, mycorrhizal, treated with *N. circulans* YRNF1 and infected, and peroxidase (POD) and polyphenol oxidase (PPO).

roots, to 673.2 mg g^{-1} f wt compared to the control plants (415.7 mg g^{-1} f wt). Phenolic content of the non-infected eggplant roots treated with *N. circulans* YRNF1 was 579.0 mg g^{-1} f wt, and for roots treated with AMF was 594.4 mg g^{-1} f wt. Combination of both bio-inoculants increased phenolic content of the non-infected eggplant roots to 763.3 mg g^{-1} f wt. Treatment of the infected eggplant roots with a combination of both bioagents resulted in increased phenolic content up to 1197.4 mg g^{-1} f wt. Application of fludioxonil increased phenolic content to 668.2 mg g^{-1} f wt.

Compared to the control plants, POD enzyme activity increased in the infected non-treated eggplant roots to 1.53 ΔA_{470} min^{-1} g^{-1} f wt, and for PPO activity increased to 1.34 ΔA_{420} min^{-1} g^{-1} f wt. Enhancements in POD and PPO potential were recorded after treatments of plants with the two bio-agents, either singly or in combination. Greatest activity of both enzymes was recorded in the infected eggplant roots treated with *N. circulans* YRNF1 (2.32 ΔA_{470} min^{-1} g^{-1} f wt) and AMF (1.86 ΔA_{420} min^{-1} g^{-1} f wt). Compared to *R. solani*-inoculated non-treated plants, activity of both enzymes increased after treatment of infected roots with both bio-inoculants. However, the increases in enzyme activity attributable to the combined treatment (B+M+P) was higher than each single treatment. Application of the fungicide resulted in a recognizable increase in the POD and PPO activity, compared to the control plants.

Mycorrhization of eggplants after treatment with Niallia circulans *YRNF1*

Effects of *N. circulans* YRNF1 on mycorrhizal colonization of eggplant roots infected with *R. solani* are summarized in Table 4. Eggplant roots un-treated with AMF did not develop mycorrhizal colonization. In contrast, all eggplant roots treated with AMF showed varying levels of mycorrhization. Greatest colonization parameters were recorded for roots treated with *N. circulans* YRNF1 and AMF, which gave means of 88.7% colonization frequency, 56.1% colonization intensity, and 39.7% arbuscule formation frequency, while the eggplants colonized by AMF only had 76.3% colonization frequency, 45.3% colonization intensity, and 27.4% arbuscule formation frequency. These results indicated the compatibility between the two potential bio-agents. However, *R. solani* inoculation of the mycorrhizal eggplants reduced the mycorrhization levels, compared to

Table 4. Mean proportions (%) of mycorrhizal colonization frequency, intensity and arbuscule frequency 45 d after treatments of eggplants with *Niallia circulans* YRNF1.

Treatment	Colonization frequency (%)*	Colonization intensity (%)*	Frequency of arbuscules (%)*
C	0	0	0
P	0	0	0
B	0	0	0
M	76.3 ± 4.12^{b}	45.3 ± 6.13^{b}	27.4 ± 3.17^{b}
B+M	88.7 ± 6.23^{a}	56.1 ± 5.41^{a}	39.7 ± 5.11^{a}
B+P	0	0	0
M+P	63.9 ± 4.55^{d}	35.4 ± 6.04^{c}	20.5 ± 4.03^{c}
F+P	0	0	0
B+M+P	70.33.4 ± 4.81^{c}	43.4 ± 5.22^{b}	24.5 ± 5.15^{bc}

*Means accompanied by the same superscript letter are not significantly different ($P \leq 0.05$), according to Tukey's HSD test. The values are means of five replicates ± SD. Treatments applied were: C, non-mycorrhizal, untreated and uninfected; P, non-mycorrhizal, untreated and infected; B, non-mycorrhizal, treated with *N. circulans* YRNF1 and uninfected; M, mycorrhizal, untreated and uninfected; B+M, mycorrhizal, treated with *N. circulans* YRNF1 and uninfected; B+P, non-mycorrhizal, treated with *N. circulans* YRNF1 and infected; M+P, mycorrhizal, untreated and infected; F+P, non-mycorrhizal, treated with the fungicide and infected; and B+M+P, mycorrhizal, treated with *N. circulans* YRNF1 and infected.

the mycorrhizal eggplants not inoculated by the pathogen. This was also the case for the mycorrhizal-infected roots treated with *N. circulans* YRNF1.

Eggplants growth in response to application of N. circulans *YRNF1 and AMF*

Results from treatments of *R. solani* inoculated eggplants with *N. circulans* YRNF1 and AMF showed increases in most of the measured plant growth parameters (Table 5). Compared to experimental controls, the *R. solani*-inoculated had 20.5% reduction in mean shoot height and 26.3% reduction in mean root length. Eggplant roots colonized by AMF had 54.6% greater shoot height and 54.7% greater root length. Treating the non-*R. solan*i inoculated plants with *N. circulans* YRNF1 and AMF increased shoot height by 71.4% and root length by 41.8%. Treatment of the *R. solan*i inoculated eggplants with both bio-inoculants, increased shoot height by 53.7% and root length 66.5%. Treatment with fludioxonil also increased shoot height and root length of the *R. solani*-inoculated eggplants. Compared to controls, inoculation of the plants reduced shoot dry weight by 41.0% and root dry weight by 60.0%. Treatment of the non-infected eggplants with *N. circulans* YRNF1 and AMF increased shoot dry weight 79.5 and root dry weight by 130%. Application of both bio-agents to infected plants increased shoot dry weight by 51.3% and root dry weight by 90%. Treatment of infected plants with fludioxonil also increased shoot and root dry weights. Inoculation of plants with *R. solani* also reduced mean leaf area by 34.3%. Application of both bio-inoculants increased the leaf area by 115.7%. Combined treatment of the infected eggplants with both bio-agents increased mean leaf area by 85.8%. Treatment of the infected eggplants with fludioxonil increased leaf area, compared to the untreated infected plants.

Influence of Niallia circulans *YRNF1 and AMF on the macronutrient contents in the eggplant leaves*

Total nitrogen (TN) contents in eggplant leaves varied across the different treatments (Table 6). Compared to experimental controls, all the treatments caused increased TN. Greatest mean TN increases resulted for *R. solani*-inoculated plants colonized by AMF (3.9%), inoculated with *N. circulans* YRNF1 (3.6%) or treated with fludioxonil (3.6%). Greatest total phosphorus (TP: 2.55%) was measured in non-inoculated plants colonized with AMF, and in *R. solani*-inoculated plants colonized with AMF (TP = 2.26%). Most of the applied treatments also increased total potassium (TK) contents in the eggplant leaves, compared to control. Measured N/P ratios were increased in the *R. solani* inoculated plants either treated with the fludioxonil (mean N/P ratio = 4.29), inoculated with *N. circulans* YRNF1 (N/P = 3.86), or colonized with AMF (N/P = 3.85).

DISCUSSION

Rhizoctonia solani is a damaging pathogen that infects several economically important host plants. In

Table 5. Mean eggplant shoot heights, root lengths, shoot and root dry weights, and leaf areas 45 d after inoculation with *Rhizoctonia solani* and treatments of *Niallia circulans* YRNF1 and/or mycorrhizal colonization.

Treatment	Shoot height (cm)*	Root length (cm)*	Shoot dry weight (g)*	Root dry weight (g)*	Leaf area (cm^2)*
C	12.50 ± 1.12^{d}	5.17 ± 0.80^{e}	0.39 ± 0.03^{c}	0.10 ± 0.02^{c}	17.99 ± 1.23^{f}
P	10.00 ± 0.99^{e}	3.81 ± 0.73^{f}	0.23 ± 0.05^{d}	0.04 ± 0.01^{d}	11.82 ± 1.01^{g}
B	13.50 ± 1.37^{cd}	7.16 ± 0.95^{bcd}	0.62 ± 0.04^{ab}	0.22 ± 0.02^{a}	24.71 ± 2.33^{d}
M	19.33 ± 1.40^{b}	8.00 ± 1.00^{ab}	0.68 ± 0.06^{a}	0.21 ± 0.04^{ab}	33.54 ± 2.15^{b}
B+M	21.42 ± 1.22^{a}	7.33 ± 0.87^{bc}	0.70 ± 0.09^{a}	0.23 ± 0.05^{a}	38.82 ± 2.56^{a}
B+P	13.17 ± 0.87^{cd}	7.33 ± 0.74^{bc}	0.39 ± 0.08^{c}	0.10 ± 0.03^{c}	21.76 ± 1.81^{e}
M+P	18.14 ± 1.13^{b}	7.30 ± 0.81^{bc}	0.58 ± 0.07^{b}	0.21 ± 0.05^{ab}	28.48 ± 1.64^{c}
F+P	15.83 ± 1.41^{c}	6.33 ± 0.89^{cd}	0.36 ± 0.06^{c}	0.11 ± 0.06^{c}	$17.43 \pm .98^{f}$
B+M+P	19.21 ± 1.42^{b}	8.61 ± 0.78^{a}	0.59 ± 0.08^{b}	0.19 ± 0.07^{b}	33.42 ± 1.11^{b}

*Means accompanied by the same superscript letter are not significantly different ($P \leq 0.05$), according to Tukey's HSD test. The values are means of five replicates ± SD. Treatments applied were: C, non-mycorrhizal, untreated and uninfected; P, non-mycorrhizal, untreated and infected; B, non-mycorrhizal, treated with *N. circulans* YRNF1 and uninfected; M, mycorrhizal, untreated and uninfected; B+M, mycorrhizal, treated with *N. circulans* YRNF1 and uninfected; B+P, non-mycorrhizal, treated with *N. circulans* YRNF1 and infected; M+P, mycorrhizal, untreated and infected; F+P, non-mycorrhizal, treated with the fungicide and infected; and B+M+P, mycorrhizal, treated with *N. circulans* YRNF1 and infected.

Table 6. Mean contents of nitrogen, phosphorus, and potassium, and N/P ratios 45 d after treatments of eggplants with *Niallia circulans* YRNF1 and/or AMF.

Treatment	Total nitrogen (%)*	Total phosphorus (%)*	Total potassium (%)*	N/P ratio*
C	2.66 ± 0.08^{e}	1.59 ± 0.04^{d}	2.15 ± 0.08^{c}	3.72 ± 0.21^{ab}
P	3.15 ± 0.04^{cd}	2.17 ± 0.03^{bc}	2.70 ± 0.15^{ab}	3.20 ± 0.02^{b}
B	3.22 ± 0.08^{cd}	2.01 ± 0.06^{bc}	2.9 ± 0.06^{a}	3.57 ± 0.20^{ab}
M	3.50 ± 0.08^{bc}	2.55 ± 0.09^{a}	2.77 ± 0.06^{ab}	3.06 ± 0.17^{b}
B+M	3.23 ± 0.02^{cd}	2.04 ± 0.02^{bc}	2.89 ± 0.07^{a}	3.52 ± 0.05^{ab}
B+P	3.64 ± 0.16^{ab}	2.15 ± 0.15^{bc}	2.81 ± 0.02^{ab}	3.86 ± 0.06^{ab}
M+P	3.92 ± 0.09^{a}	2.26 ± 0.04^{ab}	3.04 ± 0.09^{a}	3.85 ± 0.45^{ab}
F+P	3.64 ± 0.04^{ab}	1.88 ± 0.02^{cd}	3.09 ± 0.11^{a}	4.29 ± 0.08^{a}
B+M+P	3.10 ± 0.08^{d}	2.12 ± 0.06^{bc}	2.45 ± 0.03^{bc}	3.24 ± 0.08^{b}

*Means accompanied by the same superscript letter are not significantly different ($P \leq 0.05$), according to Tukey's HSD test. The values are means of five replicates ± SD. Treatments applied were: C, non-mycorrhizal, untreated and uninfected; P, non-mycorrhizal, untreated and infected; B, non-mycorrhizal, treated with *N. circulans* YRNF1 and uninfected; M, mycorrhizal, untreated and uninfected; B+M, mycorrhizal, treated with *N. circulans* YRNF1 and uninfected; B+P, non-mycorrhizal, treated with *N. circulans* YRNF1 and infected; M+P, mycorrhizal, untreated and infected; F+P, non-mycorrhizal, treated with the fungicide and infected; and B+M+P, mycorrhizal, treated with *N. circulans* YRNF1 and infected.

this study, isolation of diazotrophic bacteria from the soil samples resulted in the recovery of eight bacterial isolates. Results of *in vitro* antagonism assays of these isolates against *R. solani* showed that one strain YRNF1, molecularly identified as *N. circulans*, considerably inhibited *R. solani* growth in culture. This was probably because of antifungal compounds production (antibiosis) by *N. circulans*. Analysis of *N. circulans* YRNF1 culture filtrate using GC-MS indicated occurrence of 19 metabolites. Butyric acid and ethylene glycol were the major components of the culture filtrate. Previous studies have reported antifungal activity of butyric acid, which is a short chain fatty acid produced by bacteria (e.g. *Lactobacilli* spp.), and this compound is widely used as a bio-preservative in dairy products due to its high antifungal activity (Garnier *et al.*, 2020). Ethylene glycol is widely used as an effective antifungal component in the hand sanitizers (Vuai *et al.*, 2022). The *in vitro* antifungal potential of *N. circulans* YRNF1 may be due to additive actions of butyric acid and ethylene glycol. It is possible that ethylene glycol, thanks to its amphiphilic nature, facilitates contact between butyric acid, which is a hydrophobic and antifungal compound, and *R. solani* cells, whose contents are hydrophilic (Abouloifa *et al.*, 2022).

Nitrogen fixation is an important biological process as nitrogen (N) is a limiting nutrient for crop growth. The nitrogen fixing ability of *N. circulans* YRNF1 was confirmed by the presence of *nifH* gene in its DNA, which provides marker for N fixation ability (Young, 1992). Nitrogen fixation by *N. circulans* YRNF1 is probably the main mechanism of the observed promotion of the eggplant growth, as shown by the increases of NPK contents in leaves of the treated plants. Nitrogen is the most important element in plant nutrition, and nitrogen nutrition *via* nitrogen fixation increases plant vegetative vigor. Phosphorus and potassium are also vital for plant biochemical processes, such as photosynthesis and for the nitrogen fixation process itself (Abdelraouf *et al.*, 2020). Potassium has an important function in activating enzymes including nitrogenase (Rashad *et al.*, 2023). Coskun *et al.* (2017) highlighted that the form of available N, particularly NH_4^+ and NO_3^-, and the transformation of N in soil, also affect K uptake by plants. These authors reported that administration of NH_4^+ to barley plants enhanced K^+ uptake. Phosphorus nutrition is also important for N fixation processes, where conversion of N_2 to NH_4^+ catalyzed by nitrogenase depends on ATP (Bello *et al.* 2023). Nitrogen fertilization also positively affects P uptake by plant roots (Krouk and Kiba, 2020).

The results obtained in the present study from the greenhouse assays showed that application of *N. circulans* YRNF1 suppressed disease severity caused by *R. solani* in the treated eggplants. The bio-control efficacy of *N. circulans* YRNF1 was probably due to its ability to produce metabolites (particularly butyric acid and ethylene glycol) with antifungal potential. Other possible mechanisms of action include the ability to inhibit the growth of *R. solani* and limit severity of root rot through colonization of root infection sites, competitive exclusion of the fungal pathogen, and secretion of antifungal and/or cell wall hydrolyzing enzymes (Lugtenberg *et al.*, 2009; Berendsen *et al.*, 2012). Systemic immunity may also be triggered by *N. circulans* in treated eggplants. All the above mentioned results are promising for the potential use of *N. circulans* YRNF1 as an effective biocontrol agent. Disease severity may also be reduced by AMF colonization of eggplants infected by *R. solani*, which triggers host defense responses in plants under stress caused by plant pathogens (Rashad *et al.* 2020b). AMF can also enhance plant resistance, by promoting plant growth and vigor *via* improvement of plant nutrition (El-Sharkawy *et al.*, 2023) and production of plant hormones (Song *et al.*, 2020). In the present study, co-inoculation of infected plants with the two biocontrol agents decreased disease severity by 26%, indicating an additive action of the *N. circulans* YRNF1 and AMF.

Both potential biocontrol agents affected expression of the defense genes *JERF3, PAL1, C3H, CHI2*, and *HQT*. The *JERF3* gene was particularly expressed in response to treatment of eggplants with *N. circulans* YRNF1 and AMF. The gene *JERF3* controls several plant defense genes (Rashad *et al*., 2022b). *PAL1* regulates the main step in the polyphenolic biosynthetic pathway, through conversation of phenylalanine to *t*-cinnamic acid (Mouradov and Spangenberg, 2014). *C3H* has an important role in biosynthesis of monolignols that constitute lignin (Tao *et al*., 2015), where lignification of the infected cell walls is a pivotal physical mechanism preventing pathogen penetration of hosts and cell to cell proliferation. *CHI2* catalyzes the bioconversion of coumaroyl CoA to naringenin involved in the biosynthesis of several fungitoxic compounds, including flavonoids and phytoalexins (Zhou *et al*., 2018). *HQT* regulates biosynthesis of chlorogenic acid from caffeoyl CoA (André *et al*., 2009). Triggering expression of these defense genes indicates induction of effects of the combined application of *N. circulans* YRNF1 and AMF for eggplant resistance.

Increases in phenolics and in activities of POD and PPO were detected in response to applying *N. circulans* YRNF1 and AMF. This is similar to the results of Singh *et al.* (2016). Increments of phenolic compounds content is a plant defense response associated with induced resistance (IR) in plants infected by pathogens (Chin *et al*., 2022). POD and PPO are important antioxidant enzymes, which catalyze formation of lignin, thus contributing to structural reinforcement of plant cells and formation of physical barriers against invading pathogens (Nasr-Esfahani *et al*., 2020). In addition, POD and PPO scavenge the oxidative ROS generated within infection processes.

Results from the present study have demonstrated that the frequency of colonization of eggplant roots by AMF, the level of colonization, and the frequency of arbuscule formation were greater after treating eggplants with a combination of *N. circulans* YRNF1 and AMF. This revealed the inducing effect of *N. circulans* YRNF1 on mycorrhizal colonization, which indicates compatibility of the two potential biocontrol agents.

In the present study, the two applied potential biocontrol agents promoted eggplant growth. Colonization of plant roots by two or more AMF species has previously been reported to provide a spectrum of advantages, and has more benefits to host plants than colonization by one species (Sharma and Kapoor, 2023). The strain *N. circulans* E9 has been found to promote plant growth (Sarmiento-López *et al*., 2022), which is associated with production of IAA and promotion of growth through stimulation of cell division and increased nutrient and water uptake, resulting in increased crop yield and quality (Sarmiento-López *et al*., 2022). In the present study, increases in the growth parameters of *R. solani*-infected eggplants were detected after individual treatments with each of the two bio-inoculants. This may be due to the biocontrol potential of *N. circulans* YRNF1 and growth promotion from each of the bio-inoculants. The recorded enhancement of eggplant growth after colonization with AMF was also similar to effects reported by Han *et al.* (2023), where treating lettuce by AMF increased the plant height by 30% and root length by 64%. Colonization by AMF is known to improve the host plants through multiple modes of action, including increases in active and passive nutrient and water uptake, and increased hyphal networks on root radicles, which magnify the hydraulic conductivity of water from the soil (Rashad *et al*., 2020c). In addition, AMF can produce hydrolyzing phosphatases, pectinases, and cellulases, as well as organic acids, which assist in nutrient utilization and improve their plant availability (Liu *et al*., 2021). AMF also produce phytohormones (e.g. abscisic acid and strigolactones) that enhance plant growth (Pozo *et al*., 2015).

Niallia circulans YRNF1 also enhanced eggplant nitrogen nutrition *via* the N-fixation, which probably promoted eggplant growth, whether or not colonized by AMF. The enhancements of eggplant growth parameters were increased after applying the two bio-gents, suggesting their additive action. This observation is consistent with that of El-Sharkawy *et al.* (2022), who showed that co-treating of pea plants by *Streptomyces viridosporus* HH1 and the mycorrhizal fungus *Rhizophagus irregularis* increased pea growth, compared to individual treatments with these two microorganisms. The AMF supply host plants with mineral nutrients, mainly phosphorus, which play prominent roles in plant growth (Adolfsson *et al*., 2015). The present results also demonstrated that combined application of *N. circulans* YRNF1 and mycorrhizal colonization improved the NPK contents in eggplant leaves, which was probably due to a cumulative effect of *N. circulans* YRNF1 and AMF. These data were similar to those of Hafez *et al.* (2022), who reported that combined application of growth-promoting rhizobacteria with organic fertilizers increased total N, P, and K in treated plant tissues, compared to untreated plants, and to individual applications of the mineral fertilizers.

The present study is the first to report effective use and the additive effects of the diazotrophic bacterium *N. circulans* and AMF, as biofungicides and biofertilizers, for potential management of Rhizoctonia root rot and growth-promotion in eggplant.

ACKNOWLEDGMENTS

This research did not receive specific grants from public, commercial or not-for-profit funding agencies.

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