BIODEGRADATION OF *S*-TRIAZINE COMPOUNDS USING ACTINOBACTERIUM *FRANKIA*

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*F*rankia are an actinomycetes, filamentous and gram positive bacteria. It is capable of fixing nitrogen by converted atmospheric N_2 to ammonia and can live free in the soil or in formation a symbiotic association with actinorhaizal plants (Benson and Silvester, 1993; Schweneke and Caru, 2001; Chaia *et al*., 2010). *Frankia* forms a perennial root organ called nodule where bacteria are hosted. Two type of nodules formation occur in actinorhizal symbiosis, the intercellular and extracellular infections (Benson and Silvester, 1993; Diagne *et al*., 2013).

Frankia strains have been classified to three main groups based on host plant family. The first group includes an *Alnus* (*Betulaceae*), *Myricaceae*, *Casuariaraceae* and the second group includes an *Eleagnaceae*, *Rhamnaceae* while the third group includes *Frankia* that symbiotic with actinorhizal plants in the families, *Coriariaceae*, *Datiscaceae*, *Rosaceae* and the genus *Ceanothus* (*Rhamnaceae*) (Oakely *et al*., 2004; Benson & Silvester, 1993). At present, *Frankia* genome sequences are available for the entire four *Frankia* lineages (Tisa *et al*., 2013). Cluster 1 phylogeny include: Strains CcI3, ACN14a, and QA3 (Nor-

mand *et al*., 2007; Sen *et al*., 2013). Cluster 2 includes the uncultured *Frankia* strain *Datisca glomerata* (Persson *et al*., 2011). Moreover, cluster 3 contains EAN1pec, EUN1f, BMG5.12 and BCU110501 (Normand *et al*., 2007; Beauchemin *et al*., 2012; Nouioui *et al*., 2013; Wall *et al*., 2013). On the other hand, the genomes of *Frankia* strains CN3, EuI1c and DC12 genomes represent Cluster 4 phylogeny or atypical *Frankia* (Beauchemin *et al*., 2012; Ghodhbane-Gtari *et al*., 2013). The large-scale production of a variety of chemical compounds has caused global deterioration of environmental quality. Microbial biodegradation has taken a high attention in recent years to getaways to clean up contaminated environments. It is a natural way of recycling wastes that it can break down organic substance to smaller compound by enzyme produced by living microbial organisms such as (bacteria - fungi - algae) (Reinek, 2001; Leja and Lewandowicz, 2009).

S- Traizine is a family of herbicides called 1,3,5-triazine. The general structural composition of *s*-triazine molecule is a heterocyclic six membered ring composed of alternating carbon and nitro-

gen atoms joined by double bonds. This type of a ring system necessitates a numbering of nomenclature in which the nitrogen atoms are labeled as 1, 3 and 5 with the 2, 4 and 6 carbon atoms. *S*-Triazine herbicides have been used extensively for controlling broadleaf and grassy weeds in corn, sorghum and sugarcane crops in agriculture. *S*-Triazine herbicides are also used on residential lawns and golf courses.

Atrazine (2-choloro-4-ethyl-amino - 6 - isopropyl amino -1,3,5-triazine) is the common name for an herbicide that is widely used to kill weeds. It's an effective herbicide used worldwide for broadleaf weed control on crops corn, pineapples, sorghum, sugarcane, macadamia nuts, and on ever green tree farms and forever green forest re-growth (Madelbaun *et al.*, 1995). Biodegradation of atrazine is a complex process depends on nature and amount of atrazine in soil or water and microorganism's availability (Madelbaun *et al.*, 1995; Rousseaux *et al.*, 2001; Singh *et al.*, 2004). The major steps of atrazine degradation pathway are hydrolysis, dealkylation, deamination and ring cleavage. Process dealkylation of amino groups to give 2–chloro 4–hydroxyl -6- amino- 1,3,5-triazine is unknown. In hydrolysis, atrazine degradation occurs by hydrolytic pathways which consist of three enzymatic steps catalysed by AtzA, AtzB and AtzC that hydrolysis the bound between c-cl plus ethyl and isopropyl groups to produce cyanuric acid that convert to ammonia and carbon dioxide by AtzD, AtzE and AtzF enzymes (Crawford *et al.*, 1998; Govantes *et al.*, 2009).

The present work aim to study and confirm that *Frankia* bacterium can cleavage the s-traizine ring and may use these compounds as a sole source of carbon and/or nitrogen in addition to identify the gene(s) responsible for this process.

MATERIALS AND METHODS

Frankia strains and growth media conditions

Frankia sp EuI1c and *Frankia alni* ACN14a were grown and maintained in basal MPN growth medium with an appropriate a carbon source at 33ºC as described by Tisa *et al.* (1983) and Tisa *et al.* (1999). Under *s*-triazine exposed conditions, the growth media contains one of the selected *s*-triazine compounds (atrazine, desethyl atrazine (DEA), Desethyl desisopropyl atrazine (DEDIA)) were tested in three cases: (1) in the presence of both carbon and nitrogen sources (C^+N^+), (2) presence of carbon and absence of nitrogen sources (C^+N^-), (3) without either nitrogen or carbon sources (C^-N^-). Three different concentrations (1, 3 and 5 mM) were applied against control with the three tested compounds. In case of HPLC/MS and qRT-PCR experiments, *Frankia* strains EuI1c and ACN14a were grown in media containing atrazine or desethyl desisopropyl atrazine (DEDIA) as a sole nitrogen source except overhead nitrogen (N_2).

Chemicals

Atrazine (2-chloro-4-ethylamino - 6-isopropyamino-s-triazine), Desethyl

atrazine (DEA) (2-amino-4-isopropylamino-6-chloroatrazine), Desethyl desisopropyl atrazine (DEDIA) (2, 4-diamino-6-chloro-s-triazine) were supplied by Toronto Research Chemicals, Inc. (North York, Ontario, Canada). 2-hydroxyatrazine and ammeline from Sigma-Aldrich (Germany). Triton X-100 from Macherey-Nagel GmbH&Co.KG.

Total cellular protein assay

Frankia alni ACN14a and *Frankia* sp EuI1c were inoculated into 48-well plate in MPN medium containing one of *s*-triazine compounds at different concentrations to measure *Frankia* ability to degrade any of the compounds under this study and may be use them as a sole carbon and/or nitrogen source. After 15 days incubation at appropriate temperature, the cells were harvested and the growth yield was determined by measuring the total cellular protein assay. Protein content was measured by using Roti®-Nanoquant protein quantification assay (K880) according to Bradford method (Bradford, 1976). *Frankia* mycelia were harvested by centrifugation at 13,000 x g for 10 min at room temperature (RT) and washed once with sdH$_2$O. The cell pellet was resuspended in 100 μL of 1 N NaOH and heated at 95°C for 10 min. After incubation, the suspension was clarified by centrifugation at 13,000 x g for 10 min. Roti®-Nanoquant assay were carried out according to the manufacturer's instructions (Roth, Germany). Briefly, 20 ml of the 5X reagent were diluted with 80 ml sdH$_2$O and pipetted in 96-well plate. For each well, 200 μl of the 1X reagent was added to 50 μL from each sample (samples were diluted five times). The plate assay was incubated at RT for 5 min at least. Protein concentrations were quantified on a Tecan plate reader using a bovine serum albumin (BSA) standard (0-100 μg/mL). Growth yield was determined by subtracting the protein content of the inoculum and the experiment was performed in triplicates. Statistical analysis was completed using the Dunnett's test in Excel program.

The intermediate metabolites

To determine the metabolites produced during atrazine and desethyl desisopropyl atrazine conversion, *Frankia* EuI1c and ACN14a cells (100 μg/ml protein) were washed twice in MP buffer and grown in MP media with concentration 1 mM of atrazine or DEDIA as a sole source of nitrogen against time course (0, 3, 6 and 9 days). The media supernatants were extracted with an equal volume of ethanol and analyzed by reverse-phase high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) to identify the potential metabolic products. The HPLC was performed on an Agilent1200 series liquid chromatography apparatus equipped with a diode array UV-Vis detector and a Luna C18 (2) 5-μm, 100A column (150 x 2 mm, Phenomenex). The electrospray interfaced mass spectrometer (Agilent G1956 MSD) was used together with HPLC to identify the potential metabolites. A mobile phase contains 5 mM acetic acid (A) (pH 4.5)

and acetonitrile (B) was used at 35°C in separation. Gradient was run at 0.5 ml min^{-1} from zero to 60% B within 12 min. The HPLC method was standardized using standard atrazine and intermediate metabolite especially hydroxyatrazine and ammeline standards. Spectra were record in the range from 190 to 800 nm at ca 1 Hz. Mass spectra were recorded from 100 to 300 m/z. The atmospheric pressure electrospray ionization (AP-ESI) interface was operated in negative mode with a capillary voltage of 5,500 V.

Data mining and bioinformatics analysis

The FASTA amino acid sequences of published *Frankia* genomes (especially, (ACN14a, NCBI RefSeq: NC_00827; EuI1c, NCBI RefSeq: NC_014666) were obtained from Integrated Microbial Genome Institute from the Joint Genome Institute (JGI) (www.img.doe.gov) (Markowitz *et al*., 2006). The identified deduced amino acid sequences of several functionally analyzed chlorohydrolase genes in *s*-triazines degradation which published in the literatures were collected and used in BLASTP search of *Frankia* genomes (Sajjaphan *et al*., 2004; Boundy-Mills *et al*., 1997; Qingyan *et al*., 2008). The potential trzN (atzA) protein sequences were aligned using ClustalW (Thompson *et al*., 1997). Furthermore, for phylogenetic analysis with Maximum Likelihood (ML), Jones-Taylor-Thornton (JTT) model, Maximum Parsimony (MP) and Nearest-Neighbor-Interchange (NNI), MEGA 6.0 software (Tamura *et al*., 2013) were used to construct the phylogenetic tree from 1000 bootstrap replicates.

RNA extraction

For these experiments, all solutions and materials were DEPC (Diethyl Pyrocarbonate)-treated or RNase zap-treated to prevent RNA degradation. RNA extractions were performed by the Triton X-100 method as previously described (Niemann and Tisa, 2008). *Frankia* mycelia were harvested by centrifugation at 10,000 x g for 10 min at RT and washed with 1X TE. The washed pelleted cells were re-suspended in 400 μL TE buffer containing 0.2% Triton X-100 and incubated at 95°C for 10 min. Samples were transferred to an ice bath and cooled for 1 min. An equal volume of cold chloroform was added to the lysed samples and mixed by inversion 100 times. Cell lysates were removed by centrifugation at 13,000 x g at 4°C for 3 min. The aqueous layer was transferred to a fresh tube and the chloroform treatment was repeated. A 1/10th volume of 3 M sodium acetate (pH 5.2) was mixed with the aqueous layer in a fresh tube and 2 volumes cold absolute ethanol were added to the mixture. Samples were incubated overnight at -60°C. The RNA was recovered by centrifugation at 13,000 x g for 25 min at 4°C and the pellet washed with one mL 80% cold ethanol. The RNA pellet was air-dried and re-suspended in 50 μL RNAse-free H$_2$O.

DNase 1 treatment, RNA quantification and cDNA synthesis

RNA samples were diluted to 50 ng/µl in 100 µl and treated with DNase I (PureLink DNase, Invitrogen™) according to the manufacturer's instructions. RNA samples were quantified with Qubit® 2.0 Fluorometer (Invitrogen™) and stored at -20°C until use.

The cDNA synthesis was performed using random hexamer primers, 200 ng RNA and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, California) at 42°C for 50 min according to the manufacturer's instructions. Briefly, each sample, 1 µL of 10 mM dNTPs, 0.1 µL of random primers, and 1 µL sdH$_2$O were added and the mixture was incubated at 65°C for 5 min. The tubes were chilled on ice for 1 min and 4 µL 5x SuperScript III buffer, 1 µL DTT, 1 µL RNAseOUT, and 1 µL SuperScript. The cDNA was quantified by a Biophotometer (Eppendorf, Hamburg, Germany), diluted to 10 ng/µl working stocks in RNAse-free H$_2$O and stored at -80°C until use.

Gene expression

The mRNA expression levels were quantified using Real Time PCR with the listed primers in table 1 and 2X SYBR® Green PCR Master Mix (Rotor-Gene SYBR® Green PCR Kit (Hilden, Germany) according manufacturer's instructions. Briefly, each 24 µL reaction contained 100 ng template cDNA, 300 nM of the forward and reverse primer mix in addition to SYBR® Green PCR Master Mix. Two-step quantitative Real Time-PCR (qRT-PCR) method were applied as follows: (1) 5 min at 95°C for enzyme activation, (2) 45 cycles of 5 s at 95°C and 10 s at 55°C (acquisition of fluorescence at the end of elongation), and an additional step for production of dissociation curves from 55 to 95°C. Reactions were performed in triplicates and the comparative threshold-cycle (Ct) method was used to quantify gene expression by calculating the $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001) as fold change. The results were normalized with *rpsA* house keeping gene expression and relative to the expression levels of untreated samples. Primer efficiencies were determined using *Frankia* gDNA standard curve by plotting the log change in gDNA concentration vs. cycle threshold (Ct).

RESULTS AND DISCUSSION

Frankia growth in s-triazine compounds

With *F. alni* ACN14a (Figs. 1, 2 and 3), the growth patterns increased in presence of both nitrogen and carbon source (C$^+$N$^+$) under all tested compounds with prolonged exposure to higher concentrations from tested compound with exception of desethyl atrazine. Under the second condition (absence of nitrogen source except for overhead N$_2$ (C$^+$N$^-$)), the peaked values continued to increase under desethyl desisopropyl atrazine stress whereas the values decreased under desethyl atrazine and stayed almost close to control with atrazine exposure. In case of applying no carbon and no nitrogen

source (C⁻N⁻) condition, no significant change in the total cellular protein was detected under desethyl atrazine and desethyl desisopropyl atrazine stress whereas a detectable change was observed with atrazineexposure.

Meanwhile, *Frankia* EuI1c (Figs. 1, 2 and 3) showed an increase in growth patterns under atrazine, DEA, DEDIA at variable levels in presence of both carbon and nitrogen source (C⁺N⁺). Furthermore, a gradual increment in protein level was observed with DEDIA when variable levels were applied whereas no change or toxic effect were detected with the other *s*-triazines under C⁺N⁻ condition. This could be explained that *Frankia* EuI1c has many amidohydrolase enzymes in its genome which may help this microorganism to use NH₂-groups in DEDIA in deamination process as a sole nitrogen source. A level of growth was detected with atrazine in absence of either carbon or nitrogen source (C⁻N⁻) only, which could be due to dealkylation process.

The OD₆₀₀ of *Arthrobacter* sp. DNS10 in medium containing atrazine (100 mg L⁻¹) as the sole nitrogen source was increased obviously during the 60 h. In the same time, the OD₆₀₀ of the control treatment was slightly changed. This indicates that strain DNS10's growth might strongly rely on atrazine (Zhang *et al.*, 2011). Furthermore, García-Gonzalez *et al.* (2003) examined the effect of nitrogen on atrazine degradation. The presence of atrazine in addition to ammonium in the growth medium did not stimulate atrazine degradation which suggests that ammonium-mediated repression operates regardless of the presence of the herbicide in the culture medium.

Moreover, *Arthrobacter aurescens* TC1 can grow in liquid medium with consuming a high amount of atrazine (up to 3,000 mg/L⁻¹) as the sole source of nitrogen, carbon and energy. *A. aurescens* TC1 also metabolized compounds containing chlorine plus *N*-ethyl, *N*-propyl, *N*-butyl, *N*-*s*-butyl, *N*-isobutyl, or *N*-*t*-butyl substituents on the *s*-triazine ring. Atrazine was metabolized to alkylamines and cyanuric acid (Wang and Xie, 2012; Zhang *et al.*, 2009; Strong *et al.*, 2002).

HPLC/MS analysis for metabolites identification

The ability of *Frankia* ACN14a and EuI1c to metabolize atrazine and DEDIA was studied by samples extract with an equal volume from ethanol and analyze by reverse-phase high-performance liquid chromatography (RP-HPLC) coupled with mass spectrometry (MS). RP-HPLC-MS analysis of the supernatant after course time revealed the presence of several metabolites. A metabolite with an *m/z* of 196 was identified as hydroxyatrazine (Fig. 4) and another metabolite with an *m/z* of 126 (Fig. 5) was identified as ammeline. They were detected by mass spectrometry and their retention times were 3.8 and 1.87 min, respectively. The two metabolites showed a mass spectrum similar to spectrum recovered from analytical-grade hydroxyatrazine and ammeline standards

(Fig. 5). The previous metabolites started to appear in the culture filtrate at day 6 and after that time.

Data mining and bioinformatics analysis of Frankia genomes

Bioinformatics analysis of *Frankia* strains ACN14a and EuI1c genomes putatively showed one functional gene (putative *trzN*, FRAAL1474 and/or FraEuI1c_5874) which could play a role in *s*-triazines degradation. The identified gene is predicted to encode amido-hydrolase (trzN) which expected to hydrolyze the C-Cl bond in *s*-triazine compounds. The amino acid sequences of putative TrzN from ACN14a and EuI1c strains with reference TrzN (AtzA) proteins from *Arthrobacter aurescens* (AAS20185.1), *Pseudomonas sp.* ADP (AAK50270.1), *Arthrobacter sp.* AD26 (ABU63129.1), *Gordonia rubripertincta* (AAA90931.1), *Arthrobacter aurescens* TC1 (YP_949961.1), *Pseudomonas sp.* AD39 (ACI14306.1) and *Nocardioides sp.* AN3 (BAG55673.1) were aligned and the phylogenetic tree was constructed (Fig. 6). The deduced amino acid of TrzN from *Arthrobacter aurescens* (Accesson number, AAS20185.1) showed 34% identity and 48% similarity with putative TrzN (FRAAL1474) while in *Pseudomonas sp*. ADP, atzA (Accession number, AAK50270.1) exhibited 29% identity and 43% similarity, respectively.

Gene expression of potential TrzN gene

A reverse-genetics approach by quantitative Real Time-PCR (qRT-PCR)

was used to identify the putative *trzN* (FRAAL1474) gene activity in Frankia strain ACN14a since the same gene exist in the other strain (EuI1c). The mRNA level of FRAAL1474 gene in *Frankia* ACN14a grown under 1 mM from atrazine stress was measured after 6 days in stressed cells. The putative *trzN* (FRAAL1474) gene which encodes amidohydrolase showed more than 30 fold changes in its expression at 1 mM atrazine stress in comparison to untreated cells (Fig. 7). This result indicates that *trzN* gene over-expressed under atrazine exposure. However, the expression of gene of interest was normalized by the house-keeping gene *rpsA* (30S ribosomal protein S1) and compared with untreated cells which taken the value (1). Furthermore, a reverse transcription quantitative PCR (RT-qPCR) was used as an indicator to monitor atrazine biodegradation performances in soil (Monard *et al.*, 2012).

SUMMARY

S-triazine compounds consider as herbicides for the control of broadleaf weeds in corn, sorghum and sugarcane crops in agriculture. *S*- triazines metabolism initiate via a dechlorination reaction catalyzed by atrazine chlorohydrolase or amidohydrolase (TrzN/AtzA). *Frankia* strains ACN14a and EuI1c were exposed to different concentrations from three different *s*-triazine compounds include atrazine, desethyl atrazine and desethyl desisopropyl atrazine in media containing, carbon and nitrogen source (C^+N^+), carbon source and no nitrogen source (C^+N^-), and

absence either carbon and nitrogen source (C⁻N⁻). Hydroxyatrazine and ammeline were the first detected metabolites in ACN14a and EuI1c cultures filtrate via HPLC/MS analysis. Data mining and bioinformatics analysis of selected *Frankia* ACN14a and EuI1c genomes exhibited an amidohydrolase gene (putative *trzN*, FRAAL1474 and/or FraEuI1c_5874) which could play a role in *s*-triazines degradation (dechlorination). A reverse genetic approach (qRT-PCR) was used to evaluate the gene expression of interested gene (FRAAL1474) under atrazine stress as the sole nitrogen source. The interested gene which encode amidohydrolase showed more than 30 fold changes in its mRNA expression level under 1 mM atrazine stress after 6 days exposure in comparison to untreated cells.

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Table (1): Nucleotide sequence of primers used for quantitatively amplify the house keeping gene and the putative hydroxylase genein qRT PCR.

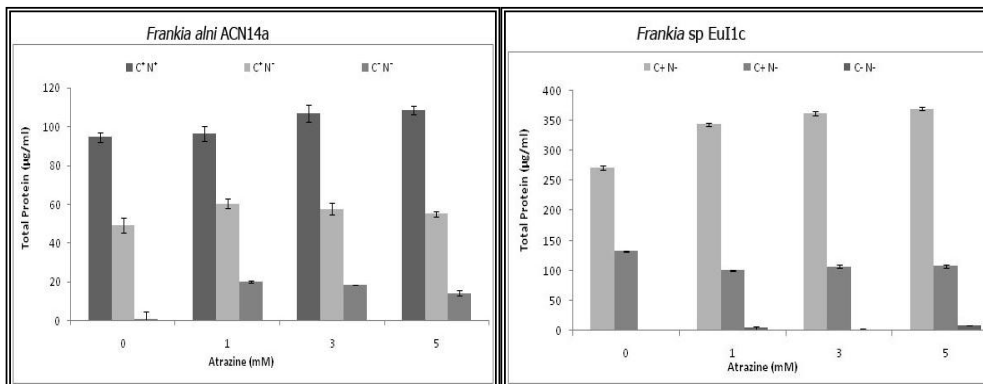| Locus ID (gene name)	Nucleotide sequences 5 ⟶ 3	Description	Size of amplified fragment
FRAAL1781	F: GCAGTCGACAAGACGATCAA R: CTCGGTCTTGTAACCGATGTC	rpsA	108 bp
FRAAL1474	F: GACCACCGACCACCTTTAC R: CTTCACCGACAGCGACAT	Putative hydrolase	127 bp

Fig. (1): Effect of different atrazine levels on growth rates of *Frankia* ACN14a and EuI1c in the presence of carbon and nitrogen source ($C^{+}N^{+}$), presence of carbon and absence of nitrogen source ($C^{+}N^{-}$), and absence of both carbon and nitrogen source ($C^{-}N^{-}$). Values shown represent the mean of three biological replicates. Error bars represent the standard deviation of the mean.
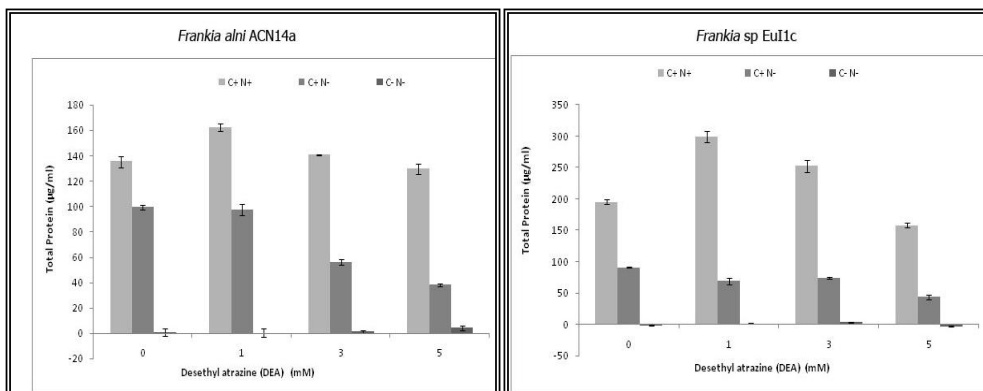

Fig. (2): Effect of different desethyl atrazine (DEA) levels on growth rates of *Frankia* ACN14a and EuI1c in the presence of both carbon and nitrogen source ($C^{+}N^{+}$), presence of carbon and absence of nitrogen source ($C^{+}N^{-}$), and absence of both carbon and nitrogen source ($C^{-}N^{-}$). Values shown represent the mean of three biological replicates. Error bars represent the standard deviation of the mean.
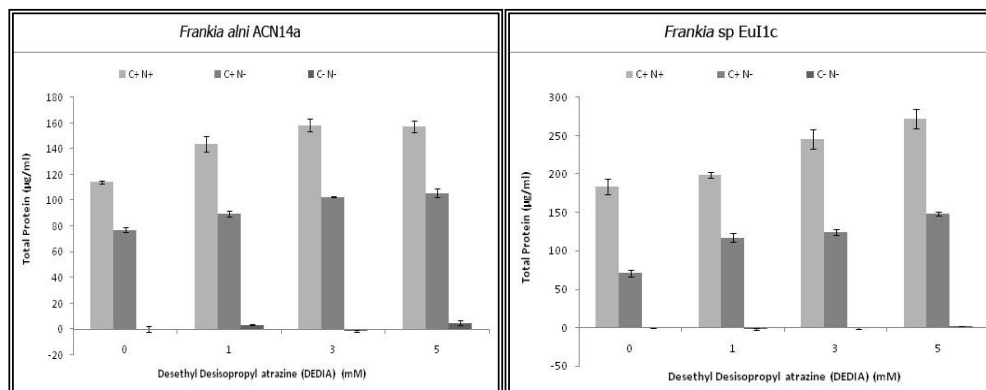
Fig. (3): Effect of different desethyl desisopropyl atrazine (DEDIA) levels on growth rates of *Frankia* ACN14a and EuI1c in the presence of both carbon and nitrogen source (C^+N^+), presence of carbon and absence nitrogen source (C^+N^-), and absence of both carbon and nitrogen source (C^-N^-). Values shown represent the mean of three biological replicates. Error bars represent the standard deviation of the mean.
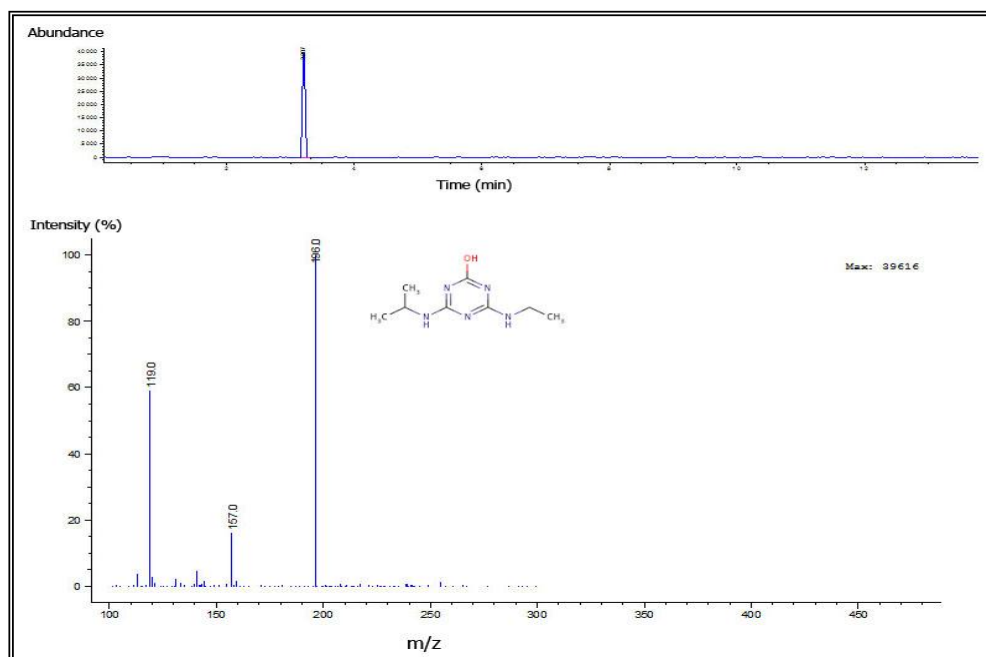
Fig. (4): Mass spectra (electron impact ionization, $E_0 = 70.1$ eV) of hydroxyatrazine formed by *Frankia* ACN14a and EuI1c, which have been incubated with atrazine as a sole source of nitrogen. The peak at *m/z* 196 represents hydroxyatrazine.
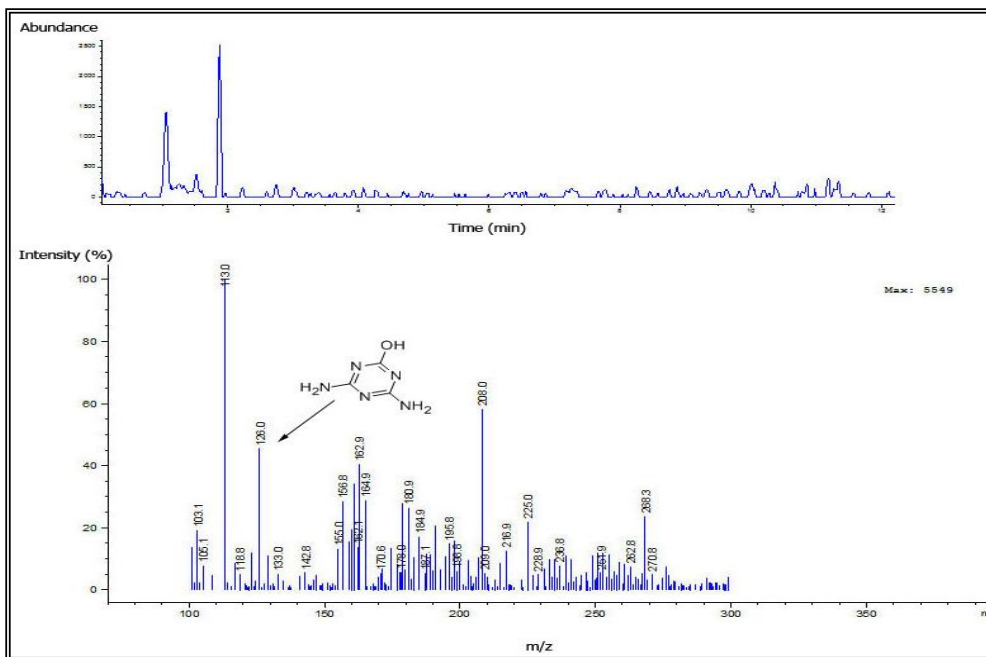
Fig. (5): Mass spectra (electron impact ionization, $E_0 = 70.1$ eV) of ammeline formed by *Frankia* ACN14a and EuI1c, which had been incubated with DEDIA as a sole source of nitrogen. The peak at *m/z* 126 represents ammeline.
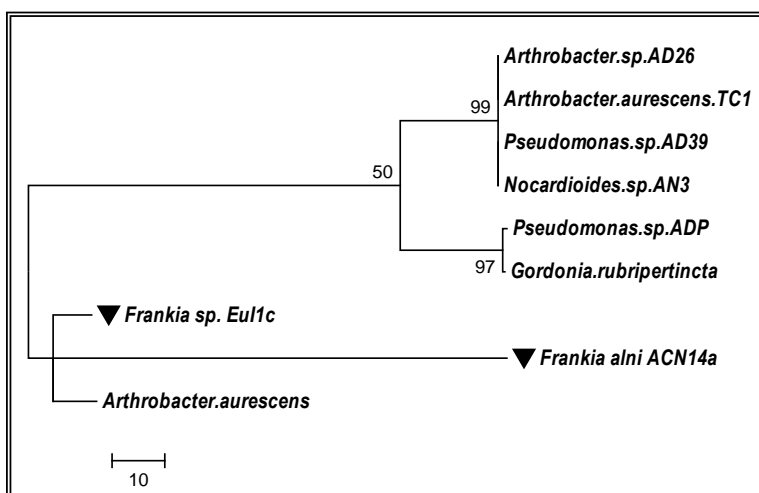

Fig. (6): Molecular phylogenetic analysis by Maximum Likelihood (ML) and Maximum Parsimony (MP) criteria. The phylogenetic tree shows the relationship between potential trzN from *Frankia* EuI1c and ACN14a (▼) with functional trzN/atzA proteins from microorganisms identified in the GeneBank database. Evolutionary analyses were conducted using MEGA6
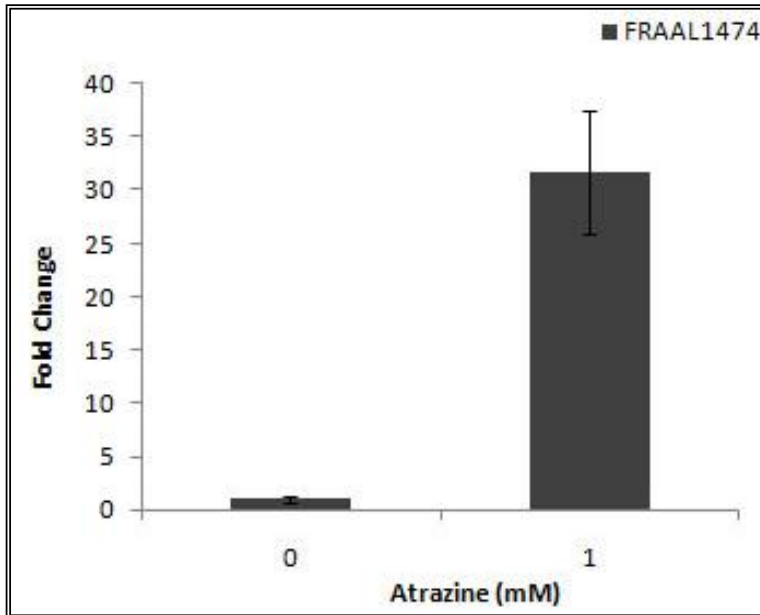
Fig. (7): Relative gene expression in response to atrazine exposure. Expression of FRAAL1474 (*trzN*) in response to atrazine stress. Cells were exposed to 1 mM atrazine treatment for 6 days. Experimental gene expression was normalized to the *rpsA* housekeeping gene and compared to the untreated cells. Data are presented as the ratio (fold change) between the values obtained with atrazine-treated and untreated wild-type cells. Mean values and standard error are presented.