Journal of Biotechnology **14**(4): 713-720, 2016

BIOLOGICAL AND TAXONOMICAL CHARACTERISTICS OF ENDOPHYTIC *STREPTOMYCES* TQR8-14 AND ITS PRODUCTION POTENTIAL OF ANTIMICROBIAL SUBSTANCE

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Received: 09.11.2016
Accepted: 30.12.2016

SUMMRY

Endophytic actinomycetes colonize in living plant tissues without causing harm to the host plant. In fact, they are regarded as effective bio-control agents and plant growth promoters due to their ability to activate plant self-immunity and produce biologically active secondary metabolites. Recent studies reported higher rates in finding new strains and antimicrobial substances among endophytes than actinomycetes from soil and plant surface. In this work, endophytic actinomycetes were isolated from Ham Yen orange trees, a famous specialty of Tuyen Quang province, and screened for antimicrobial activity against plant pathogenic bacteria and fungi. The isolate TQR8-14, showing strong activity, was studied with regard to its biological and taxonomical characteristics and production of antimicrobial substance. Based on the mycelial morphology, this isolate was placed in yellow group of streptomycetes. The isolate developed milky to yellow aerial mycelia on all test media and long straight spore chains bearing smooth cylinder spores. The isolate could grow at wide range of temperature 15 to 45^{o}C, of pH 4 to 10; on substrates such as carboxy methyl cellulose, starch, protein and chitin; and tolerated up to 3% salinity. Its 16S rDNA nucleotide sequence (1,404 bp) shared 99% similarity to *Streptomyces parvus*. Therefore, the isolate was named as *Streptomyces parvus* TQR8-14. The highest antimicrobial activity was achieved in culture on medium AH4 containing soybean meal and glucose, at 30^{o}C and pH 7.0.

Keywords: *Endophytic actinomycetes; citrus plant; actinomycete identification; 16S rDNA*, *Streptomyces parvus*

INTRODUCTION

Endophytic actinomycetes live inside the host plant, such as stems, roots, leaves, fruits, ovules, seeds and tubers etc. (Shimizu, 2011). Nearly every plant species hosts a certain endophytic microorganism. Endophyte receives nutrients from its host, and in turn, the endophyte supports plant health through enhancement its immune system and systemic resistance to pathogenic agents, as well as reduces adverse effects of the environment on the host plant (Hasegawa *et al.*, 2006). It was found that endophytes produce antifungal substances, such as antibiotics or enzymes degrading the fungal cell wall, or agents enhancing the immune system, which help to protect plant from pathogenic fungi. There are reports on production of phytohormones like indole-acetic-acid (IAA) by endophytic microorganisms. In such cases, the growth of host plant was improved via gains in leaft and root dry weights and lengths (Malfanova *et al*., 2013; Sharma, 2014). Over the years, various endophytic microorganisms, especially the streptomycetes, were isolated from different plant species and exploited (Gangwar *et al.,* 2014; Shutsrirung *et al.,* 2013). A number of biologically active compounds produced by the endophytes can have abnormality in chemical structure that is important for development of new chemicals with new properties (Joseph *et al.,* 2012). In this work, an endophytic actinomycete TQR8-14 isolated from citrus trees of Tuyen Quang province was studied on its classification and activity against plant pathogenic bacteria and fungi.

MATERIAL AND METHODS

Materials

Endophytic actinomycete TQR8-14 was obtained from the Culture Collection of Soil

Microbiology Laboratory, Institute of Biotechnology (IBT) of Vietnam Academy of Science and Technology (VAST).

Test (indicator) microorganisms: *Colletotrichum truncatum, B. subtilis* ATCC 6633 (Culture Collection of Soil Microbiol. Lab., IBT, VAST), *S. aureus* ATCC25922 and *P. aeruginosa* ATCC10145 (National Institute of Drug Quality Control). Primers for amplification of 16S rDNA (27f (5'-TAACACATGCAAGTCGAACG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3')) were purchased from Invitrogen (Hongkong).

Morphological and cultural characterization

Morphological and cultural characteristics of the isolate TQR8-14 were studied according to the protocols of the International *Streptomyces* Project (ISP) (Shirling, Gottlieb, 1966a,b; Nomomura, 1974). The spores were studied under Scanning Electron Microscope FESEM S4800.

Qualitative evaluation of enzyme activity was done by plate assay method. Carboxy methyl cellulose, chitin, oat spelt xylan, casein and starch were used to detect cellulase, chitinase, xylanase, protease, and amylase respectively (Johnsen, Krause, 2014). The activity of an enzyme was evaluated as a clear zone (degraded substrate) around the colony.

The thermal and pH tolerance of the isolate were evaluated on medium ISP2 at different temperatures from 15 to 50^{o}C, and pH from 3.0 to 11.0. The salinity tolerance was tested on medium ISP2 supplemented with NaCl to the final concentrations of 3÷7 % (wt/vol). In all the experiments mentioned above, the results were recored after 14 days.

DNA extraction and 16S rDNA gene amplification

The biomass for DNA extraction was obtained by growing the isolate on a non-sporulating agar medium according to Ishikawa *et al.* (2000). Genomic DNA was isolated using NucleoSpin® Tissue extraction kit (Macherey-Nagel, Germany) according to the manufacturer's instruction. The 16S rDNA gene were amplified from the genomic DNA using primers 27f and 1492R. The themal cycle for PCR was described by Shutsrirung *et al.* (2013). The obtained 16S rDNA nucleotide sequence (1,404 bp) was registered at the National Center for Biotechnology Information (NCBI) GenBank database under accession number KX712241. The 16S rDNA nucleotide sequence of isolates was aligned with the relevant known sequences from GenBank using http://www.ncbi.nlm.nih.gov/BLAST. Phylogenetic analysis was done by CLC DNA Workbench 6.6 program.

Assay of antibacterial activity

Agar block method

Three indicator bacteria were grown separately in LB broth for 24 hours. A 100 µl of an indicator bacterial culture was thoroughly spread on each of three LB agar plates. Agar blocks (Φ 5mm) of actinomyces isolates previously grown on ISP2 were cut by a cork borer and placed on the LB plates with indicator bacteria. After overnight incubation at 37ºC, growth inhibition was evaluated by measuring diameters of halo surrounding the agar blocks.

Agar well diffusion method This assay was conducted as described by Sharon *et al.* (2014).

Cultivation conditions for production of antimicrobial substance(s)

Actinomycete TQR8-14 was cultivated in the following traditional media: Gause I, Gause II, ISP2, ISP4, and starch casein (SCA). Additionaly, media such as AH4 (glucose, 15 g/l; soybean meal, 15 g/l; NaCl, 5 g/l; $CaCO_3$, 1 g/l; pH 7.0) and 79 broth (glucose, 10 g/l; peptone, 10 g/l; casein hydrolysis, 2 g/l; NaCl, 6 g/l; pH 7.2) were used. The effects of cultivation temperature and pH were studied in the range of 25 to 37^{o}C and pH of 6 to 9 to find the values most favourable for the actinomycete TQR8-14. In all experiments, shake flask cultures were performed on rotary shaker at 150 rpm for 5 days at 28°C.

RESULTS AND DISCUSSION

Biological characteristics of endophytic actinomycete TQR8-14

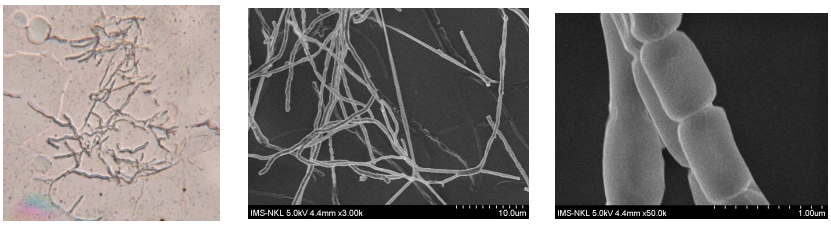
On all eight test agar media, the isolate TQR8-14 grew as convex colonies of 1-2 mm diameter forming tufted powdery mass. Growth and colouring characteristics of the isolate TQR8-14 on different agar media are summarized (Table 1). The aerial mycelia were milky to yellow, while the substrate mycelia were yellowish. Orange pigmentation was observed on ISP3 and ISP6 agar media, but in shake flask cultures with all test media, the broth was

coloured yellow to orange. According to colour grouping manual by Shirling, Gottlieb (1966), the endophytic actinomycete TQR8-14 was placed in yellow group.

Figure 1 shows morphology of aerial mycelia of 7-day cuture on ISP2 agar medium. The spore chains are straight; each bears up to 50 smooth cylinder spores.

Table 1. Growth and colouring characteristics of actinomycete TQR8-14 on test media.

Media	Growth	Aerial mass colour	Subtrate mass colour
Tryptone Yeast extract agar (ISP 1)	Moderate	Light yellow or milky	Light yellow
Yeast extract malt extract agar (ISP 2)	Poor	Light yellow or milky	Yellow
Oat meal agar (ISP 3)	Good	Light yellow or milky	Brownish yellow
Inorganic salt agar (ISP 4)	Good	Light yellow	Yellow
Glycerol – Aspargine agar (ISP 5)	Poor	Light yellow	Yellow
ISP6	Moderate	Light yellow or milky	Orange
Tyrosine agar (ISP 7)	Good	Light yellow	Yellow
ISP8	Moderate	Milky	Light yellow



A **B** **C**

Figure 1. Mycelia of actinomycete TQR8-14: Light microscope image (x40) of mycelia and spore chains (A); scanning electron images: spore chain (B) and spores (C). Bar = 1 µm.

Table 2. Biochemical characteristics of actinomycete TQR8-14. **Note:** (-): no growth; (+) Moderate; (++) Good.

Test	Results
Gram staining	+
Hydrolysis of:	
Starch	18 mm
Casein	30 mm
CMC	24 mm
Chitin	35 mm
Xylan	36 mm
Utilization of carbon source (1,0 %, w/v):	**Growth**
D-Glucose	+
L-Arabinose	+
Sucrose	-
D- Xylose	++
D-Manitol	++
D- Fructose	++
D-Cellulose	++
D-Rafinose	-
Negative control (no sugar)	-

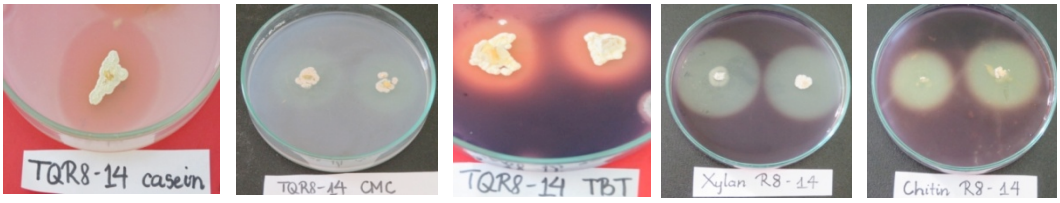


Figure 2. Enzyme activity of actinomycete TQR8-14: Degradation of casein (A); carboxymethyl cellulose (B); starch (C); xylan (D), and chitin (E).

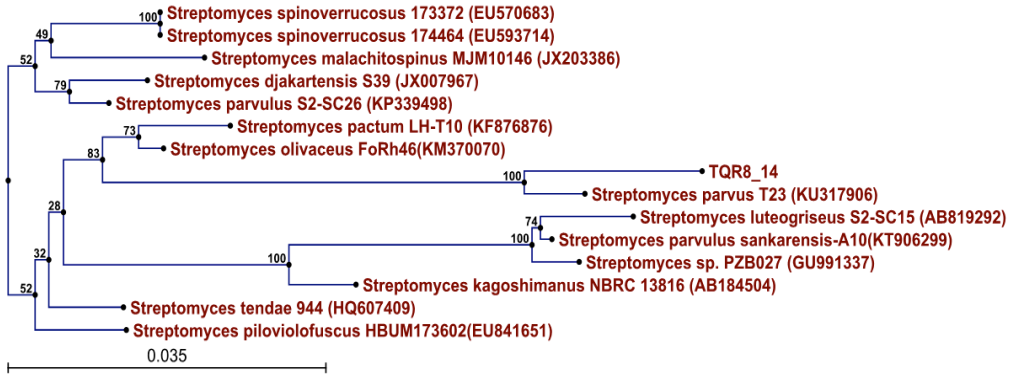


Figure 3. Neighbor-joining tree showing the phylogenetic relationships based on analysis of the 16S rRNA gene sequences of the isolate TQR8-14 and closest species.

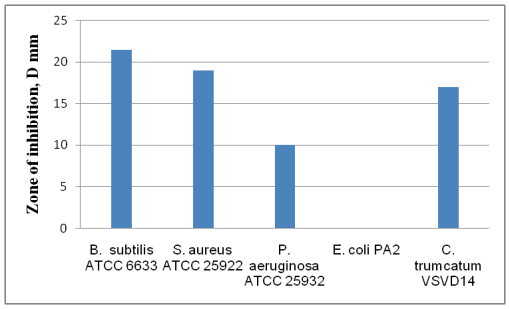


Figure 4. Antimicrobial activity of *S. parvus* TQR8-14.

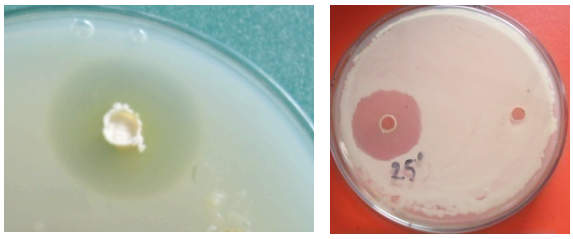


Figure 5. Inhibition of *B. subtilis* by *S. parvus* TQR8-14. (A) agar block method; (B) agar well diffusion method.

In shake flask cultures, isolate TQR8-14 could grow at temperatures from 15 to 40°C and pH of 4 to 10. However, the most favorable temperatures were 25 to 30°C and pH 6.0 to 8.0. The isolae tolerated up to 3% salinity (Table 3). A strong enzyme system enabled it to degrade chitin, protein, starch, carboxymethyl cellulose and xylan (Fig. 2). The strain utilized carbon sources, such as D-glucose, L-arabinose, D-xylose, D-manitol, D-fructose, D-cellulose, but did not use D-raffinose and sucrose (Table 2).

Based on the study of biological and physiological characteristics, using the Bergey's Taxonomical manual and ISP Protocols, isolate TQR8-14 was placed in phylum *Actinobacteria*, class *Actinobacteria*, order *Actinomycetales*, family Streptomycetaceae, genus *Streptomyces*.

As the results of sequence analysis of 16S rDNA nucleotide, the obtained sequence (1,404 bp) from isolate TQR8-14 was registered at the NCBI GenBank database under accession number KX712241. The sequence was compared with

relative sequences available in GenBank database, showing high similarity with that of *Streptomyces parvus* T23 (KU317906) and *Streptomyces parvus* MJM 10108 (KT906299) (Fig. 3).

In conclusion, the endophytic isolate TQR8-14 can be named as *Streptomyces parvus* TQR8-14.

Table 3. Cultural characteristics of actinomycete TQR8-14. ***Note:*** *"-" not growth, "+"* Poor, *"++"* Moderate, *"+++" Good.*

Temperature (°C)	Growth
10	-
20	++
25	+++
30	+++
37	++
40	+
45	-
pH	***Growth***
3	-
4	+
5	+
6	++
7	+++
8	++
9	+
10	+
11	-
NaCl (%)	***Growth***
0	+++
1	+++
2	+++
3	++
5	-
7	-

Production of antimicrobial substance(s)

The antimicrobial activity of *S. parvus* TQR8-14 was briefly evaluated in cultures on medium ISP 2. Four bacteria and a fungus were used as test (indicator) strains. The highest activity was detected against two Gram-positive bacteria, i.e. *B. subtilis* ATCC 6633 (Fig. 5), *S. aureus* ATCC 25922, with inhibition zone of 21.5, 19 mm diameter, respectively. The inhibition against *P. aeruginosa* ATCC 25932 was rather weak (10 mm). Gram-negative *E. coli* PA2 was not inhibited by our strain. In the case of pathogenic fungus *C. trumcatum* VSVD14 (causing anthracnose of citrus plants), the inhibition zone was 17 mm diameter (Fig. 4).

Effects of medium composition and cultivation conditions on antibiotic production by *S. parvus* TQR8-14

The growth and production of antibiotics is strongly affected by medium compositions and cultivation conditions. In this work, seven media, specifically Gauze I, Gauze II, ISP2, ISP4, SCA, AH4, 79, were used to study the growth of *S. parvus* TQR8-14 and antibacterial production. Also, the effects of cultivation temperature and pH were studied.

Media ISP4, AH4, SCA and 79 were most effective for growth of *S. parvus* TQR8-14. Concerning the antimicrobial activity, except medium Gause I that gave fair results, other seven test media were suitable for production of antimicrobial substance. The best results were obtained in cultures on medium AH4 (Table 4), where the inhibition zone against *B. subtilis* ATCC 6633 and *S. aureus* ATCC 25922 was of 26 mm and 24.5 mm diameter, respectively.

In general, actinomycetes are highly sensitive to the changes of cultivation temperature, especially regarding the production of secondary metabolites. The most favourable temperatures for *S. parvus* TQR8-14 were from 25 to 30^{o}C, however, in order to study the effect of temperature on the antimicrobial activity, *S. parvus* TQR8-14 was cultivated at temperatures of 25, 30, 35 and 37^{o}C. The highest activity was achieved at 30^{o}C, at which the inhibition zone against *B. subtilis* ATCC 6633 and *S. aureus* ATCC 25922 was of 27 mm and 26 mm diameter, respectively (Fig. 6). Higher cultivation temperatures did not favour the antibiotic production, as the activity at 37^{o}C was about 30% lower.

Strain *S. parvus* TQR8-14 exhibited remarkably high antimicrobial activity at the medium pH of 6 to 8.5. However, the maximum activity was observed at pH 7.0, at which the clear zone of *B. subtilis* ATCC 6633 and *S. aureus* ATCC 25922 was of 31.3 mm and 29.5 mm diameter, respectively (Table 5). In conclusion, the medium pH of 6.5 and 7.0 are most favourable for *S. parvus* TQR8-14.

Table 4. Antimicrobial activity of *S. parvus* TQR8-14 on different media.

Medium	Zone of inhibition (D, mm)	
	***B. subtilis* ATCC 6633**	***S. aureus* ATCC 25922**
Gause I	18 ± 0,50	17 ± 1,53
Gause II	24 ± 0,68	23 ± 1,04
ISP2	21,5 ± 0,58	21 ± 1,00
ISP4	22 ± 0,50	21,5 ± 1,00
AH4	**26** ± 0,79	**24,5** ± 1,00
SCA	24 ± 0,46	23 ± 0,76
79	23 ± 0,79	22 ± 1,04

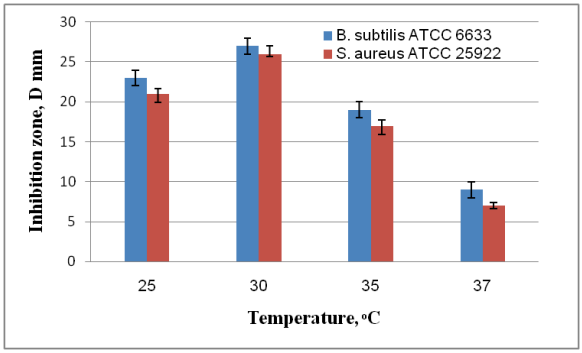


Figure 6. Effect of cultivation temperature on antimicrobial activity of *S. parvus* TQR8-14.

Table 5. Effect of medium pH on antimicrobial activity of *S. parvus* TQR8-14.

pH	***Inhibition zone*, (D, mm)**	
	***B. subtilis* ATCC 6633**	***S. aureus* ATCC 25922**
6	24.50 ± 0,71	23.00 ± 0,35
6,5	27.00 ± 0,35	26.25 ± 1,06
7	**31.25** ± 0,71	**29.50** ± 0,49
7,5	25.75 ±1,41	24.75 ± 0,49
8	25.25 ± 0,35	24.00 ± 0,85
8,5	25.50 ± 0,71	23.50 ± 0,28

In nature, species of *Streptomyces parvus* can be isolated from different habitats, such as marine environment (Abd-Elnaby *et al.,* 2016), soil (Rao *et al.,* 2013), or as plant endophytes (Gholamia *et al.,* 2014). Recent publications reported the high application potential of *S. parvus* in production of bioactive compounds, e.g. cholesterol oxidase (Praveen *et al.,* 2011), L-asparaginase (El-Naggar, Noura, 2015), arylomycins (Rao *et al.,* 2013), and substances for cancer treatment (Abd-Elnaby *et al.,* 2016) and biocontrol. Our findings confirmed the value and promise of this endophytic streptomycete in the production of antimicrobial substance(s), and possibly, other active compounds as well.

Traditionaly, ISP2 is one of the most frequently used media to study the biological characteristics of actinomycetes. Moreover, this medium is often used in screening of the actinomycetes producing antimicrobial substances (Badji *et al.,* 2006; Sharon *et al.,* 2014). Besides, media containing soybean meal and soluble starch are commonly favourable (Yan *et al.,* 2010). Regarding the medium pH, different authors reported that high antibiotic activity is often achieved at neutral to basic pH (6.5 to 8.0), with most favourable values of 7.0-7.5. The most suitable temperatures vary within 28-37^{o}C depending on the specific strain (Thakur *et al.,* 2009; Attimarad *et al.,* 2010). In this paper, it was found that the most favourable cultivation conditions of the endophytic strain *S. parvus* TQR8-14 include: medium AH4 containing soybean meal and glucose, pH 7.0, temperature 30^{o}C, rotary shake speed 150 rpm, and cultivation time 120 hours. The established conditions enabled the strain to express highest antimicrobial activity.

CONCLUSIONS

The endophytic actinomycete TQR8-14 was isolated from the roots of Ham Yen orange, an elite citrus cultivar of Tuyen Quang province. This isolate strongly inhibited pathogenic bacteria, such as *B. subtilis, S. aureus*, and *P. aeruginosa* and fungus *C. trumcatum* causing anthracnose of citrus plants. Based on the biological and taxonomical study, this

isolate was placed in genus *Streptomyces* of family *Streptomycetaceae*. Sequence analysis of 16S rDNA gene of streptomycete TQR8-14 revealed 99% similarity to *Streptomyces parvus*, therefore it was named as *Streptomyces parvus* TQR8-14. In shake flask cultivation using medium AH4 at pH 7.0 and temperature 30^{o}C, *S. parvus* TQR8-14 achieved highest antimicrobial activity after 120 hours. Further study will be focused on production and recovery of the antimicrobial substance, its chemical structure and aspects of application.

Acknowledgement: *This work is financially supported by Vietnam Academy of Science and Technology to the Project "Studies on diversity of endophytic streptomycetes isolated from elite citrus cultivars of the Northern Vietnam and their potentials in biosynthesis of antimicrobial and plant growth regulator compounds" (VAST.DLT.12/15-16). The National Key Laboratory of Gene Technology, Institute of Biotechnology, provides facilities for this study.*

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NGHIÊN CỨU ĐẶC ĐIỂM SINH HỌC CỦA XẠ KHUẨN NỘI SINH *STREPTOMYCES* TQR8-14 VÀ TIỀM NĂNG SINH TỔNG HỢP CHẤT KHÁNG KHUẨN

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Viện Công nghệ sinh học, Viện Hàn lâm Khoa học và Công nghệ Việt Nam

TÓM TẮT

Xạ khuẩn nội sinh cư ngụ trong mô sống của thực vật nhưng không gây bệnh cho cây chủ. Ngược lại, chúng được đánh giá là có tiềm năng trong kiểm soát sinh học, kích thích tăng trưởng thực vật, kích hoạt hệ thống miễn dịch của cây chủ. Ngày nay nhóm xạ khuẩn này được quan tâm nghiên cứu nhằm thu nhận các chất có hoạt tính sinh học mới sử dụng cho sản xuất nông nghiệp và Y-Dược. Một số nghiên cứu gần đây cho thấy tỷ lệ phát hiện ra các chất kháng khuẩn mới và loài xạ khuẩn mới từ các thể nội sinh cao hơn so với xạ khuẩn phân lập từ đất và bề mặt thực vật. Trong bài báo này, các chủng xạ khuẩn nội sinh được phân lập từ cây cam Hàm Yên (Tuyên Quang) và được kiểm tra hoạt tính kháng khuẩn đối với vi khuẩn Gram-dương *Staphylococcus aureus* ATCC 25922, *Bacillus subtilis* ATCC 6633 và nấm gây bệnh trên thực vật *Colletrichum trumcatum*. Trong số đó, chủng TQR8-14 thể hiện hoạt tính mạnh nhất đã được nghiên cứu về đặc điểm sinh học, phân loại và điều kiện sinh chất kháng khuẩn. Xạ khuẩn TQR8-14 được xếp vào nhóm vàng, khuẩn ty khí sinh có màu trắng sữa đến vàng nhạt, khuẩn ty cơ chất có màu vàng trên hầu hết các môi trường kiểm tra. Chủng TQR8-14 sinh ra các chuỗi bào tử dài dạng thẳng, bào tử hình trụ có bề mặt nhẵn. Chủng TQR8-14 có thể sinh trưởng ở khoảng nhiệt độ 15-45^{o}C, pH từ 4-10, và chịu được nồng độ muối đến 3%; có khả năng phân hủy một số cơ chất như carboxy methyl cellulose, tinh bột, protein và chitin. Gen 16S rDNA (1.404 bp) của chủng TQR8-14 có độ tương đồng 99% với loài *Streptomyces parvus* nên được đặt tên là *S. parvus* TQR8-14. Chủng TQR8-14 sinh chất kháng khuẩn cao nhất trên môi trường AH4 có thành phần chính là bột đậu tương và glucose, ở pH 7,0 và nhiệt độ 30^{o}C.

Từ khóa: *Xạ khuẩn nội sinh; cây cam; phân loại xạ khuẩn; 16S rDNA, Streptomyces parvus*