ISOLATION AND IDENTIFICATION OF *BRENNERIA NIGRIFLUENS* AS CAUSAL AGENT OF BARK CANKER DISEASE ON WALNUT IN IRAQ

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ABSTRACT
Bark canker disease has recently found in Sulaimani province. Disease incidence reached to 17.2% in Tawella. Bacterial isolates were identified by their morphological and biochemical characteristics using the API 20E system. Fifty-one and half percent of the isolates were identified as *Brenneria nigrifluens* and 36.3% as *Pantoea spp*. *B. nigrifluens* isolates formed circular colonies with entire margins and creamy color on nutrient agar. Biochemical tests classify *B. nigrifluens* isolates into seven groups. Vitek GN system further used to confirm the identification. The isolates produced necrotic lesion of different size on artificial inoculated walnut branches, but no symptoms on detached leaves. Isolates 22, 28 and 31 induced typical symptoms on two years old seedlings. *B. nigrifluens* successfully re-isolated from the inoculated seedlings. All *B. nigrifluens* isolates were resistant to erythromycin and cephalexin, most of them (16/17) were resistant to ampicillin, (13/17) resistant to vancomycin, (12/17) resistant to rifampin and amikacin; while (10/17) were moderately resistant to penicillin; (7/17) were moderately susceptible to streptomycin and (6/17) to gentamicin. Chloramphenicol, tobramycin, and tetracycline showed high efficiency in bacterial growth inhibition. Minimum inhibitory and minimum bactericidal concentrations of five chemicals against 17 bacterial isolates showed high efficiency of Kocide in killing 94.1% and inhibiting of 100% of the isolates at 0.31g/L *In vitro*, followed by Nordox which kill 70.6% and inhibit 76.4% of the isolates at 0.65 and 0.32 g/L respectively. *In vivo* studies confirm high efficacy of Kocide in disease control and restriction of vertical and horizontal extension of the cankers followed by Nordox.

Key words: *Juglans regia*, Bark kanker, *Brenneria nigrifluens*, Bacterial diseases.

مجلة العلوم الزراعية العراقية -2019: 50(3):814-826 **المعروف وآمين**

عزل وتشخيص البكتريا *Brenneria nigrifluens* كمسبب لمرض تقرح سيقان وذبول اشجار الجوز في العراق

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المستخلص:
تم ظهور مرض تقرح سيقان الجوزفي محافظه السليمانيه وبلغت نسبه الاصابه 17.2% في منطقه تويله.عزلت العديد من العزلات البكتيريه وشخصت اعتماداً على الصفات المظهرية والكيموحيوية وقابليتها على النمو في بعض الاوساط الانتخابية والتفريقية و نظام الـ API 20E .. تم تشخيص البكتريا *Brenneria nigrifluens* في 51.5 % من المجموع الكلي للعزلات، والبكتريا *Pantoea spp* في 36. 3 % من العزلات، تميزت عزلات البكتريا *B. nigrifluens* بنموها على الوسط الغذائي (NA) على شكل مستعمرات فردية دائرية ذات حواف متكاملة ولون حليبى ابيض ، بناءاً على الصفات الكيموحيوية تم تقسيم العزلات الى سبع مجاميع. استخدم جهاز Vitek2 GN لتأكيد تشخيص العزلات البكترية. اظهرت نتائج اختبار القدرة المرضية للعزلات البكترية، وجود اختلافات كبيره بينها في انتاج البقع النخريه على اغصان الجوز الملوثه اصطناعيا بينما لم يتم الحصول على اي نتائج موجبة بأستخدام الاوراق. اظهرت العزلات 22، 28 و 31 اعراض نموذجيه للتقرح على شتلات بعمر سنتين. أبدت جميع عزلات البكتريا B. nigrifluens مقاومة مطلقة للمضادات الحيويه Cephalexin و Erythromycin ومعظمها (17\16) ذات مقاومة عالية للمضاد الحيوى Ampicillin و (17\13) منها مقاومه للـ Vancomycin و (17\12) للـ Rifampin و للـ Amikacin ، في حين اظهرت (17\10) منها مقاومة متوسطة للمضاد الحيوي Penicilli ، و (7 \ 17) منها حساسيـة متوسطـة للStreptomycin و (17\6)، منها gentamicin بينما أبدت المضادات الحياتية Tetracycline،Chloramphenicol و Tobramycin كفاءة عالية في تثبيط نمو جميع العزلات البكترية بصورة مطلقة. اسفرت نتائج اختبار اقل تركيز مثبط للبكتريا (MIC) واقل تركيز قاتل للبكتريـا (MBC) لخمس مبيدات كيمياويه باستخدام 17 عزلة من البكتريا *B. nigrifluens* كفاءه عالية للمبيد Kocide نتيجة لقتل 94.1% وتثبيط 100% من العزلات في تركيز 0.3 غم/لتر ، يليه المبيد Nordox super75 الذي قتل حوالي 70.5% وثبط 76.4% من العزلات في التراكيز 0.65 و 0.32 غم/لتر على التوالي. أكدت نتائج الدراسات داخل الجسم الحي الكفأءه العالية للمبيد Kocide في مقاومة المرض وتحديد أنتشاره التقرحات افقياً وعمودياً ، يليه المبيد Nordox .

كلمات مفتاحيه: امراض بكتيريه، تقرح الساق *Juglans regia*, *Brenneria nigrifluens*

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INTRODUCTION

Walnut (*Juglans regia*) is a traditional important tree, wide distributed in Iraqi Kurdistan mountains. It belongs to Juglanaceae, a family of 6 genera and 50 species. Kurdistan region produce 2500t of walnut nuts (13). Many biotic and abiotic stresses affect walnut production. The absence of specific measures and new technologies in disease management increase the risk of the diseases in the region. Symptoms similar to those caused by bark canker disease have been observed in Tawella in the last few years. Bacterial canker was first reported in California on Persian walnut in 1957. In Europe, the disease was first reported in Spain in 1994 on adult Persian walnut trees (26). The pathogen was also observed on Persian walnut trees in Mandanzarian, Iran (21). During the period 1994–2007, outbreaks of shallow bark canker disease of walnut incited by *B. nigrifluens* have been recorded in Veneto, Piedmont region, north and central Italy (27, 34 40). The first report of the disease in France was in 2004 (32). During 2001-02, high disease incidence was detected in south Iran (49). In addition to North America, both *B. nigrifluens* and *B. rubrifaciens* have been reported as a causal agent of the disease across Europe (19 and 29). Disease importance was studied in Sacrameno Valley on 3680 trees within 27 orchards during 1968 to 1972. In 1968, only 1.7% of the trees showed disease symptoms, while disease incidence increased to 4% in 1969, 9% in 1970 and 11.6% in 1971respectively. Disease incidence reached up to 70% in some San Joaquin orchards (15). Occurrence and widespread of the disease in Italy, confirms *B. nigrifluens* among the resident microflora that infect the plant under stress conditions (35). The optimum temperature for *B. nigrifluens* growth is 27-30ºC, while the minimum and maximum temperatures are 4ºC and 40ºC respectively. The causal agent was described under the name of *Erwinia nigrifluens* (47). Later on it was re-described as *B. nigrifluens* and the recent phylogenic reconstruction based on 16S rDNA sequence analysis confirmed that (22). *Brenneria* is closely related to *Pectobacterium*, *Erwinia*, *Pantoea*, and *Enterobacter.* As it is very difficult to differentiate them phenotypically, genomic methods are recommended for differentiation (6). According to the former studies, *Erwinia* species are grouped into three clusters, and then placed in *Erwinia, Brenneria* and *Pectobacterium*, and the fourth cluster is *Pantoea* (16). The study was aimed to identify the causal agent of walnut decline, estimate disease incidence at the region and control the disease

MATERIALS AND METHODS

Eextensive survey was conducted during June-September 2009 to determine the incidence and distribution of walnut bark canker disease in 11 walnut orchards in Tawella, Sulaimani. Canker size, number, type on walnut bark was recorded and the external bark removed by sterilize knife. Cankers dimension, rate of extension to the inner bark and the brown necrotic area's were mapped. Disease incidence and severity was measured according to (27). Thirty-three samples of walnut branches and trunks with typical symptoms were collected. Samples were cut in to 5×5mm pieces, surface sterilized by 1% NaOCl for five min, then placed in Potato Dextrose Agar (PDA) plates and incubated at 25-27ºC, for seven days (44). Other samples were cut into 5×5 cm sections and washed for 30 min by running tap water. Small pieces from the margin of necrotic and healthy tissues were aseptically excised, disinfected for 2min in 0.5 %NaOCl, rinsed with sterile distilled water and placed in test tubes with 10mL Sterile Distilled Water (SDW) and left to soak for 12-18h. After vigorous shaking of the test tube, 1-2 drop of the suspension was streaked on the nutrient agar (NA) (34). Bacterial cultures were maintained on NA slants and kept in refrigerator at 4ºC as stock culture. The long-term storage was conducted by transferring two single colonies of the bacteria to 1ml nutrient broth (NB) test tube (15 % glycerol), well shaken and stored at 20ºC (3)

Identification of the isolates

Identification of the bacterial isolates was based on colonies morphology; pigment production, fermentation and gram stain tests (6, 30 and 39). Biochemical tests were performed for further identification of *B. nigrifluens* isolates .The tests include solubility of the bacterial cells in KOH (38), oxidase and

catalase test (28), Indole production test (25), methyl red test (6), Citrate utilization test (10), Urea activity test (10) ,Kligler iron-agar (14) , Motility test (23), and Aerobicc test (38). API 20E analytic system was also used to confirm the identification and typing of 29 bacterial isolates from walnut tissues (2, 33 and 34). Isolates 1, 18, 22, 24, 28 and 31 (Table 2) were separately used for comparing with the biochemical identification obtained by Vitek2 GN according to Pincus, 2006 (37).

Pathogenicity tests

Uniform healthy walnut leaves and branch pieces (7-9cm) were placed in petri-plates contains two layers of sterilized filter papers wetted with SDW. Bacterial inoculum was prepared by growing the isolates on NA at 28ºC for 24h, suspended in SDW and equivalents to McFarland 0.5 standard then adjusted to10^8 cfu ml^1, the leaves were punctured by sterile needle, and artificially inoculated by depositing 0.5ml of the bacterial suspension. The branch surface was disinfected with 0.5 %NaOcl and inoculated by placing 0.5ml of the bacterial suspension in one cm long wound in the branches. The holes covered with Para film to avoid contamination and keep moisture. SDW was used in control treatment. Plates were incubated at room temperature for until symptoms appeared (1). Tobacco leaves also wounded and artificially inoculated with *B. nigrifluens* isolates and left in the greenhouse until appearance of necrotic lesion symptoms (4). Pathogenicity test for the highest virulent isolates were conducted in Naqshbandi nursery in Tawella. Two-year old seedlings were artificially inoculated by placing 0.5mL of the bacterial suspension in one cm long wound in the stem. The wounds were covered by parafilm to avoid contamination and keep the moisture. SW used for control treatment (34). Data were collected after 60-90 days using 0-4 scale described by (5).

Antimicrobial susceptibility test by disc diffusion method.

The test was performed according to (28), Single colony of the bacterial isolates inoculum transferred to test tube contains 5ml of NB and incubated at 28ºC for 24h. A sterile cotton swab dipped in the inoculum and swabbed across Muller-Hinton agar plate surface, the antimicrobial discs applied within 15 minutes of inoculation. The plates were inverted and incubated at 28ºC for 18h. Inhibition zone diameter was measured using calipers and the isolates were interpreted as susceptible, intermediate or resistant to particular antimicrobial by comparing with standard inhibition zone.

Physiochemical analyses

The tests were included effect of sodium chloride (NaCl concentrations 0, 2.5, 5, 7.5 and 10), pH (pH 5, 6, 7, 8 and 9) and temperatures (4 and 40ºC) on *B. nigrifluens* growth (18).

Chemical control

This experiment was conducted to define efficiency of five chemicals (Kocide ($CuSO_4$), Champion, (Copper hydroxide), Nordox super75 (Cu_2O), Melody-R (Copper Csclour) and Coure (Molecular Copper) against the causal agent of walnut bark canker disease both *in vivo* and *in vitro.*

Determination of MIC and MBC

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of all the chemicals were measured against *B. nigrifluens* in the lab by using broth-diluting method (46), Seven fold dilutions of each bactericide (field dose, 1⁄2, 1⁄4, 1⁄8, 1⁄16, 1⁄32 and 1⁄64 field dos) were prepared, The field doses were equal to 2.5g/L in Kocide; 1.3g/L in Nordox super 75 and Champion; 20 mL/L in Coure and 2g/L in Melody-R. Each tube examine for presence/absence of turbidity in the bacterial growth to recoded the MIC. MBC used to determine the bactericidal or bacteriostatic activity of each bactericide by transferring a loop full of the MIC test tubes and streak on NA. After incubation time for 24h at 28ºC, if no bacterial growth appears at the used concentration, it is good indication for the MBC for the bacterial isolates, which is a lowest concentration of antimicrobial agent that had bactericidal activity. Data were recorded in the charts, which indicate the MIC and MBC of the tested chemicals to bacteria isolates. Data analyzed using SAS. Results are expressed as mean ± standard error. LSD determined statistical differences for multiple comparisons after analysis of variance (ANOVA).

Field experiments

Fifty-four walnut trees characterized with typical symptoms of bark canker disease were selected from six orchards in Tawella .The trees were homogeneous in size, shape and age. All the cankered tissues shaved and surgically removed by sterilized knife with 95 %ethanol ,all the trunks were treated with a mixture of recommended dose of Kocide (2.5g/L), Nordox super75 (1.3g/L), Champion (1.3g/L), Coure (20mL/L) and Melody-R (2g/L) with emulsified in water at a ratio of 1:1. SDW used for control treatment. The lower and upper parts of the entire diseased trunks were brushed completely with the mixture. All the treated trees were tagged with particular labels indicating the details of the treatment. Treated plants were monitored and any development of new canker areas from the original ones was recorded. Throughout a year, the infected areas were measured; also rate of canker extension to the inner bark was recorded. The experiment carried out using RCBD with three replications; each replicate contains three trees as experimental units.

RESULTS AND DISCUSSION

Disease Survey

Extensive survey results revealed that the overall mean of bark canker disease incidence and severity were 17.2% and 1.4 respectively in 11 walnut orchards in Sulaimani (table1). Out of 759 walnut trees, 131 trees showed bleeding, cracking and bark discoloration associated with canker symptoms, which represented 17.2% of the total number of the trees. The high disease incidence accompanied with low severity in Mam Idres orchard may be a good indication for the new introduction and well distribution of the disease in the orchards. Walnut orchards in Tawella characterized with chronic and debilitating decrease in yield and tree stands, lead to great fluctuations in walnut production from recent years. The disease caused progressive losses vigor, foliage reduction, and early leaf senescence. Yellowing and lagging of the leaves on the upper branch are usually noticed. The typical symptoms usually appear on mature trees characterize by development and localization the trunks; main branches and rarely extended to the scaffold branches of the old trees. On young trees, canker symptoms appear as external dark brown oval area about 3-4 cm long. Removal of the outer bark exposes the dark brown to blacken streak tissue of varying width, which develops vertically, and extend to the inner bark and occasionally reach the cambium. The infected areas exuded dark color watery sap, which stain the affected inner bark. This type of symptom was mentioned formerly by (40). Bark canker symptoms are variable in depth and show necrosis of the affected tissues and copious exudation usually appears on bark surface of the trunks. Other kind of symptoms appeared as longitudinal lesions on the trunk of infected trees. The lesions normally produce dark reddish, often slimy exudates. The exudates abundantly produced in summer, run down on the surface of bark and die, leaving discolor deposits. By removing the bark surface, irregular longitudinal canker observed as brown to necrotic area. The variable size of lesions on the trunks and main branches of Persian walnut are similar in appearance and location to those of shallow-bark canker caused by *E. nigriflues* which was first described by Wilson (47) in California. Result of direct isolation of the pathogens from 33 infected bark tissue samples shows no fungal growth on PDA, except for some saprophytic or contamination fungi. All the fungal pathogen showed negative results in pathogenicity tests on walnut. Streaking of 33 infected sample suspensions on NA plates resulted in detection of 29 bacterial isolates. The bacterial colonies were creamy in color and circular with entire margins. Biochemical characterizations shows that all the bacterial isolates are gram negative with positive reaction to catalase and negative to oxidase.

Table 1. Disease severity and incidence of bark canker on walnut at different locations during 2009 in Tawella, Sulaimani, IKR, Iraq.

No.	Owner Name	Location	Disease Severity	Disease Incidence %	Isolate No.
1	Government	Naqshbendi Nursery	0.9	12.5	6, 12, 30
2	Fakhraddin Mustafa	Qaqwlee	0.8	5.7	10, 17
3	Dyari Tawfiq	Aweser	1.1	5.5	16, 24, 27
4	Sulaiman Osman	Gryiat	1.9	14	8, 11. 31
5	Isa Jaafer	Sarcan	0.8	23	4,15, 19
6	Anwar Abdulkhaliq	Qaqulee	1.0	13.2	9, 20, 26
7	Mam Idris Karim	Dowleban	0.6	40	13, 25
8	Ahmad Sulaiman	Bach geria	1.5	12	5,18, 29
9	Ayub Ebdulqadir	Qaqwlee	3.0	3.5	14, 28
10	Nasraddin AbdulWali	Deray Zawer	3.2	37.7	22, 33
11	Yunis Abudulkhaleq	Khawer gryiat	0.7	22.8	1, 7, 23
	Mean	-	1.4	17.2	

Necrotic test

Artificial inoculation revealed, none of the bacterial isolates showed symptoms after leaf inoculation. Out of 29 bacterial isolates, 12 isolates were pathogenic on walnut branches, which represent 41% of the tested isolates. Isolate 10, 22, 28 and 31 are highly virulent, they produce more necrosis and exudates on the branch segments in short time, While isolate 16 and 24 were less virulent and isolate 6, 7, 9, 15, 18 and 25 were pathogenic only (Table 1). Analytical profile index of API 20E system Identification results of API 20E system reveal various biochemical properties of the 29 bacterial isolates. Isolate 5, 6, 7, 9, 10, 11, 15, 16, 17, 22, 25, 26, 27, 28, 29, 30 and 31 generate the 7-digit code number 0005773, which is specific code for identification of *B. nigrifluens* as mentioned earlier by (34) when they use the type strain LMG2694, 5107-5953 of *B. nigrifluens*. The code is also similar to identification of the type strain NCPPB 564 and reference strain NCPPB 564 of *B. nigrifluens* by using the same system (33). While strain number 1, 4, 8, 12, 13, 14, 18, 19, 20, 23 and 33 generate the 7-digite code number 0005520, which is refer to *Pantoea spp* possibility "*Erwinia spp*" at accuracy of 97.7% according to API 20E system. Isolate 24 generate the 7-digite code numbers 0007520, which is not known by the system (Table 1).

Physiological and biochemical characteristics of *Brenneria nigrifluens*

Biochemical characteristics of *B. nigrifluens* are similar to those obtained by API20E system. All *B. nigrifluens* isolates are gram negative at smear preparation; rod shaped, forms in single, pairs or short chain. All the isolates colonies are circular, dark purple with green metallic sheen, not mucous, with entire margins on Eosin Methyl Blue (EMB) Figure1a. Colonies diameter reach to 2-4mm after 4 days. The green metallic sheen appearance of the isolates on EMB are used as criteria by many scientists for identification and distinguishing of *B. nigrifluens* from other phytopathogenic bacteria before PCR identification (39, 49). EMB containing lactose and glycerol is the best medium for identification of *B. nigrifluens* isolates because the colonies will show stable green metallic sheen. Production of green metallic sheen depends on pH value around the colonies as a result of acid production from glycerol (40). All *B. nigrifluens* isolates shows white and circular entire margin colonies on Yeast Dextrose Calcium Carbonate Agar (YDCA), Figure 1b. Culturing of *B. nigrifluens* isolates on MaCconky agar (MA) explored forming of circular colonies with entire margin, smooth, small in size, pink in color. MA contains lactose and pH indicator, which allows differentiating between lactose fermenters (red colonies) and non-fermenters (white-pale pink colonies)(46). Biochemical and physiological characteristics test results show that all *B. nigrifluens* isolates has positive reaction to solubility in KOH 3%, glucose fermentation, motility, anaerobic fermentation and methyl red reaction except Isolate 15 and 11 which showed negative and weak reaction to methyl

red respectively, while all the isolates had negative reaction to produce tryptophanase and citrase, pigments on NA and YDCA, acid from lactose, kligler H_2S, hypersensitive reaction on tobacco and gas production from lactose except Isolate 17 for the last trait. All the isolates show red alkaline and acid alkaline on kligler iron agar slope and bottom respectively. Urease production was negative to the isolates except 9, 15 and 19 that were positive and isolate 10 and 28 showed weak reaction. The isolates tolerance to NaCl was positive at 5% and negative at 7.5% except isolate 17. Growth of all the isolates was negative at 4C and positive at 40C except isolate 15 that show weak reaction. All the isolates grown at pH 9 while isolate 6, 7, 22, 25 and 31 showed negative growth reaction and isolate 5 weak growths at pH5.

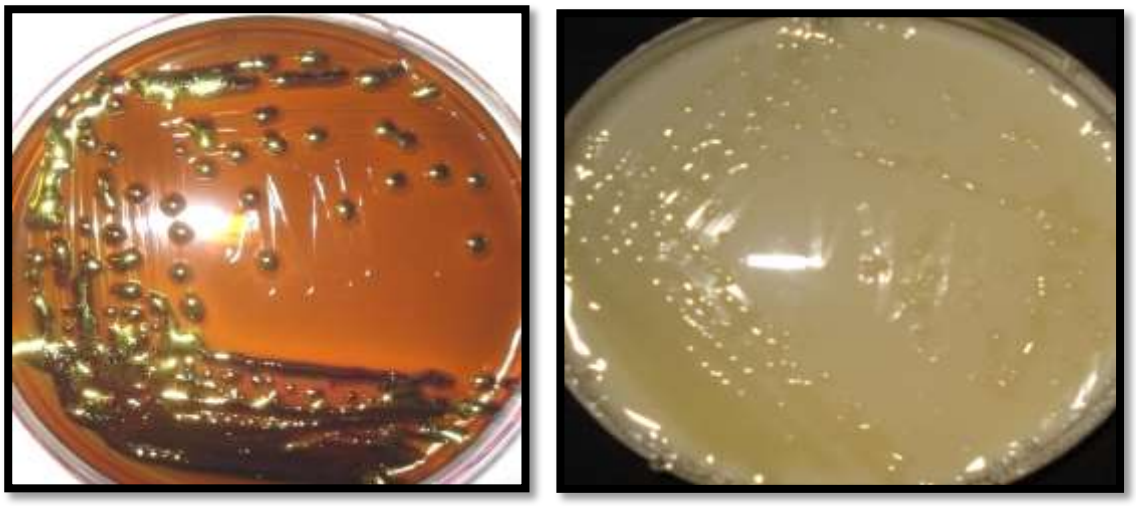
Pathogenicity tests

Among the virulent and pathogenic bacterial isolates used in artificial inoculation of walnut seedlings (table 2), *B. nigrifluens* isolate 22, 28 and 31 are only able to produce disease symptoms, While isolate10 from *B. nigrifluens* and 18 and 24 from *Pantoea* spp fail to produce any symptom. No any significant differences are detected between the treatments and control. *B. nigrifluens* successfully re-isolated from the symptoms appeared on all bark tissue of the inoculated seedling, while the bacteria was re-isolated only from 10% of the symptomless bark tissues (Mainly *Pantoae* sp). All the re-isolated bacteria show the 7-digit code number 0005773 by using API 20E system similar to the original bacterial isolates used in artificial inoculation. Pathogenicity test results of walnut seedlings in the field recognize *B. nigrifluens* as a causal agent of bark canker disease in walnut. Disease symptoms appears as small necrotic areas on the bark of walnut trunks above and below the inoculation points after 2-3 months in the field, by removing barks of inoculated seedlings, brown necrotic areas (0.5-2cm) in diameter appears. Analytical profile index test of Biomerieux Vitek GN Results of Vitek GN identification system shows that the bacterial isolate 22, 28 and 31 generate the Bio-number profile 4441151012501020, which is unidentified by the system (Chart1). While isolate 1 and 18 generate the Bio-number profile 4605510652520010 which refer to *Pantoea* spp and isolate 24 generate the Bio-number organism 4601510652520010 which also refer *to Pantoea spp*. The system could not identify isolate 22, 28, and 31 because the procedure has been developed for rapid identification of clinical Enterobacteriaceae. So the bio-number profiles 4441151012501020 can be used as identification code for *B. nigrifluens*, since it has been identified by different system and biochemical test and show similar data to this system. The chart papers also presents results of an extensive biochemical and enzymatic characteristics of six bacterial isolates from walnut bark canker tissue, Result of physiological and biochemical characteristics obtained by other scientists using Vitek system are similar and confirm our identification results (6, 34). The system also provide some other physiological and biochemical characteristics of *B. nigrifluens* which are not been reviewed by other scientists before (Chart 1).

Table 2. Artificial inoculation of walnut seedlings with different Bacterial isolates at Naqshbendee nursery

No.	Treatment	Disease severity*
1	Control	0.0
2	B. nigrifluens No. 10	0.0
3	B. nigrifluens No. 22	2.1
4	B. nigrifluens No. 28	1.6
5	B. nigrifluens No. 31	0.6
6	Pantoea spp No. 18	0.0
7	Pantoea spp No. 24	0.0
	L.S.D P≤ 0.05	0.37

*** Each number represents the mean of disease severity of three replicates**



A B

Fig1.Morphology of *B. nigrifluens* isolates on EMB(a) and YDCA(b) after two days of inoculation

bioMerieux Customer ADT — Laboratory Report — April 25, 2011, 08:42

System#: — Printed by Labadmin

Isolate Grou; 5s-1 — Bench: Ahmed

Bionumber: 4441151012501020

Selected Organism: Unidentified Organism

Comments:	

Identification Information:	Card: GN	Lot Number: 241125240	Expires: May 19, 2011 13:00 ADT
	Completed: Apr 24, 2011 16:50 ADT	Status: Final	Analysis Time: 5:75 h
Selected Organism:	Unidentified Organism Bionumber: 4441151012501020		
SRF Organism:			
Analysis Organisms and Tests to Separate:			
Analysis Massages:			
Contraindicating Typical Biopattern(s)			

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	+	5	IARL	-	7	dCEL	-	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	+	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	+	18	dMAL	-	19	dMAN	-	20	dMNE	+	21	BXYL	-	22	BAlap	+
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	-
33	SAC	+	34	dTAG	-	35	dTRE	-	36	CIT	-	37	MNT	+	39	5KG	-
40	ILATK	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	+	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	-	57	BGUR	-
58	O129R	-	59	GGAA	+	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Installed VITEK 2 Systems Version:04.01

MIC Interptetation Guideline: — Therapeutic Interpretation Guideline

AES Parameter Set Name: — AES Parameter Last Modified

Chart 1. Identification information, biochemical and enzymatic details of the bacterial Isolate Nos. 22, 28 and 31 isolated from walnut tissues in Sulaimani, Iraq

Antimicrobial susceptibility of *B. nigrifluens*

Antimicrobial susceptibility test result of *B. nigrifluens* isolates revealed different susceptibility reactions of the isolates to the tested antimicrobial (table 3). All the tested isolates were resistant to erythromycin and cephalothen, and 16/17 to ampicillin, while all the isolates were highly susceptible to tetracycline, tobramycin and chloramphenicol. The isolates demonstrate various reactions to vancomycin, amikacin, rifampin, penicillin, streptomycin and gentamicin. Results also revealed sensitivity of *B. nigrifluens* isolates to most of the tested antimicrobial agents, which are formerly confirmed by other scientist except cephalothen, ampicillin, streptomycin, gentamycin which showed different reactions (6).

Table 3. Antimicrobial susceptibility of *B. nigrifluens* isolates

No	Antimicrobial	No. of resistant Isolates	Ratio
1	Erythromycin	17	17/17
2	Cephalothen	17	17/17
3	Ampicillin	16	16/17
4	Vancomycin	13	13/17
5	Amikacin	12	12/17
6	Rifampin	12	12/17
7	Penicillin	10	10/17
8	Streptomycin	7	7/17
9	Gentamicin	6	6/17
10	Tetracycline	0	0/17
11	Tobramycin	0	0/17
12	Chloramphenicol	0	0/17

Chemical control

Results of different bactericidal concentration effect against *B. nigrifluens* isolates exhibit differences in the MIC and MBC activities after incubation, with difficulties to standardize between MIC and MBC, which are usually very similar. The results were previously confirmed by (38). Some antibacterial has high bactericidal effect. In this case, the MIC and MBC are usually similar (Table 4). In general, number of bacteria cells decrease with the increase of bactericide concentrations in the media. It is quite clear that Kocide completely inhibits100% and kill 94.1% of *B. nigrifluens* isolates at 1/8 field dose (0.31mg/ml), followed by Nordox super75 which inhibit 76.4% and kill 70.6% of the isolates at 1/4 field dose (0.32mg/ml) and 1/2 field dose (0.65mg/ml) respectively, while Champion inhibit 70.4% and kill 100% of the isolates at 1/2 field dose (0.65mg/ml), whereas efficiency of Melody-R occur at 1mg/ml (1/2 field dose) resulting in MIC and MBC of 82.3% and 100% respectively in the isolates, Coure chemical inhibit and kill 88.2% of *B. nigrifluens* isolates in half and complete field dose respectively.

Field experiments

Efficiency of five bactericides to control bark canker disease on walnut trees in Tawella orchards is illustrated in table 5. Results revealed that all the treatments significantly surpass the control in restriction both vertical and horizontal extension of the canker. Kocide completely inhibit the vertical and horizontal extension of the treated cankers and significantly surpass all other treatments in disease control followed by Nordox super 75, which are significantly, decrease 94% and 62% of vertical and horizontal canker extension compared with the control. No significant differences are detected among Champion, Courey and Melody-R in restriction of canker extension. They successfully decrease vertical and horizontal canker expansion between 20–55% and 40–50% respectively and significantly surpass the control.

Table 4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of different bactericides against *B. nigrifluens* isolates *in vitro*.

Treatments	Mean of MIC				Mean of MBC			
	1	1/2	1/4	1/8	1	1/2	1/4	1/8
Kocide							5.8%	94.1 %
Nordox super75		23.6 %	76.4 %			70.6 %	29.4 %	
Champion		70.6 %	29.4 %			100%		
Melody-R		82.3 %	17.7 %			100%		
Coure		88.2 %	11.8 %		88.2 %	11.8 %		
Positive control								
Negative control								

***Green cells= 100% bacterial growth inhibition; Red cells= bacterial growth is 100%.**

*** Field doses of the chemicals are 2.5g/L in Kocide; 1.3g/L in Nordox super 75 and Champion; 20 mL/Lin Coure and 2g/L in Melody-R**

Table 5. Effects of some chemicals in bark canker disease control of walnut trees in Tawella, Kurdistan region, Iraq after one year

No.	Treatment	Canker expansion and extension 'mm'	
		Horizontally	Vertically
1	Kocide	0.0	0.00
2	Nordox super75	111.1	0.12
3	Champion	141.11	0.88
4	Courey	148.8	1.25
5	Melody_ R	171.1	1.55
6	Control	286.6	1.95
L.S.D (P≤ 0.05)		29.76	0.37

Disease monitoring in walnut orchards shows that all the observed symptoms of bark canker disease in Tawella are similar and agree with previous scientific findings (27, 34 and 49) and disagree with (31) who report other canker type called deep bark canker (Phloem canker) caused by *B. rubrifaciens*, which produce dark brown pits on the sapwood. Detection of the symptoms on few young walnut trees disagrees with (40) who detect severe infection on young trees. Many scientists describe *Brennera sp* as causal agent of bark canker disease on different trees (5, 6). The disease is mostly active and rapidly increases at high temperature in summer. Schaad and Wilson (41) mention to the rapid increase of canker size during summer, extension rate depend on temperature rise from spring to summer. The bacteria may remain viable in the dried exudates for 123 days. Observing of large amounts of exudates associated with bark cankers particularly in spring and releasing of abundant sap in summer contains millions of bacteria cells are similar to (24). Detection of high disease incidence in Tawella orchards is hazardous since the disease will properly disseminate and increase with time to obstacle walnut production. On the other hand frequent surgical remove of bark tissues from trunk can't eradicate the disease, that's why the local farmers name the disease 'Saratan' (cancer). Kado (24) reported that bark canker diseases symptoms may appear only at infection site but with time will develop elsewhere on the tree. The study result agrees with Gardner (15) who confirm systemic movement of the causal agent and remain latent in other parts of the infected trees. Therefore, surgical removal of visually infected canker area will effectively reduce bacteria population in the orchards. The high disease severity and incidence in Deray Zawer orchards (37.7% and 3.2 respectively) may attribute to first detection of the disease in this area, which is close to Iranian border. The source of water used for walnut orchards irrigation in Deray Zawer comes directly from

Iran and flow to other orchards. So we believe that these orchards may be a source of inoculum to other orchards. Many scientists referred to the widespread occurrence and distribution of bark canker disease in Iran (21, 39, 49). Charkhabi (8) isolated 24 different strains of *B. nigrifluens* from Persian walnut in five locations of Iran. From this review we can conclude that bark canker disease probably transmit by water flow from Iran to establish the disease in walnut orchards in Iraq. Furthermore, irrigation type used in walnut orchards in Tawella is suitable for the pathogen dissemination form infected trees to healthy ones. This conclusion strongly agrees with other scientists, who confirm water stress as a key factor in eliciting disease symptoms appearance (43). Primarily water, animals, and human carry out plant pathogenic bacteria from one plant to another or to other parts of the same plant. Even the bacteria possess flagella to move easily for short distance by active transport. The random distribution of commercial walnut seedlings introduced from Iran in Tawella orchards directly play important role in distribution of the disease. Morphological characteristics of *B. nigrifluens* isolates on YDCA agree with Yousefikopaei (49), who confirmed appearance of *B. nigrifluens* isolates as white colonies surrounded by a clear zone indicating acid production with dissolution of the calcium carbonate. Colony characterization of the bacteria isolates on YDCA also illustrates and detects all the isolates that generate the 7-digite code number 0007553 as *B. nigrifluens*. YDCA as selective media resolve *B. rubrifaciens* from all close related *Brenneria sp* (30), Production of red pigment by *B. rubrifaciens* on YDCA is the key of phenotypic feature which distinguish *B. rubrifaciens* from other related walnut pathogens (31), this is very important due to the similarities between canker symptoms incited by *B. rubrifaciens* and *B. nigrifluens* (32). According to the biochemical characteristics and pathogenicity of *B. nigrifluens* isolates on branches we can group the virulent isolates in two groups, G1 include isolate 10 and 28 which have similar biochemical reaction and differs from isolate 22 and 31 (G2) in Urease production and growth at pH 5. The pathogenic isolates can be classified in three groups, G3 include isolate 6, 7 and 25 that explore the same biochemical reaction and differs from isolate 9 and 16 (G4) in Urease production and from isolate 15(G5) in Methyl red reaction, while the main differences between isolate 9 and16 (G4) and isolate 15 (G5) is in Methyl red reaction. Biochemical characteristics of the non-pathogenic isolates recognize them in two groups. G6 include isolate 5, 11, 26, 27, 29, and 30 and G7 include isolate 17. The main differences between them are in tolerance to NaCl at 7.5% and production of gas in glucose. Out of four *B. nigrifluens* isolates used in artificial inoculation of the seedlings, three isolates were only virulent and able to develop canker symptoms on trunks of walnut seedlings. The detected symptoms are similar to the natural infection symptoms; small area of necrotic tissue appears in the inner bark. Our results strongly agree with other scientists who use wounds made in bark of walnut stem for artificial inoculation of *B. nigrifluens*, they also found that the external canker appears after three months (34). Difficulties in reproducing external cankers have been frequently reported by artificial inoculating of woody plants with pathogenic bacteria including *B. nigrifluens* (19). Success of reproducing external cankers may depends on the physiological status of the host tissues at inoculation time and the prevalent environmental condition thereafter (49). Seedlings inoculated with *Pantoea spp*, and sterile water not shows any visible symptoms. Our results are supported by (34) who found that all *Pantoea* and *Pectobacterum spp* isolates were a virulent to expose bark canker symptoms on walnut seedlings. However, *P. herbicola* identified as secondary parasites and some *Pantoea* species are frequently isolated from the environment and found as epiphytes on a range of host plants. *P. anantis* was recovered from 25 asymptomatic weed species and crop plants (17). Furthermore, several *Pantoea* species are known as plant pathogens, *P. ananatis* causes a variety of disease on a wide host range including bacterial blight and dieback of Eucalyptus (11) and brown stalk rot of maize (20). The wide distribution of *B. nigrifluens* as a causal agent of walnut decline

in many parts of the world like Iraq, Iran, Italy, France, Spain, USA and other places, needs further systemic investigation, therefore using the rapid protocol of Vitek GN system for identification of the bacterial isolates is very essential since the system can rapidly and reliably identify *B. nigrifluens*. So we suggest the system as new protocol for quick identification of the bacteria.The absolute resistance of all *B. nigrifluens* isolates to erythromycin may attribute to alteration of ribosomal protein. Erythromycin produced by *S. eyrthreus*, contains large lactone ring linked through glycoside bonds with amino sugars to inhibit protein synthesis (42). Resistance of *B. nigrifluens* isolates to penicillin and cephalothin may be due to production of some enzymes like penicillenase and cephalothinase, which is common in Enterobacteriaceae (36). Resistance of Enterobacteriaceae isolates to cephalosporin group is mostly relate to one gene locate on plasmid, which produce extend-spectrum of B-lactamase (12). Aminoglycoside group used in this study like, gentamycin, amikacin, tobramycin and streptomycin, demonstrate great variation in resistance levels of the isolates to each antimicrobial agent. Aminoglycoside is one active bactericide group produced by *Streptomyces spp*, it inhibit the connected 30s RNA protein synthesis therefore the bacteria can't use it (9). All *B. nigrifluens* isolates are absolutely sensitive to tetracycline and chloramphenicol. Tetracycline produce by *S. aurofaciens* block protein synthesis on the ribosomes (9). Chloramphenicol produce by *S. venezuelae* inhibits the translation during protein synthesis while rifampin produced by *S. mediterrnei* inhibits the transcription in eubacteria RNA polymerase (9).Chemical control results of bark canker disease strongly agree with (48). Application of Bordeaux mixture and copper compounds gives effective control of the pathogen in the exudates. Copper sulphate is the active ingredient of kocide; the role of copper sulphate pent-hydrate gave interesting result since copper concentration is 10 times less than other copper compounds (9g $hl^{-1,}$ as opposed to 90g hl^{-1} of copper oxychloride). This is very important since it compliance with the new European union regulation in restriction of copper compounds in organic crops (45). By comparing application strategies of the latter studies to our study, we conclude that application of copper may successfully contribute in reducing efficacy of walnut bark canker disease in Iraq. On the other hand, kocide have been used successfully to control walnut bacteria blight incited by *X. campestris* pv *juglandis* in California, Kocide application reduce disease incidence from 36.7% in control treatment to 5.4 and 9.6% in kocide 3000 and kocide 2000 respectively. Disease reduction accompanied with yield increase from 0.89 t/Ac in 2005 to 2.64 t/Ac in 2006 as compared to the last six-year average in the same location. This indicates that even under high conducive environment as in spring 2006, effective control measures can be obtained even after high disease incidence in the previous season (7)

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