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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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ARTICLES

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Full Length Research Paper

Ciliate ectoparasites (Ciliophora: Trichodinidae/Chilodonellidae) on gills of *Pelteobagrus fluvidraco* from the Yangtze River, China, with the description of *Trichodina fluvidraco* sp. n

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This study aimed at investigating the parasitic ciliates on commercial fishes in Luzhou, Sichuan Province, China. Several ciliates were collected from gills of *Pelteobagrus fluvidraco*. Three trichodinids and one chilodonellid were collected from the gills of the *Pelteobagrus fluvidraco*. These are *Trichodina fluvidraco* sp. n., *Tripartiella dactylodentata, Tripartieila nana* and *Chilodonella hexasticha*. Descriptions presented here were obtained by examinations of specimens prepared using the dry silver impregnation and Wright-Giemsa staining. For the new species comparisons with closely related species are provided.

Key words: Trichodinid, chilodonellid, *Trichodina fluvidraco* sp. n., *Tripartiella dactylodentata*, *T. nana*, *Chilodonella hexasticha*, *Pelteobagrus fluvidraco.*

INTRODUCTION

Pelteobagrus fluvidraco (Chordata: Pisces: Bagridae) is an important commercial fish in China. Some trichodinids have been found on *P. fluvidraco* in China such as *T. domerguei* (Wallengren, 1897) (*T.domerguei* f.latispina Dogiel, 1940, *T.domerguei* f.sinensis Chen et Hsieh, 1964) (Chen, 1973), *T.nobilis* Chen, 1963 (Chen, 1973), *T.nigra* Lom,1960 (Chen, 1984), *T. oviformis* Chen,1955 (Chen, 1984), and *Trichodinella myakkae* Raabe,1950 (Chen, 1984). However, trichodinids on *P. fluvidraco* have not been reported in other parts of the world. No.

The present paper deals with three trichodinids (Ciliophora: Trichodinidae) and one chilodonellid (Ciliophora: Chilodonellidae). One of the trichodinids belongs to the genus *Trichodina* Ehrenberg, 1838, and the other two belong to the genus *Tripartiella* Lom, 1959; the chilodonellid belongs to the genus *Chilodonella* Strand, 1926. They are *Trichodina fluvidraco* sp. n., *Tripartiella dactylodentata*, *T.nana* and *Chilodonella hexasticha*, respectively. This study aimed to extend our knowledge on the geographic distributions and diversity of these ciliates.

Descriptions presented here were obtained by

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examinations of specimens prepared using the dry silver impregnation and Wright-Giemsa staining. Modern methods are used for descriptions of these ciliates.

MATERIALS AND METHODS

The host *P. fluvidraco* that was more than one year old was obtained during 2009-2013 from the Yangtze River at Luzhou, Sichuan, China (28°51N, 105°23'E), which is the longest rivers in China and one of the longest river in the world. Out of 50 *P. fluvidraco* collected, 20% were infected by *T. fluvidraco* sp. n., 6% by *Tripartiella dactylodentata* and *T. nana*, and 10% by *Chilodonella hexasticha*.

Gill scrapings were made from the hosts. Smears with ciliates were air–dried and then the slides with ciliates were impregnated with Klein's dry silver impregnation technique (Klein, 1958). The nuclear apparatus was shown using Wright-Giemsa staining. All photomicrographs and illustration drawings were made with the help of a camera (Motic DM–BA300–B, provided by Motic China Group CO., LTD.) at 1000 magnification with oil immersion lens and software Motic Images Advanced 3.2 and CorelDraw X4. The statistic analysis was conducted with Microsoft Excel 2010.

All measurements are presented in micrometers (µm). In each case minimum and maximum values are given, followed in parentheses by the arithmetic mean and standard deviation. In the case of the number of radial pins/denticle and denticles of the trichodinid, number of kineties of the chilodonellid, and the mode is given rather than the arithmetic mean with the number of specimens examined given in parentheses. The body diameter of trichodinid is measured as the adhesive disc plus the border membrane. The measurements of trchodinid follow the uniform specific characteristics proposed by Lom (1958) while the method proposed by Basson and Van As (2002) was followed for denticle description, as shown in Figures 1a and b. Y+2 -axis is added to the left side of Y+1 -axis for better describing denticles of some *Tripartiella*.

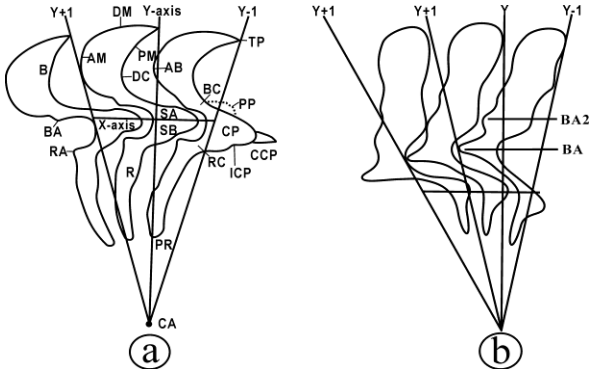

Figure 1. Schematic drawings of trichodinid denticle. **(a)**. *Trichodina* (after Basson and Van As 2002); **(b)**. *Tripartiella* AB, apex blade; AM, anterior blade margin; B, blade; BA, Blade apophysis; BA2, Second blade apophysis; BC, blade connecting; CA, central of adhesive disc; CCP, central conical part; CP, central part; DC, deepest point of curve; DM, distal blade margin; ICP, indentation in lower central part; PM, posterior blade margin; PP, posterior projection; PR, point of ray; R, ray; RA, ray apophysis; RC, ray connecting; SA, section of central part above X-Axis; SB, section of central part below X-Axis; TP, tangent point.

The position of the micronucleus of trichodinid is given relative to the arch-shaped macronucleus, according to the format described by Lom (1958), which was based on the system originally proposed by Dogiel (1940). In this system, the micronucleus is situated in one of three positions relative to the terminations of the arms of the macronucleus: (1) externally, near the right termination (+y); (2) externally, between the two terminations (–y); and (3) internally, near the right termination (–y1).

RESULTS AND DISCUSSION

Trichodina fluvidraco sp. n.

Taxonomic summary

Species: *Trichodina fluvidraco* sp. n.
Family: Trichodinidae Claus, 1874
Type Host: *Pelteobagrus fluvidraco* Richardson, 1846
Fish Family: Bagridae
Type Locality: Luzhou, Sichuan, China (28°51N, 105°23'E)
Location: Gills
Date of Sampling: 7/2012
Etymology: The specific epithet "fluvidraco" is coined from the name of host.

Reference material: Holotype, slide LZY109/2012, and paratype slides LZY112/2012, LZY113/2012 are deposited in the Biological Laboratory of Luzhou Vocational & Technical College.

Descriptions

Small-sized, freshwater trichodinid. Body diameter 25.9–38.8 (34.5±3.5). Adhesive disc 20.7–31.6 (27.9±3.1). Denticulate ring 12.8–20.9 (17.5±2.1). Border membrane 2.6–4.0 (3.5±0.4). Denticle number 19–23(22) (Figures 2 a, b; Table 1). Radial pins per denticle 8–9(8). Denticle span 6.4–10.7 (8.9±1.1). Denticle length 3.2–6.8 (5±0.8). Blade broad, filling most of space between Y -axes and with sharp tangent point. Distal blade margin straight, higher than tangent point. Blade length 2.7–2.9 (3.9±0.5). Anterior blade margin approximately parallel to curve of posterior blade margin, forming almost L-shape, apex of blade touches *Y*+1-axis, blade apophysis present and barely seen, blade connecting long. The central part well developed with rounded point fitting tightly into preceding denticle, extending towards more than 2/3 *Y*–1 -axis. The section of central part above *X* -axis was more than section of central part below *X* -axis in shape (Figure 3a). The central part width was 1.0–2.0 (1.6±0.3). The ray connecting very short. The ray apophys is present and barely seen. The ray directed towards backward. The point of the ray was sharp or rounded. Ray length was 1.4–3 (2.3±0.5). Adoral ciliary spiral makes a turn of

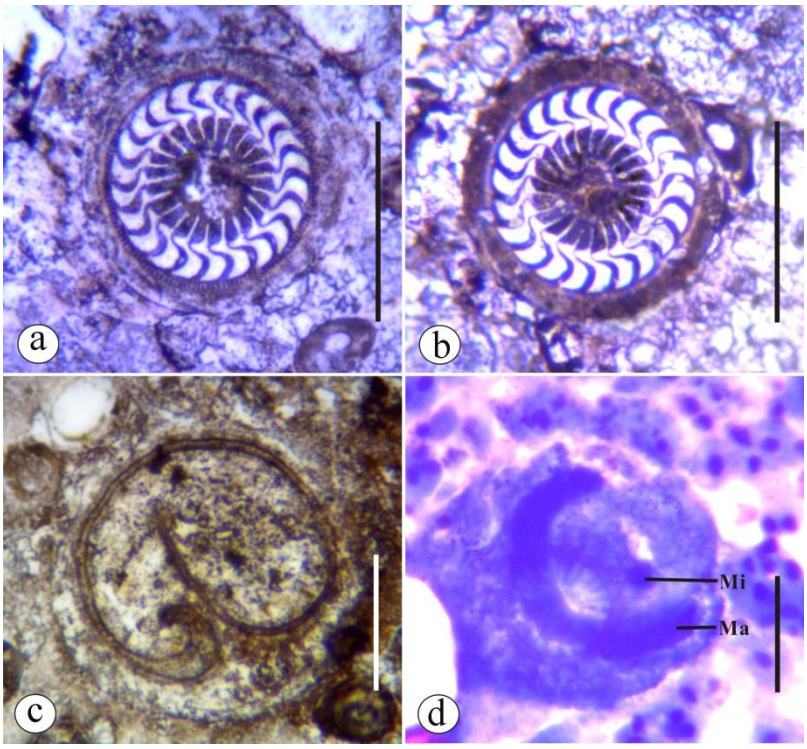

Figure 2. Photomicrographs of *Trichodina fluvidraco* sp. n. from Pelteobagrus fluvidraco, after dry silver impregnation (a–c) and Wright-Giemsa staining (6). **(a–b).** adhesive discs. **(c).** Adoral ciliary spiral. **(d).** Nuclear. MA, macronucleus; MI, micronucleus. (Scale bars = 20 µm)

Table 1. Morphometric comparison of *Trichodina fluvidraco, T.minuta* and *T.microdenticulata* (measurements in micrometers)

Trichodinid species	***T.fluvidraco* sp.n.**	***T.minuta***	***T.microdenticulata***
Host	*Pelteobagrus fluvidraco*	*Oreochromis mossambicus,Tilapia sparrmanii,Barbus trimaculatus*	*Dorosoma petenensc*
Locality	Luzhou,China	South Africa	America
Site	gills	Skin, fins ,gills	Body and gills.
References	Present study	Basson & Van As, 1983	Thomas and Wellborn, 1967
No. of specimens measured	40	23	–
Body diameter	25.9–38.8(34.5±3.5)	28.2–38.0(33.3±3.2)	22.0–37(26)
Adhesive disc	20.7–31.6(27.9±3.1)	22.4–33.7(27.2±3.0)	12.0–15.5(13)
Denticulate ring	12.8–20.9(17.5±2.1)	12.2–18.2(15.5±1.7)	6.0–8.0(6.9)
Border membrane	2.6–4.0(3.5±0.4)	2.1–4.1(3.3±0.3)	1.5–2.5(2)
Denticle number	19–23(22)	19–22(21)	15–18(16)
Radial pins / denticle	8–9(8)	6–7(6)	5
Denticle span	6.4–10.7(8.9±1.1)	–	–
Denticle length	3.2–6.8(5±0.8)	3.2–5.4(4.2±0.5)	2.0–3.0(2.6)
Blade length	2.7–2.9(3.9±0.5)	3.1–4.6(3.7±0.4)	2.0–3.0(2.6)
Central part width	1.0–2.0(1.6±0.3)	1.7–2.7(2.2±0.2)	0.5–1.0(0.9)
Ray length	1.4–3(2.3±0.5)	2.6–4.4(3.4±0.5)	1.5–2.0(1.9)
Macronucleus external diameter	33.0–34.3(33.5±0.5)	18.4–36.1(29.4±4.6)	13–22(17)
Macronucleus thickness	6.1–6.7(6.4±0.2)	3.8–7.6(5.1±0.2)	2.0–3.0(2.5)
Micronucleus diameter	3.7–4.8(4.4±0.4)	–	–
Adoral ciliary spiral	430–448(437.5±6.5)°	400–410°	330–350°

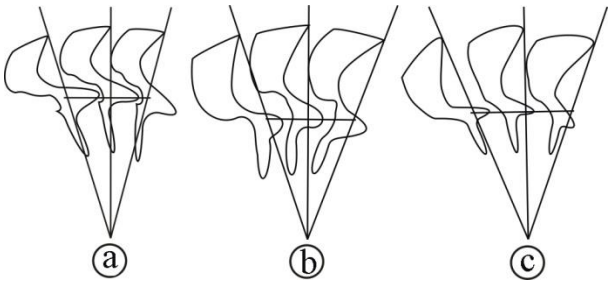

Figure 3. Diagrammatic drawings of the denticles of *Trichodina fluvidraco* sp. n., *T. minut* and *T. microdenticulata*. **(a)**. *Trichodina fluvidraco* sp. n.; **(b).** *T. minuta* (redrawn from Basson et al., 1983); **(c).** *T. microdenticulata* (redrawn from Thomas and Wellborn, 1967).

430–448 (437.5±6.5)° (Figure 2c; Table 1). Macronucleus was horseshoe-shaped, elongated and with characteristic dilations at both ends; external diameter was 33.0 – 34.3 (33.5 ± 0.5). Micronucleus was spherical; diameter, 3.7 – 4.8 (4.4 ± 0.4), situated in – y1 position (Figures 2d; Table 1).

Remarks

About five trichodinds have been found from *P. fluvidraco* so far, but *T. fluvidraco* sp. n. is obviously different from them. *T. fluvidraco* sp. n. only resembles *T. minuta* Basson and Van As, Paperna 1983 obtained from skin, fins of *Oreochromis mossambicus, Tilapia sparrmanii, Barbus trimaculatus* in South Africa, and *T. microdenticulata* Thomas and Wellborn, 1967 obtained from the body and gills of *Dorosoma petenensc* found in America.

T. fluvidraco sp. n. is different from *T. minuta* by the shape of denticle and some other measurements Figures 3a, b, Table 1). (1) It has more radial pins per denticle than *T. microdenticulata* (8 – 9 vs. 6–7). (2) The blade length is shorter in the new species than in *T. minuta* (2.7–2.9 vs. 3.1–4.6). (3) The blade connecting is thicker and longer in the new species than in *T. minuta*. (4) In the case of the new species the posterior tip of the central part extends towards more than 2/3 to *Y*–1 -axis, but in the case of *T. minuta* less than halfway to *Y*–1 -axis. (5) The blade apophysis is present in the new species but absent in *T. minuta*. (6) The ray apophysis is present in the new species but absent in *T. minuta*. (7) The ray directs backward in the new species, but forward in *T. minuta*. (8) The macromucleus external diameter and macronucleus thickness are different for both species (33.0–33.4 vs. 18.4–36.1; 6.1–6.7 vs. 3.8–7.6). (9) The adoral ciliary spiral is also quite different for both species (430–448 vs. 440–410).

T. fluvidraco sp. n. is clearly distinguished from *T. microdenticulata* too (Figures 3a, b; Table 1). (1) The connecting is longer in the new species than in *T. microdenticulata*. (2) In the case of the new species the posterior tip of the central part extends towards more than 2/3 to Y-1 -axis, but in the case of *T. microdenticulata* it towards just over Y -axis. (3) The blade apophysis is present in the new species but absent in *T. microdenticulata*. (4) The ray apophysis is present in the new species but absent in *T. microdenticulata*. (5) The ray directs backward in the new species, but parallels *Y*-axis and directs towards the center of adhesive disc in *T. microdenticulata*. (6) The morphometic data of the new species also varies when they are compared with those of *T. microdenticulata*, for instance, the adhesive disc (20.7 – 31.76 vs. 12.0 – 15.5), the denticulate ring (12.8 – 20.9 vs. 6.0 – 8.0), the denticle number (19 – 23 vs. 15–18), The central part width (1.0 – 2.0 vs. 0.5 – 1.0), the Macronucleus external diameter (33.0–34.3 vs. 13–22), the macronucleus thickness (6.1–6.7 vs. 2.0–3.0), the adoral ciliary spiral (430–448 vs. 330–350).

According to the comparison with the related species, *T. fluvidraco* is considered to be a new trichodinid species.

***Tripartiella dactylodentata* Al-Rasheid, Ali, Sakran, Baki and Ghaffar, 2000**

Descriptions

Small-sized freshwater trichodinid. Body diameter, 19.0–25.2(23.3±2.3). Adhesive disc, 17.1–2.6 (20.8±2.0). Central area not clear. Denticulate ring, 6.5–10.2(9.0±1.3). Border membrane, 1.0–1.6 (1.3±0.2). Denticle number 22–28 (24). Radial pins per denticle 5–9 (7). Denticle span, 7.3–8.1(7.6±0.3). Denticle length, 3.2–4.2 (3.8±0.4). Blade length, 3.7–5.4(4.9±0.7). Blade finger like, long and narrow, with two bulbous parts, sloping from tip downwards and slanting forwards. Distal blade margin round. Tangent point is relatively large, round and slightly lower than distal blade margin. Anterior and posterior blade margins convergent with no apex. Blade more than half the distance between the Y+1-axes. Blade and second blade apophysis present, blade apophysis conspicuous and abrupt. Posterior projection present. Central part concave, traverses the whole area between Y+2 and Y-axes and obliquely backwards. Denticles loosely connected to each other at the central part. Central part width, 1.2–2.3 (1.4±0.8). Ray connection indistinct. Ray straight, thin, tapering to a round end and parallel to *Y*-axes. Ray length, 1.1–2.0(1.5±0.3).

Adoral ciliary spiral makes a turn of 142.0–168.0 (153.2±9.8)° (Figure 4b; Table 2). Macronucleus horseshoe-shaped, elongated and with characteristic dilations at both ends; external diameter, 25.7–25.9 (25.8±0.1), Macronucleus thickness 3.7–4.4 (4.1±0.4).

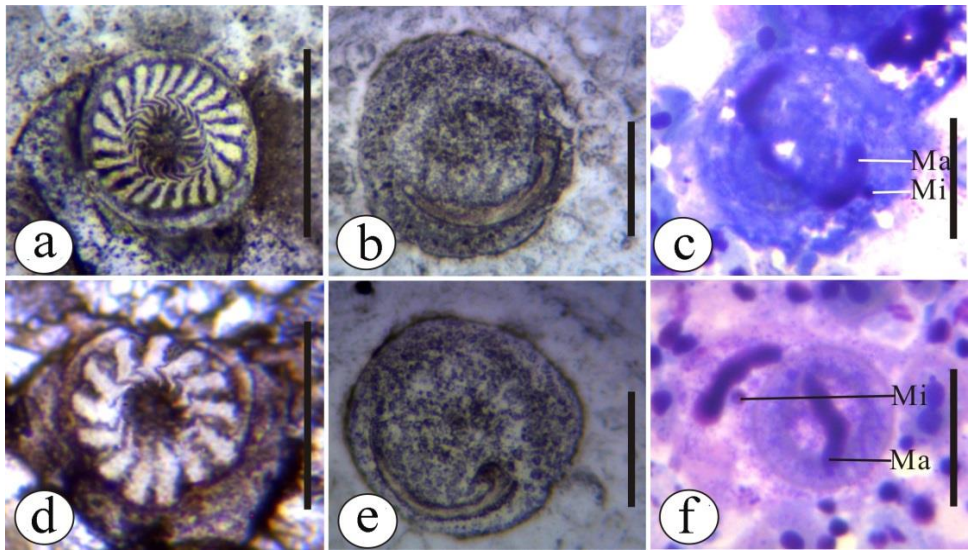

Figure 4. Photomicrographs of *Tripartiella dactylodentata* and *T. nana* from *Pelteobagrus fluvidraco*, after dry silver impregnation (a–b, c–d) and Wright-Giemsa staining (e, f). (Scale bars = 20 µm). **(a–c).** *Tripartiella dactylodentata*: **(a).** adhesive discs; **(b).** Adoral ciliary spiral; **(c).** Nuclear. Ma, macronucleus; Mi, micronucleus. **(e–f).** *Tripartiella nana*: **(d).** adhesive discs; **(e).** Adoral ciliary spiral; **(f).** Nuclear. MA, macronucleus; MI, micronucleus.

Table 2. Morphometric and meristic data of *Tripartiella dactylodentata* and *T.nana* (measurements in micrometers).

Trichodinid species	***T. dactylodentata***			***T. nana***	
Host	*Pelteobagrus fluvidraco*	*Mormyrus kannume* (large numbers)	*Schilbe mystus* (moderate numbers)	*Pelteobagrus fluvidraco*	*0reochromis mossambicus*
Locality	Luzhou,China	Egypt		Luzhou,China	Venda
Site	gills	gills		gills	gills.
References	Present study	*Al-Rasheid et al.*,2000		Present study	Basson & Van As, 1987
No. of specimens measured	20	–	–	20	50
Body diameter	19.0–25.2(23.3±2.3)	29.7–31.7(30.2±1.0)	23.0–27.8(25.1±1.8)	19.9–20.6(20.2±0.3)	16.8–26.1(20.6±2.4)
Adhesive disc	17.1–26(20.8±2.0)	22.7–27.7(24.8±2.1)	19.1–23.8(21.0±1.9)	16.2–18.7(17.2±1)	13.3–21.6(16.9±2.4)
Denticulate ring	6.5–10.2(9.0±1.3)	11.9–14.9(13.4±1.3)	8.2–10.7(9.6±1.2)	6.5–6.9(6.7±0.1)	6.7–10.8(8.7±1.0)
Border membrane	1.0–1.6(1.3±0.2)	2.5–3.9(3.7±0.7)	1.2–1.9(1.6±0.2)	1.0–1.9(1.5)	1.0–2.5(1.7±0.3)
Denticle number	22–28(24)	27–32(29)	27–32(29)	13–14(14)	16–19(17)

Table 2. Contd.

Radial pins / denticle	5–9(7)	7–9(8)	6–8(7)	4–6(5)	5 (5)
Denticle span	7.3–8.1(7.6±0.3)	8.9–9.9(9.3±0.7)	6.2–8.3(7.2±0.8)	5.2–6.5(5.7±0.4)	5.2–6.5(5.7±0.4)
Denticle length	3.2–4.2(3.8±0.4)	2.9–4.9(3.9±0.5)	2.4–3.2(2.8±0.5)	2.8–2.9(3.3±0.4)	2.2–3.6(3.0±0.3)
Blade length	3.7–5.4(4.9±0.7)	4.0–5.9(4.6±0.5)	3.2–5.0(3.6±0.3)	3.6–4.4(4.1±0.3)	1.7–3.3(2.4±0.4)
Central part width	1.2–2.3(1.4±0.8)	1.6–2.2(1.8±0.4)	1.3–1.5(1.4±0.1)	0.5–1.2(0.8±0.2)	0.8–1.5(1.1±0.2)
Ray length	1.1–2.0(1.5±0.3)	2.8–3.3(2.9±0.4)	2.0–2.9(2.4±0.3)	0.4–1.1(0.8±0.3)	0.6–1.6(1.0±0.2)
Macronucleus external diameter	25.7–25.9(25.8±0.1)	–	–	21.0–22.5(21.8±0.7)	14.3–24.7(19.4±2.6)
Macronucleus thickness	3.7–4.4(4.1±0.4)	–	–	3.2–4.5(3.9±0.7)	3.4–6.0(4.7±0.6)
Micronucleus diameter	2.0–2.3(2.1±0.2)	–	–	2.2–2.3(2.3±0.1)	2.7–13.2(6.6±2.6)
Adoral ciliary spiral	142.0–168.0(153.2±9.8)°	–	–	158.0–164.0(161.0±3.0)°	190–260°

Micronucleus spherical; diameter 2.0–2.3 (2.1±0.2), situated in + y position (Figure 4c; Table 2).

Remarks

In the present study, the denticle morphology and morphometric data of the trichodinid species from gills of *P. fluvidraco* is extremely close to *Tripartiella dactylodentata* (Figures 5a, b, Table 2). *T. dactylodentata* was originally described by Al-Rasheid *et al*. (2000), and the specimen were obtained from gills of *Mormyrus kannume* and *Schilbe mystus* in the year of 2000 in Egypt. A complete series of photo-micrographs, which includes the photo-micrographs of the adhesive disc, the adoral ciliary spiral and the nuclear (Figures 4a, b, c), is provided in this paper. *T. dactylodentata* is a new record in Asian and *P. fluvidraco* is a new record host for *T*. *dactylodentata*. The present study extends the geographic range and host range of *T*. *dactylodentata*.

Descriptions

Small-sized freshwater trichodinid. Body diameter, 19.9–20.6 (20.2±0.3). Adhesive disc, 16.2–18.7 (17.2±1). Denticulate ring, 6.5–6.9 (6.7±0.1). Border membrane, 1.0–1.9 (1.5). Denticle number, 13–14 (14). Radial pins per denticle, 4–6 (5). Denticle span, 5.2–6.5 (5.7±0.4). Denticle length, 2.8–2.9(3.3±0.4).

Blade broad, well developed, filling most of the space between *Y*-axis. Distal blade margin round. Tangent point blunt, lower than distal blade margin. Anterior blade margin curve gradual, blade and second blade apophysis present, blade apophysis rounded and well developed, fitting well into notch of preceding denticle. Posterior blade margin curve prominent. Posterior projection present and prominent. Blade length 3.6–4.4 (4.1±0.3). Central part delicate and sharply pointed. Central part width 0.5–1.2 (0.8±0.2). Ray short, needle-shaped, straight and directed slightly posteriorly. Ray length 0.4–1.1 (0.8±0.3). Adoral ciliary spiral makes a turn of 158.0–164.0 (161.0±3.0)° (Figure 4e; Table 2). Macronucleus horseshoe-shaped, elongated and with characteristic dilations at both ends; external diameter 21.0–22.5(21.8±0.7), Macronucleus thickness 3.2–4.5(3.9±0.7). Micronucleus spherical; diameter 2.2–2.3(2.3±0.1), situated in – y1 position (Figure 4f; Table 2).

Remarks

The denticle shape and morphometric data of the trichodinid in this paper are extremely close to *Tripartieila nana* reported by Basson and Van As (1989) (Figures 5c, d, Table 2). *T. nana* was originally described by Basson and Van As with the sample obtained from gills of *Oreochromis mossambius* in South Africa in 1987. After that it was reported by Asmat in India in 2002 and the specimen were collected from gills of *Boleophthalmus boddarti.* A complete series of photomicrographs, which includes the photo-micrographs of the adhesive disc, the adoral ciliary spiral and the nuclear (Figures 4d,

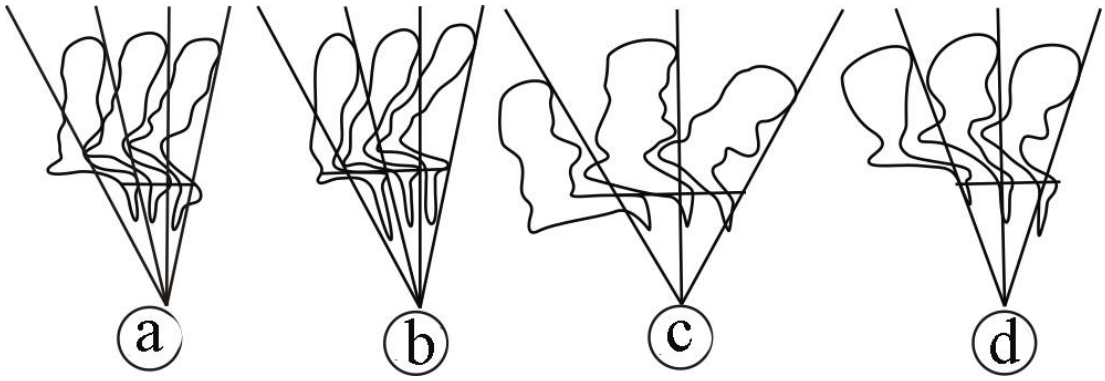

Figure 5. Diagrammatic drawings of the denticles of *Tripartiella dactylodentata* and *T. nana*. **(a).** *Tripartiella dactylodentata* from *Pelteobagrus fluvidraco*; **(b).** *T. dactylodentata* (redrawn from Al-Rasheid et al., 2000); **(c).** *T. nana* from *Pelteobagrus fluvidraco*; **(d).** *T. nana* (redrawn from Basson and Van As, 1987).

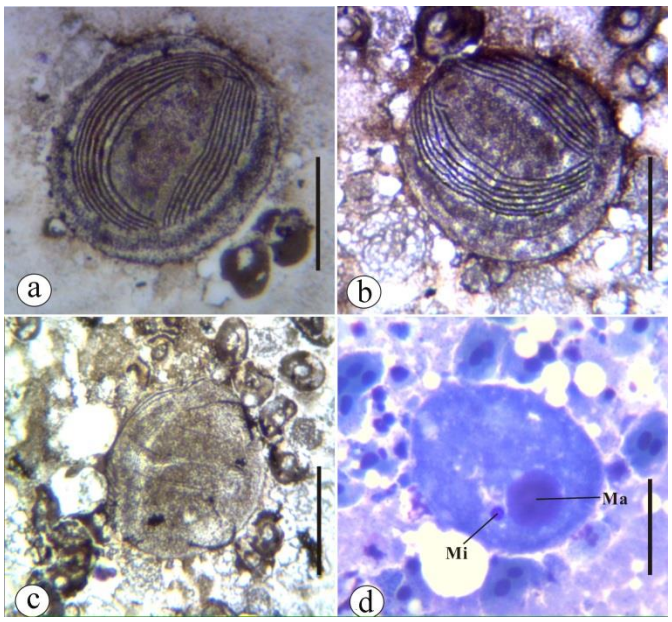

Figure 6. Photomicrographs of the silver nitrate. impregnated of *Chilodonella hexasticha* (Scale bars = 20 µm) after dry silver impregnation (a–c) and Wright-Giemsa staining (d). **(a-b)** ventral (oral) side; **(c)** dorsal side; **(d)** Nuclear. Ma, macronucleus; M.

e, f), is provided in this paper. *T. nana* described here is a new record in China and *P. fluvidraco* is a new record host. This extends its known geographic and host range.

Chilodonella hexasticha Kiernik, 1909

Descriptions

The chilodonellid body flattened at the ventral (oral) side, dorsal side rounded in shape. Body length, 26.0–44.6 (35.5±5.7) µm, width, 23.4–37.0 (28.8±4.7) µm. The ciliature of the ventral body side composed of a short preoral kinety and two systems of kineties. The left and right system of kineties conspicuously separated by a glabrous area. In the anterior part of this zone the oral opening. Kineties loosely arranged and the distances between them not equal. The right system consisting of 5–8 (6) kineties, 1–3 (2) postoral kineties. Two outermost rows beginning more anteriorly surrounding at front the kineties of the left system. The left system consisting of 8–9 (9) kineties. In the system short rows at the inner side in the posterior as well as outer side in the anterior part of the body. Others existing between the two kineties (Figures 6a, b, c).

Macronucleus large, spherical, diameter 9.6–12.0 (10.9±1.0) µm，Micronucleus small, spherical, diameter 2.8–3.4(3.1±0.3) (Figure 6d).

Remarks

C. hexasticha was originally described by Kiernik in 1909 and the specimens were collected from skin and gills of *Tinca tinca (L). Ch.hexasticha* has been reported from Former USSR, Germany, Poland, Czechoslovakia, USA and China (Kiernik 1909, Prost 1952, Kazubski & Migala 1974, Lom et al.1976, Wierzbicka 1997, Hu 2012), with samples obtained from various fish species including *Tincta tinca*, *Cyprinus carpio*, *Leuciscus idus*, *Carassius carussius*, *C. auratus, Giardinius januarius*, *and Gambusia affinis*. Photomicrographs of the ventral side as well as the dorsal side and the nuclear are provided in this paper (Figures 6a-d). *P. fluvidraco* is a new record host for *Ch.hexasticha*, therefore this chilodonellid's host range has been extended.

Conflict of interests

The author did not declare any conflict of interests.

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Full Length Research Paper

Microbiological quality of selected street-vended foods in Coimbatore, India

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Quality analysis of street-vended food enterprises and street food vendors from the Coimbatore city, Tamil Nadu, India was carried out. This research reveals that, handling and trading practices of street vendors are not hygienic enough to obtain a safe food. These street-vended foods are easily contaminated by food borne pathogens, due to the poor quality of water used and the poor sanitary procedures in preparation and storage of foods. Street-vended foods (10 items) sold by street vendors obtained from the locale were found to have microbial load representing *E. coli* and *Salmonella* sp. 70% of the food samples collected from street vendors have shown high bacterial load. Results have demonstrated the non-hygienic quality of most popular types of street vended foods. The pressing need as indicated by WHO is that, there must be measures imposed by the Food Safety Authorities, to follow proper control measures to improve microbial quality of the street vended foods.

Key words: Microbiological food quality, street vended foods, food safety indicators.

INTRODUCTION

In India, street vended foods are gaining overwhelming momentum; hence strong efforts with broader dimensions are needed, to explore the safety of street food establishments (Chadrasekhar et al., 2003). In recent years, street foods have become part of urban modern day life. They provide inexpensive, economic and often nutritious foods for urban and rural poor. They are the major source of income and opportunity for self-employment for a great number of people (Chadrasekhar et al., 2003). These foods satisfy the people, serving specially to the taste of the consumer, with little attention bestowed on hygiene, food safety or nutritional aspects (Bhat, 2004).

However among all other kinds of foods consumed by human being today, the street foods are exposed to abundant sources of contamination, as from unclean water, unhygeinic preparation and storage, space constraint, raw food, infected handlers and inadequately cleaned equipment etc. Hence, the chances of food borne illness associated with these street foods are increasing (WHO/FNU/FOS/96.7, 1997). WHO and Food safety organization's studies on street foods in developing countries have shown a high microbial count and are commonly contaminated with pathogens such as *E. coli, Salmonella, Shigella, Vibrio cholerae* and *Staphylococci* which are responsible for serious food poisoning outbreaks (Bryan et al., 1992). Hence the present study was planned to assess the microbiological

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quality and safety of selected street foods.

METHODOLOGY

Selection of the area and outlets

Five busy areas of Coimbatore city, Tamil Nadu, India, where the common street vended foods are available were selected for the study. The number of outlets in each area ranged from a minimum of five to a maximum of fifteen and the total number of outlets selected for the present study were fifty outlets.

Collection of samples of food items sold at street food outlets

Ten (10) different food items sold in the outlets were collected for the microbial analysis. Two samples each were collected from the selected outlets based on different methods of cooking and medium used (Table 1).

Table 1. List of food items selected for the study.

Food item	Method of cooking
Idli	Steaming
Idiyappam	Steaming
Bhelpuri	Ready mixing of raw ingredients
Panipuri	Ready mixing of raw ingredients
Bonda/Bhajji	Deep Frying
Fish fry	Deep Frying
Tandoor chicken	Sauteing and Deep frying
Chilly mushroom	Sauteing and Deep frying
Fried rice	Cereal based / starch food
Vegetable noodles	Cereal based / starch food

Microbiological examination of the selected food items

Being ubiquitous in distribution, microorganisms can gain entry into the food chain from various sources during different stages of their processing, storage and serving. Besides providing a suitable nutritional and physical environment for growth and multiplication of microorganisms, the food possesses the inherent capacity to sustain them in rare numbers. Representative samples of street foods were collected from the selected outlets in recyclable LDPE (Low-density polyethylene) covers. The samples were immediately taken to the laboratory. They were analyzed for the following criteria; all the analyses were carried out following FAO 1992 procedures (FAO, 1992; Tambekar et al., 2011).

Total plate count

The test was used to determine the total number of viable bacteria in street foods which is an indication of the sanitary conditions in which these foods were prepared and stored.

Identification and counting of coliform bacteria

The process of identification of the disease causing contaminant bacteria from foods and especially counting of coliforms was done for assessing the adequacy of sanitation of these street vended foods. Coliform counting becomes important as it indicates the contamination of food by disease causing bacteria from fecal contamination. Among the coliforms, *E. coli* is a valid index organism indicating faecal contamination of water that is being used for food preparations. This indicates inadequate processing and/or post process contamination due to cross contamination by raw materials, improper cleaning, and use of contaminated water for washing and preparation, use of dirty equipment or poor hygienic handling (Mbah et al., 2012).

Fungal count

The presence of yeasts and moulds is indicative of improper storage of food stuffs or long term storage of food stuffs. The presence of which indicates that food is unfit for consumption.

Salmonella count

This was used to determine the *Salmonella* group of bacteria which if present in food substances at levels of 10^5/g is highly suggestive of food poisoning to occur. *Salmonella* also enters through contaminated water for use in cooking, improper cleaning and unhygienic handling and storage practices in food items.

RESULTS AND DISCUSSION

Microbiological quality of street foods

Routine examination of foods for the complete range of pathogenic microorganisms is impractical. In order to assess the microbiological safety of food borne pathogens, indicator organisms are used which indicate the presence of pathogens of intestinal origin as a result of direct or indirect faecal contaminations and they are usually used to assess food hygiene (FAO, 1997; Chandi and Sonali Patra, 2012; WHO, 2002).

The identification criteria for indicator organisms have been done in four methods in the present study to determine the contaminating conditions to which the foodstuffs were exposed to during handling. (i) Total plate count, (ii) Enteric Indicator bacteria (iii) *E. coli and Salmonella* sp. and (iv) yeasts and molds were the tests done to identify the microbial quality of foods.

Table 2, Plates 1 and 2 showed the bacterial count of different street foods. The highest plate count for different products were as follows, chilly mushroom (8.4×10^5), fish fry (9.1×10^5), kuska rice (6.4×10^5). Average standard plate count is 4.0×10^5 cfu/gm. High counts in foods indicate contaminated raw materials and also indicate inappropriate time/temperature storage conditions.

The next order of microbiological contamination was found in vegetable noodles (2.3×10^5), Egg bonda (2.1×10^5), Idly (1.3×10^5), and Idiyappam (2.5×10^5). Standard plate count of these food samples were found to be less, presumably due to heat treatment (frying, steaming) during its preparation. Plates with different colonies on nutrient agar and EMB agar have been presented with

Table 2. Bacterial counts of different street foods

Food sample	Standard plate count cfu/g
Bhelpuri	1.4×10^5
Chilly Mushroom	8.4×10^5
Tandoori Chicken	4.8×10^5
Idiyappam	2.5×10^5
Idli	1.3×10^5
Panipuri	1.8×10^5
Fish Fry	9.1×10^5
Kuska Rice	6.4×10^5
Egg Bonda	2.1×10^5
Vegetable Noodles	2.3×10^5

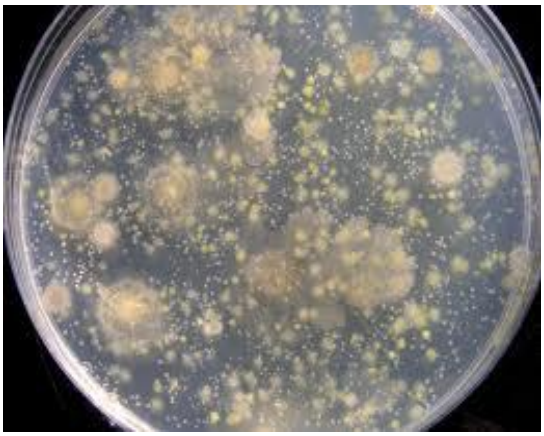

Plate I. High microbial counts in Tandoori chicken.

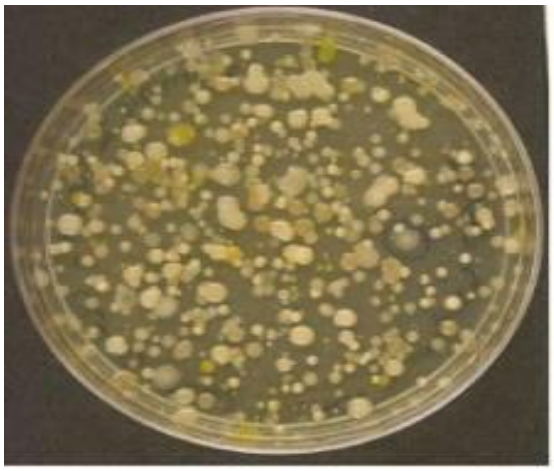

Plate II. High microbial counts in Kuska rice.

different pathogens found in street foods. Standard plate count has been done on street foods at Tirumala and similar results have been observed in a study done by Suneetha et al. (2011). The study by Suneetha et al.

Table 3. Testing for the presence of indicator organisms

Food sample	*E. coli*	*Salmonella* sp	Fungus
Bhelpuri	-	-	+
Chilly mushroom	-	-	-
Tandoori chicken	+	-	+
Idiyappam	-	-	-
Idli	-	-	-
Panipuri	-	-	+
Fish fry	+	-	-
Kuska rice	+	-	+
Egg Bonda	-	+	-
Vegetable noodles	-	-	-

Plate III. *E. coli* isolated from Tandoori chicken

(2011) stated that breakfast items and fruit juices among street foods were contaminated with Coliforms, *Bacillus cereus*, *Staphylococcus aureus* and Yeast / Moulds as indicated by standard plate count. Lack of awareness on requirement for basic safety issues by vendors add up the microbial loads. Unhygeinic practices such as use of rudimentary stands and carts, nonavailability of running water for dilution and washing, extended preservation without refrigeration, unhygienic ambiance with teeming flies and airborne dust (Lewis et al., 2006) result in severe contamination of food.

From Table 3, it can be interpreted that presence of *E. coli* which has been widely accepted as indicator of faecal contamination of water, denoted the possible presence of pathogens of enteric origin. However substantial numbers of *E. coli* in foods such as Tandoor chicken, Fish Fry and Kuska Rice (Plate III, IV and V) suggested lack of hygiene and cleanliness in handling and improper storage. While the presence of large numbers

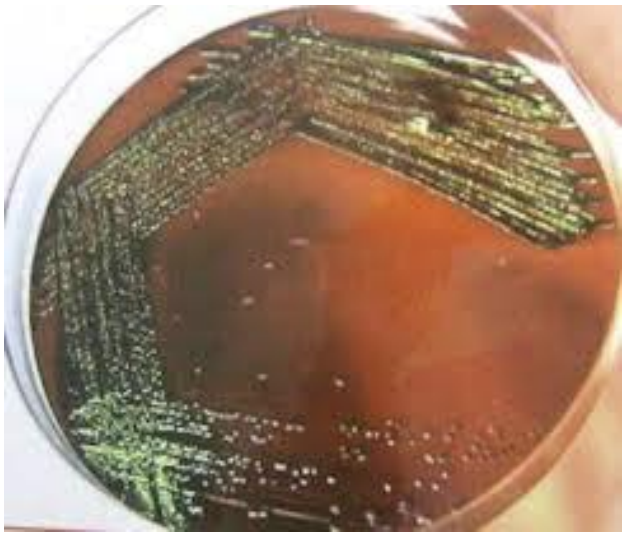

Plate V. *E. coli* isolated from Kuska Rice.

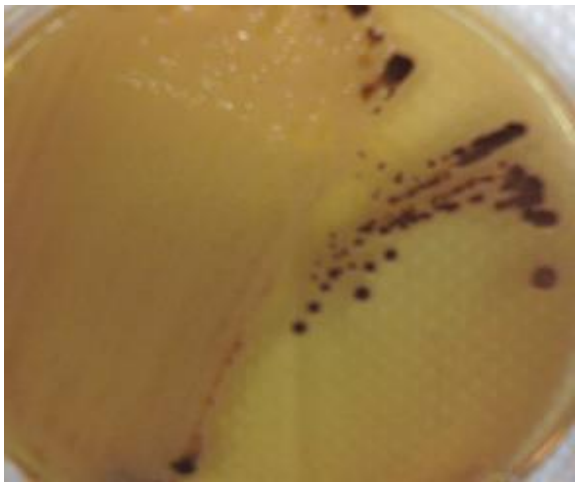

Plate VI. *Salmonella* isolated from egg bonda.

Plate IV. *E. coli* isolated from fish fry.

of *E. coli* in foods is highly undesirable, it would be virtually impossible to eliminate all of them. From the standard plate count, characteristic colonies were picked from each food sample and plated on Eosin Methylene Blue agar and Salmonell Shigella agar for identification of *E. coli* and *Salmonella* sp. Eosin Methylene Blue (EMB) agar was used to selectively identify *E. coli* that showed greenich metallic sheen of colonies, Salmonella showed colonies with black pointed centre on Salmonella Shigella (SS) agar (Plate VI). A similar study on hazard analysis of street vended foods was carried out by Sharmila (2011).

Though low numbers of coliforms are usually permitted in sensitive foods at numbers ranging from 1 to not exceeding 100/g or ml of food quantity (Frazier 1994), the presence of enterobacteriacea or coliforms indicate inadequate processing and or post process contamination due to cross contamination by raw materials, dirty equipments or poor hygienic handling and thus microbial proliferation could have allowed multiplication of a wide range of pathogenic and toxigenic forms. It is a common practice to handle both cooked and raw samples alternately during the roasting process thereby increasing the possibility of transmitting enteropathogens to cooked products (Mbah et al., 2012).

The proposed microbial specification to be used as guidelines for foods has been presented in Table 4. By comparing the bacterial counts in the street foods obtained in present study in Table 5 to the proposed food standard specifications given in Table 4, it was inferred that Fish fry and Kuska rice were poorest in Microbiological quality with high microbial load, indicating inadequate cleaning during preparation, unhygienic preparation procedures, use of contaminant water for cooking and washing that carries these contaminant indicator organisms and unhygienic storage conditions of these food items. Tandoori chicken was also poor in microbial quality, which prepared by mixing the ingredients with hands. The vendors who dip their hands in the vessel for rolling the chicken in the batter, and impure water used for the purpose were the sources that contaminate this food item.

Based on these findings, the study signifies the need for maintanance of proper hygiene in street food outlets and has suggested that provision of suitable facilities and training are prime requisuites for the food vendors. Critical control points to avoid contamination should be identified and proper initiatives should be taken to reduce the contamination. Local authorities should take measures to go through planning, investment, mass media and also campaign on food safety and regulations.

Table 4. Microbiological specification of foods

Food type	Total viable count	*E. coli*	*Salmonella* sp	Yeast and molds
Fish	1000000/g	<500/100 g	0/20 g	-
Cooked Poultry	1000000/g	0/20 g	0/20 g	-
Egg and Egg products	<20000/g	<50/g	0/25 g	<200/g
Deep Fried Food Products	<10000/g	0/g	0/25 g	-
Starchy cereal cooked foods	<200000/g	0/g	0/25 g	>10000/g
Food items requiring further cooking	-	<10/g	0/25 g	-
Raw vegetables	-	0/g	0/25 g	-

Source: Guidelines for environmental health officers on the interpretation of microbiological analysis of food, Department of health directorate, food control, 1992.

Table 5. Microbial Load in different types of street vended foods

Food Sample	Standard plate count cfu/gm	*E. coli*	*Salmonella* sp	Fungus
Bhelpuri	1.4×10^5.	-	-	0.5×10^5
Chilly mushroom	8.4×10^5.	-	-	-
Tandoori chicken	4.8×10^5.	1×10^5	-	0.8×10^5
Idiyappam	2.5×10^5.	-	-	-
Idli	1.3×10^5.	-	-	-
Panipuri	1.8×10^5.	-	-	0.1×10^5
Fish Fry	9.1×10^5.	1×10^5	-	-
Kuska rice	6.4×10^5.	4×10^5	-	1.2×10^5
Egg Bonda	2.1×10^5.	-	5×10^5	-
Vegetable noodles	2.3×10^5.	-	-	-

Conclusion

Street foods are source of inexpensive, convenient and delicious food, providing significant quantities of nutrient level of calories, protein and fat. But they raise concern with respect to the potential hazards due to microbiological contamination adulterants and poor environmental sanitation and hygiene involved for their preparation. Simple precautions like keeping cooked and raw food separate and covered, minimizing handling with unhygienic hands and utensils, keeping the surroundings of stall clean, using clean water and holding foods at appropriate temperature for not longer than its required time period can ensure safe food delivery to the customers.

Conflict of interests

The authors did not declare any conflict of interest.

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Improving physico-chemical and microbiological quality of compost tea using different treatments during extraction

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Compost tea is gaining much interest due to its nutritional, biostimulation and disease suppression benefits to the plants. Four experiments were carried out to determine the effect of incubation temperature, incubation periods, dilution rate and nutritional sources on the microbial populations, physico-chemical properties and indole acetic acid (IAA) levels. Incubation at 28°C showed the highest number of bacteria and aerobic N_2-fixing bacteria (ANFB), along with the highest levels of total nitrogen and IAA. All the microbial populations increased in proportion with increase in the incubation period; however, increasing the incubation more than 48 h did not show any significant improvement ($P \leq 0.05$) in the physico-chemical properties or IAA levels. All microbial numbers and chemical properties decreased by increasing the dilution rate. Adding a mixture of the three used nutrients (molasses "0.5% v/v", ammonium nitrate "0.5 g/L" and di-potassium phosphate "0.5 g/L") resulted in the highest microbial populations (except for ANFB) as compared to the molasses, or double the amount of each other chemical alone. This study show the optimum conditions for preparing compost tea with high microbiological and physico-chemical properties. The study includes ANFB, important for soil application, and IAA, important for foliar application, which have not been studied before in compost tea.

Key words: Compost tea extraction, factors, microbiology, physico-chemical, indole acetic acid (IAA), aerobic N_2-fixing bacteria (ANFB).

INTRODUCTION

The use of liquid organic fertilizers and biofertilizers containing beneficial microorganisms for supporting organic farming has gained much global interest (Naidu et al., 2010). Compost tea is a compost extract that is brewed, either aerobically or anaerobically, with microbial food sources of carbohydrate and protein. Such trend is

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commercially increasing, and is resulting in microbiologically enriched compost extract, commonly called “compost tea”. Compost tea is used in agriculture either as soil amendment or as foliar application, and is a safe replacement for the potential hazards of costly chemical fertilizers and pesticides (Ingham, 1999; Pant et al., 2009; Radovich and Arancon, 2011).

The benefits of using compost teas in agriculture are: biostimulation and improvement of crop yield and quality, suppression of plant pathogenic microorganisms and, supplying the plant with water-soluble nutrients (Anonymous, 2007; Hegazy et al., 2013; Pane et al., 2014). In addition, compost extract enriches soil with microorganisms and "brings the soil back to life" (Martens, 2001). Such enrichment has a positive effect on the microbial selection, where the typical application rate of 150-200 L/ha changes the microbial composition towards more beneficial groups for the soil and plant (Hoitink and Boehm, 1999). Some of the benefits of these microbial groups are the production of plant growth promoters and chemicals such as siderophores, which reduce availability of iron for phytopathogens in the rhizosphere, tannins and phenols which inhibit most plant pathogens (Antonio et al., 2008). Other microbes are capable of nitrogen fixation and/or phosphate solubilization, making it available to plants (Glick et al., 1999). It is also reported that compost extract increases the carbon level and organic matter in soil, which help in building soil aggregates, and thus, improve soil structure and water holding capacity (Ha et al., 2008).

The production of compost extract with high quality depends on several factors, such as: adding nutritional sources, dilution rate and water quality, level of aeration, compost quality and age, and incubation time. Compost quality is also an important factor, which depends on the consistency of the used materials and the methods used for compost preparation. Some researchers consider the definition of compost "quality" is open-ended, because of the wide variety of benefits that compost provides (Shrestha et al., 2011). Generally, the extraction process should continue until most of the compost contents of soluble nutrients and microorganisms are extracted into the solution, where some authors adopt times of extraction and fermentation of one or two weeks (Pane et al., 2012).

Many compost tea producers include additives in order to increase the populations and diversity of microorganisms and to increase the level of plant disease suppression (Pane et al., 2012). Molasses, kelp extract, fish emulsion and rock dust have been used as cheap and commonly available nutritional sources (Ingham, 2005; Naidu et al., 2010). The additives would affect the C : N ratio and the forms of carbohydrates or nitrogen source in the extraction medium, which should change the composition of the microbial group such as bacteria, actinomycetes and fungi; however, not enough research has been done in this area (Pant et al., 2009; Naidu et al., 2010). Even though many studies have focused on the disease suppression aspect as affected by compost-to-water ratio, this ratio obviously affects the nutritional and microbial composition of the produced extract (Radovich and Arancon, 2011; Weltzien, 1990). A wide range of dilution rate was used in the extraction process, ranging from 1:1 to 1:50 (Weltzien, 1990; Zhang et al., 1998) with the most commonly used ration of 1:3 to 1:10 (Scheuerell and Mahaffee, 2004). It is also expected that the highly diluted extract could pose the risk of microbial contamination.

IAA is one of the most active auxins in plants, controlling many important physiological processes including cell development and division, playing a role in plant resistance to different pathogens, and microorganism-plant signaling. It is a product of several microorganisms, some of which are natural components of compost tea, and is proposed to contribute to plant growth and defense against pathogens (Lynch, 1985; Gravel et al., 2007; Hardoim et al., 2008).

In this study, we aimed to optimize the four major production variables, namely, incubation temperatures, incubation periods, compost dilution rate and additives of microbial food sources during compost extraction. In addition, we investigated the effect of these variables on compost extract aspects that has not been addressed before, which is the numbers of ANFB and the levels of IAA.

MATERIALS AND METHODS

Source of compost and preparation of compost teas

Garbage compost was obtained from the Cairo Organic Fertilizers Company, Cairo, Egypt, and was composed mainly of a household biodegradable wastes, after sorting out plastics, glass and metals at the site of composting. Based on the company’s analyses, it has a 30% organic carbon, 1.0% total nitrogen, 35% moisture and pH 7.5±0.2. Compost teas were prepared according to the method of Ingham (2005) with some modifications. Compost extractions were done in four different experiments to measure the effects of: incubation temperatures, incubation periods, compost-to-water ratio and addition of microbial food sources. Each experiment consisted of four treatments, in a 50 L plastic bucket, and a working volume of 20 L for brewing. Garbage compost was sealed in a cotton bag and submerged into tap water in plastic bucket, and each was amended with microbial food source. The water used was pump aerated for 30 min to remove chlorine before addition to the compost. Compost soaking was done in the laboratory, while being aerated continuously (10 L/min air delivery per bucket through air stones). Samples were taken in three replicates for the microbiological and chemical analyses.

Experimental design and treatments

Four experiments were carried out indoors in the Agricultural Microbiology Department Laboratory, Faculty of Agriculture, Zagazig University, Egypt. Each experiment consisted of four

treatments with three replications and the experimental design was a complete randomized. In all experiments, unless otherwise is mentioned, molasses was used at 0.5% (v/v), compost-to-water ratio was 1:20, incubation temperature was 28°C, and the incubation period was 48 h.

The first experiment involved aerated incubation of compost extracts at four different temperatures, namely 20, 28, 37 and 45°C for 48 h. The second experiment consisted of compost extracts incubated at different periods (12, 24, 48 and 72 h). The third experiment consisted of compost extracts incubated in different dilution ratios (1:10, 1:20, 1:30 and 1:40, compost: water on a weight/volume basis). The forth experiment involved aerated incubation of compost extracts with four different microbial food sources. In addition to molasses, ammonium nitrate and potassium phosphate were tested as cheap and available sources for the major nutritional elements required for plants (NPK), and sources for nitrogen and phosphorus required for microorganisms; also potassium phosphate contains a soluble form of phosphate, unlike other commercial chemical fertilizers. The tested treatments contained: 1) molasses (0.5% v/v), 2) ammonium nitrate (1 g/L), 2) di-potassium phosphate (1 g/L) and 4) molasses (0.5% v/v) plus ammonium nitrate (0.5 g/L) and di-potassium phosphate (0.5 g/L), which were added to each treatment in a bulk amount for the three replicates, followed by distributing them, and submerging the compost bags.

Physico-chemical analyses of compost teas

Physico-chemical analyses of compost teas (CT) were determined at the end of incubation in accordance with AOAC (2002). A pH meter (ORION 720A, Boston, USA) was used for determining the pH values, and a conductivity meter (HORIBA - HE-960CW) for the EC. Total nitrogen was measured using micro-Kjeldahle, and total phosphorus was measured colorimetrically using JENWAY 6305 UV-vis Diode Array Spectrophotometer, by the hydroquinone method. Total potassium was determined using a Carl Zeiss flame-photometer with acetylene burn.

Determination of indole acetic acid (IAA) in compost teas

IAA in the compost teas was assayed by modified procedure of Glickmann and Dessauxa (1995). CT were centrifuged at 5000 rpm for 15 min, then 1 ml of CT supernatant was added to 1 ml of Salkowski´s reagent, well mixed in a 3 l spectrophotometer cuvette, and the mixture was left in the dark for 30 min at room temperature. Rosy color developed means the presence of IAA and color density was measured using JENWAY 6305 UV-vis Diode Array Spectrophotometer at 530 nm. The level of IAA was estimated by standard IAA graph, and the results were expressed as µg IAA /ml.

Microbial analyses

Microbial populations in the garbage compost and compost tea, that is, bacteria, aerobic N_2-fixing bacteria, actinomycetes and fungal populations, were determined using plate count or most probable number (MPN) technique. Bacteria were enumerated on nutrient agar (Difco, 1985) and incubated at 30°C for 2 days. Enumeration of potential N_2-fixing bacteria as strict aerobes was done using the most probable number (MPN) technique of Abd-El-Malek (1971) on Ashby modified medium and incubated at 30°C for 7 days. It was enumerated as surface pellicle formation. Actinomycetes were enumerated on starch casein agar (Conn and Leci, 1998) and incubated at 28°C for 7 to 14 days, while fungi were enumerated on Martin's Rose bengal agar (Martin, 1950), and incubated at 25°C for 3–5 days, with three replicates of all microbial counts.

Statistical analysis

Data recorded in three replicates for the parameters in various treatments were subjected to the analysis of variance (ANOVA) according to Snedecor and Cochran (1980), using SPSS statistical package version 16.0 (SPSS Inc., Chicago, IL, USA) to quantify and evaluate the sources of variation. Duncan's multiple range test (DMRT) was applied to compare the mean performances of different treatments for the specific parameters under study and the rankings were denoted by superscripts in the relevant tables. Differences in means were compared at $P \leq 0.05$.

RESULTS AND DISCUSSION

In this study, four major microbial groups were determined, namely, bacteria, aerobic N_2-fixing bacteria, actinomycetes and fungi, as a measure for the biological activity and quality of the produced compost tea. Previous reports have shown that increasing the population and activity of microorganisms improve quality of compost tea, since it increases its effectiveness, support its binding to the foliage of the plant and contribute to plant health and systematic resistance to diseases (Kavroulakis et al., 2005; Scheuerell and Mahaffee, 2002; Naidu et al., 2010). In addition, certain bacterial groups, like the nitrogen fixing bacteria are important for soil fertility and as a safe alternative for the potential hazards of chemical nitrogen fertilizers (El-Aidy et al., 2012).

The effect of incubation temperatures during the preparation of compost tea on the microbial populations of bacteria, aerobic N_2-fixing bacteria, actinomycetes and fungi, is shown in Table 1. At an incubation temperature of 28°C, the numbers of bacteria was at the highest level, and numbers of aerobic N_2-fixing bacteria was signify-cantly higher ($P \leq 0.05$) than the numbers at 37 and 45°C. In contrast, the number of actinomycetes and fungi were at the highest level at incubation temperature of 45°C, being significantly higher ($P \leq 0.05$) than all other temperatures. Such higher number of actinomycetes and fungi is probably due to the survival of their thermophilic spores during the cooling stage of the compost. Also, increasing the temperature causes water evaporation, which results in more concentration of the nutrients, and therefore, promotes the growth of some microbial species (Pant, 2011). On the other hand, the low temperature may slow down the growth rate of the actinomycetes and fungi. Apparently, the best incubation temperature was 28°C, since it has the highest number of nitrogen fixing bacteria that are important for soil fertility.

Table 2 shows the effect of incubation periods during the preparation of compost tea on the previously mentioned microbial groups. All microbial groups showed the same trend by increasing their numbers in proportion

Table 1. Microbial populations of bacteria, aerobic N_2-fixing bacteria, actinomycetes and fungi, in compost teas under different incubation temperatures.

Incubation temperature	Total bacteria (log10 CFU/g)	ANFB (log10 CFU/g)	Actinomycetes (log10 CFU/g)	Fungi (log10 CFU/g)
20 °C	6.77 ± 0.57[b]	3.31 ± 0.09[c]	2.12 ± 0.06[a]	2.52 ± 0.09[a]
28 °C	7.07 ± 0.61[c]	3.42 ± 0.11[c]	2.29 ± 0.09[ab]	2.54 ± 0.09[a]
37 °C	6.72 ± 0.58[b]	3.2 ± 0.09[b]	2.38 ± 0.09[b]	3.11 ± 0.09[b]
45 °C	6.24 ± 0.54[a]	2.5 ± 0.08[a]	2.58 ± 0.05[c]	3.5 ± 0.09[c]

Different letters ([a] through [c]) represent significant differences ($P \leq 0.05$) among numbers in the same column.

Table 2. Microbial populations of bacteria, aerobic N_2-fixing bacteria, actinomycetes and fungi, in compost teas under different incubation periods.

Incubation period (h)	Total bacteria (log10 CFU/g)	ANFB (log10 CFU/g)	Actinomycetes (log10 CFU/g)	Fungi (log10 CFU/g)
12	6.40±0.47[a]	2.24±0.10[a]	1.86±0.05[a]	2.11±0.12[a]
24	6.69±0.50[b]	2.58±0.13[b]	2.08±0.08[b]	2.32±0.13[b]
48	7.09±0.55[c]	3.35±0.21[c]	2.32±0.08[c]	2.58±0.11[c]
72	7.32±0.55[d]	3.9±0.25[d]	2.66±0.09[d]	3.21±0.14[d]

Different letters ([a] through [d]) represent significant differences ($P \leq 0.05$) among numbers in the same column.

Table 3. Microbial populations of bacteria, aerobic N_2-fixing bacteria, actinomycetes and fungi, in compost teas under different compost dilution ratios.

Dilution ratios	Total bacteria (log10 CFU/g)	ANFB (log10 CFU/g)	Actinomycetes (log10 CFU/g)	Fungi (log10 CFU/g)
1:10 w/v	8.41±0.71[d]	5.23±0.31[c]	3.04±0.13[c]	3.41±0.15[c]
1:20 w/v	7.07±0.63[c]	3.34±0.30[b]	2.48±0.11[b]	2.5±0.08[b]
1:30 w/v	6.39±0.68[b]	2.88±0.45[a]	2.12±0.10[a]	2.34±0.07[ab]
1:40 w/v	6.16±0.67[a]	2.70±0.35[a]	2.04±0.08[a]	2.22±0.09[a]

Different letters ([a] through [d]) represent significant differences ($P \leq 0.05$) among numbers in the same column.

with the incubation period, with incubation period of 72 h being significantly higher ($P \leq 0.05$) than all other periods for all microbial groups. This is most likely due to the increasing extraction of the soluble material and microorganisms from the compost into the liquid, which is generally beneficial for agricultural application. Extraction of a larger amount of soluble material from the compost, allows more nutritional sources for the beneficial microorganisms, and also, more nutrients available to the plants (Anonymous, 2007). On the other hand, long incubation period is not always preferred, as it may allow the oxygen to be consumed by the aerobic micro-organisms, especially if pump aeration is not performed, hence, the anaerobic microorganisms will grow. In addition, it would not be economical to increase the incubation period more than 72 h.

The populations of the different microbial groups as affected by the dilution rate during the compost tea incubation, were determined and presented in Table 3. Generally, the numbers of all microbial groups have decreased as the dilution rate increased. The decreasing rate between the first two dilutions (1:10 and 1:20) was much higher, being always significantly different ($P \leq 0.05$) in all microbial groups, than the decreasing rate between the other dilutions (e.g., the difference between 1:30 and 1:40 is not significant ($P \leq 0.05$) in the three microbial groups ANFB, actinomycetes and fugi). Even though dilution 1:10 has the highest microbial

Table 4. Microbial populations of bacteria, aerobic N_2-fixing bacteria, actinomycetes and fungi in compost teas under different nutritional sources.

Nutritional sources	Total bacteria (log10 CFU/g)	ANFB (log10 CFU/g)	Actinomycetes (log10 CFU/g)	Fungi (log10 CFU/g)
Molasses	7.06±0.61^{c}	3.44±0.21^{c}	2.32±0.15^{b}	2.55±0.18^{b}
NH_4NO_3	6.48±0.45^{a}	1.53±0.13^{a}	2.24±0.15^{b}	2.40±0.19^{b}
K_2HPO_4	6.21±0.44^{b}	4.50±0.23^{d}	2.08±0.11^{a}	1.89±0.15^{a}
Molasses + NH_4NO_3+ K_2HPO_4	7.54±0.44^{d}	2.42±0.20^{b}	2.56±0.14^{c}	3.34±0.19^{c}

Different letters (^{a} through ^{d}) represent significant differences ($P \leq 0.05$) among numbers in the same column.

populations, it would not be recommended since it may be so concentrated that not all the nutrients and microorganisms are extracted. Dilution rate of 1:20 could be the best, as it should allow a full pull-out of the compost extractable components, and at the same time, keep the produced tea with enough level of nutrients and microorganisms (Anonymous, 2007; Ingham, 1999). In addition, it was reported that tea dilution rate of 1:50 has less efficiency in disease suppression as compared to the rates of 1:3 and 1:10 (Weltzien, 1990). It is also expected that higher levels of dilution would render the produced tea with so little nutrients, that it would not allow a normal growth or survival of the microorganisms.

As microorganisms are fundamental component of the compost extract, and are influenced by the nutritional additives during the extraction process (Bess, 2000), determining the effect of some nutritional sources was carried out in the study. Table 4 shows the effect of adding different nutritional sources on the four microbial groups. Molass (0.5% v/v) as a commonly added component during the extraction, was used as a control. Adding ammonium nitrate alone resulted in a considerable decrease in the numbers of ANFB, which is reasonable since this group of bacteria is known to be negatively affected by the presence of any nitrogen source in the media. The presence of potassium phosphate alone, however, increase the numbers of ANFB to its maximum level as compared to all other treatments, and signify-cantly decreased ($P \leq 0.05$) the numbers of all other groups to their lowest levels. Obviously, the reason for the decreasing numbers of the other groups is that they require an external source of nitrogen to grow and proliferate, which is absent in this treatment. The mixture of the three nutrients (molass, ammonium nitrate and potassium phosphate) resulted in a significant increase ($P \leq 0.05$) in the population of all microorganisms (except for the ANFB) for the reasons mentioned before. These results coincide with previous reports (Naidu et al., 2010) stating that adding the microbial nutrients is helping beneficial microorganisms to be active, and more likely to survive and be transferred to the soil or plant surfaces. Additionally, introducing nutrients and the consequent increase of microbial population can boost the biological control efficiency, but the selection of the additives must be done with extreme caution (Scheuerell, 2003). For instance, the addition of molasses alone was found to support the growth of human bacterial pathogens if they already exist in the compost to begin with (Duffy et al., 2004), and thereby abolishing the ability for disease suppressive characteristics of compost tea (Scheuerell, 2003). Molass was significantly better ($P \leq 0.05$) nutrient for bacteria as compared to either ammonium nitrate or potassium phosphate alone. It was also better than potassium phosphate alone for fungi and actinomycetes. That might be due to the chemical composition of the molass, which is a complex carbohydrate containing a variety of minerals (Castle and Watson, 1985).

In all these experiments, the numbers of total aerobic bacteria were the highest among other microbial groups, represented in the average of $\log_{10}$ 6.84 cfu/ml. Such number is well appropriate for offering the treated plants with enough microbial coverage for foliar pathogen suppression. On the other hand, actinomycetes were the lowest group in its population (average of $\log_{10}$ 2.31 cfu/ml) which is in accordance with previous reports stating that compost teas are not appropriate media for extraction and growth of actinomycetes (Anonymous, 2014).

Physico-chemical properties are very important factor in determining the value of the produced compost tea. Most important are the major nutritional elements that are necessary for all plant in large amounts. Determining the changes in IAA as a result of the different treatment is also important, especially for the foliar plant treatment, even though it has not been studied before.

Table 5 shows the effect of different incubation temperatures on physico-chemical analyses (pH, E.C., total N, P and K) and IAA content in compost teas after48 h. Total nitrogen was significantly higher ($P \leq 0.05$) under the incubation temperature of 28°C as compared to the other incubation temperatures. Similar behavior was shown in the IAA, with significantly higher level detected at the same temperature as compared to the others. These results correlates very well with those of the

Table 5. Effect of different incubation temperatures on physico-chemical analyses and IAA content in compost teas after 48 h.

Treatments (°C)	pH	E.C. (ds/m)	Total N (mg/L)	Total P (mg/L)	Total K (mg/L)	IAA (µg/L)
Incubation at 20	7.4[a]	1.51[a]	1.70[a]	16.73[a]	450.0[a]	2.51[a]
Incubation at 28	7.4[a]	1.43[a]	1.86[b]	16.73[a]	452.0[a]	3.17[b]
Incubation at 37	7.3[a]	1.47[a]	1.76[a]	16.66[a]	455.5[a]	2.73[a]
Incubation at 45	7.2[a]	1.51[a]	1.74[a]	16.73[a]	451.0[a]	2.65[a]

Different letters ([a] and [b]) represent significant differences ($P \leq 0.05$) among numbers in the same column.

Table 6. Effect of different incubation periods on physico-chemical analyses and IAA content in compost teas incubated at 28°C.

Treatments (h)	pH	E.C. (ds/m)	Total N (mg/L)	Total P (mg/L)	Total K (mg/L)	IAA (µg/L)
Incubation for 12	7.7[b]	1.54[a]	1.72[a]	16.59[a]	450.0[a]	1.51[a]
Incubation for 2	7.5[ab]	1.50[a]	1.88[b]	16.73[a]	455.0[a]	2.87[b]
Incubation for 48	7.4[ab]	1.57[a]	1.94[b]	16.66[a]	460.5[a]	3.34[c]
Incubation for 72	7.2[a]	1.57[a]	1.96[b]	16.73[a]	467.0[a]	3.65[c]

Different letters ([a] through [c]) represent significant differences ($P \leq 0.05$) among numbers in the same column.

Table 7. Effect of compost dilution ratios on physico-chemical analyses and IAA content in compost teas incubated at 28°C for 48 h.

Treatments (w/v)	pH	E.C. (ds/m)	Total N (mg/L)	Total P (mg/L)	Total K (mg/L)	IAA (µg/L)
Compost dilution ratios 1:10	7.6[b]	1.62[b]	3.14[c]	25.83[d]	634.0[d]	3.41[b]
Compost dilution ratios 1:20	7.4[a]	1.50[b]	1.86[b]	16.73[c]	451.0[c]	3.17[b]
Compost dilution ratios 1:30	7.3[a]	1.34[ab]	1.31[a]	11.48[b]	366.5[b]	2.83[a]
Compost dilution ratios 1:40	7.3[a]	1.16[a]	0.97[a]	7.56[a]	280.0[a]	2.77[a]

Different letters ([a] through [d]) represent significant differences ($P \leq 0.05$) among numbers in the same column.

microbial growth, where the high population of ANFB were shown at 28°C, explaining the increase of total nitrogen. Also, the higher levels of IAA at 28°C may be due to the highest numbers of bacteria shown at this incubation temperature. The ability of several bacterial species to synthesize IAA has been reported before (Ali et al., 2009). All other parameters did not show any significant difference due to the temperature variations.

Table 6 shows the effect of different incubation periods of extraction on physico-chemical analyses and IAA content in compost teas. The factors that were affected clearly were the total nitrogen and IAA, where both of them increased significantly ($P \leq 0.05$) in response to increasing the incubation period. Forty eight hours, however, seem to be the most suitable, since further increase in the incubation period did not result in significant increase in both of them, making such increase not economically valuable. The pH values were also affected, as they slightly decreased with increase in the incubation period, however, all other parameters were not affected. Also, the results for the pH were close to those obtained by Pant et al. (2012), who recorded a pH value of 7.4 for the compost tea produced from food waste vermicompost in a 1:10 dilution ratio. Conversely, the E.C. level in their work (1 ds/m) was clearly lower than ours (1.6 ds/m for the same dilution rate), which is likely due to their use of deionized water in their experiment, while we used tap water.

The effect of the compost dilution ratios on physico-chemical analyses and IAA content was studied in compost teas incubated at 28°C for 48 h (Table 7). All values of the studied parameters were decreased with increase in the dilution rate. It is not practical, however, to produce a highly concentrated compost extract, as it would not be able to extract all the nutrients and microorganisms from the compost, so, a dilution of 1:20 is probably the most suitable to use. Previous studies (Pant, 2011) have shown that the application of

Table 8. Effect of different microbial nutritional sources on physico-chemical analyses and IAA content in compost teas after 48 h.

Treatments	pH	E.C. (ds/m)	Total N (mg/L)	Total P (mg/L)	Total K (mg/L)	IAA (µg/L)
Molasses	7.4[a]	1.67[a]	1.73[a]	16.80[a]	453.0[a]	3.60[a]
NH_4NO_3	7.3[a]	2.65[b]	35.22[c]	16.73[a]	451.0[a]	3.64[a]
K_2HPO_4	7.5[a]	2.68[b]	1.76[a]	156.94[c]	875.5[c]	3.71[a]
Molasses + NH_4NO_3 + K_2HPO_4	7.4[a]	3.36[c]	19.56[b]	79.73[b]	641.0[b]	5.65[b]

Different letters ([a] through [c]) represent significant differences ($P \leq 0.05$) among numbers in the same column.

vermicompost tea increased plant yield and root growth, and that the result of the extract dilution (1:10 to 1:100) was generally linear in decrease in plant growth. Almost the same trend was shown for the levels of tissue nitrogen, phytonutrient and microbial activities in soil. The decreasing rate of the different parameters was obviously faster in N, P and K, than the three other studied parameters. Edwards et al. (2006) found that there was no significant difference on tomato seedlings growth response with the applications of vermicompost teas with 1:25 and 1:10 ratios. Other researchers (Touart, 2000; Scheuerell and Mahaffee, 2002), however, reported effective results on disease suppression and yield improvement when limiting compost to water ratio to 1:10.

The effect of microbial nutritional sources on the physico-chemical characteristics and IAA content in compost teas were also studied (Table 8). As expected, total nitrogen was at its highest level (316.7) when ammonium nitrate was added to the extraction solution, and with lesser extent when a mixture of the three chemicals was added. The same trend was observed with total phosphorus and total potassium where their levels reached their peaks (224.2 and 875.5, respectively) when potassium phosphate was added to the extraction solution. IAA and E.C. levels increased with the presence of the mineral nutrients and reached their peaks with the mixture of all components. IAA was also increased in all treatments containing nutritional sources as compared to the molasses alone, with the collective nutritional mixture reaching the maximum level. Such higher IAA levels are likely due to its synthesis by the extracted microbial population and the supporting nutrients. It is already reported that several strains of bacteria such as *Bacillus, Pseudomonas, Escherichia, Micrococcus* and *Staphylococcus* genera are able to synthesize IAA (Ali et al., 2009). At a temperature of 28°C, the highest population of bacteria (Table 1) was accompanied by the highest level of IAA concentration (Table 5). In the experiments of incubation periods and dilution rate, the correlation is even more obvious. In the experiment of the nutritional sources, the number of bacteria decreased by using NH_4NO_3 and K_2HPO_4 when compared with the molasses alone (Table 4), while the IAA levels did not change significantly (Table 8). Such deviation from the previous trend might be explained by a positive selection for the two mineral compounds toward promoting the growth of IAA-producing bacterial species, while molass increase the bacterial population in a general manner. When molass and the two mineral compounds were combined together, both the general bacterial population and the IAA-producing species (reflected by the much higher level of IAA) were increased significantly.

Conclusion

This study demonstrated the optimum conditions for compost extraction which enhances the microbial activity, physico-chemical characters and IAA content. Incubation temperature of 28°C was most suitable for growing important microbial groups such as bacteria (producing IAA and biological activity) and aerobic nitrogen-fixing bacteria. Also, at this temperature, the highest levels of IAA and nitrogen content were shown. The number of all microbial groups increased due to increasing incubation periods up to 72 h, and similar trend was established in the levels of IAA and nitrogen content; however, in our study, there was no significant increase in both of them after 48 h. Among the tested dilution rates, the dilution of 1:20 (w/v) seems to be most appropriate in terms of microbial and nutritional contents. Enriching the extraction solution with molasses, NH_4NO_3 and K_2HPO_4 resulted in the highest number of all microorganisms, except for the ANFB. The same result was true with the IAA, where it reached its maximum level with the mixture of the three additives.

Conflict of interests

The authors did not declare any conflict of interest.

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Production of extra cellular enzymes by microbial strains in molasses and additives supplemented fermentation media

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This work investigates the use of molasses (a cheap substrate) together with additives in fermentation medium to produce extra cellular protease, amylase and lipase, with the aid of *Aspergillus oryzae* and *Bacillus subtilis.* The concentration of molasses (0.1 – 1.0%) used for enzyme production by *A. oryzae* significantly influenced the extracellular lipase, amylase and protease biosynthesis/ secretion. The highest extracellular enzyme activities were found at 96 h of processing (72, 52 and 65 U/ml for lipase, amylase and protease, respectively). As additives, two different carbon sources: *Avena sativa* and *Cicer arietinum* were tested for production of extracellular enzymes by *A. oryzae.* Among the different concentrations of *A. sativa,* high enzyme activity (61, 83 and 121 U/ml, respectively for lipase, amylase and protease) was noted at concentration of 2.5 g/L. For another additive, *C. arietinum,* highest extracellular enzyme activity was found at 96 h (71; 61, 80 U/ml for lipase, amylase and protease). In this study, it is concluded that the production of extracellular enzyme using molasses with additives had maximum value compared to using molasses alone in 96 h fermentation by *Aspergillus oryzae.*

Key words: Molasses, extracellular enzyme, *Aspergillus oryzae.*

INTRODUCTION

Enzymes are proteins that function as specialized catalysts for chemical reactions. They have contributed greatly to the traditional and modern chemical industry by improving existing processes (Rao et al., 1998). The use of enzyme-mediated processes can be traced to ancient civilizations. Today, more than 3000 enzymes are known, and of these, about 600 are in commercial use; the majority of the industrial enzymes are of microbial origin. Until the 1960s, the total sales of enzymes were only a few million dollars annually, but the market has since grown spectacularly (Godfrey and West, 1996; Wilke, 1999; Gilbert and Dupont, 2011). Because of improved understanding of production biochemistry, the fermentation processes, and recovery methods, an increasing number of enzymes can be produced affordably. Also, advances in methods of using enzymes have greatly expanded demand. Furthermore, because of the many different transformations that enzymes can catalyze, the number of enzymes used in commerce continues to multiply (Haq et al., 2006). The world enzyme demand is satisfied by

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12 major producers and 400 minor suppliers. Around 60% of the total world supply of industrial enzymes is produced in Europe (Hasan et al., 2006). Global enzymes market is estimated to rise by 7% at a healthy pace to $8.0 billion in 2015. Gains will reflect a continued world economy rebound from the global financial crisis of 2009. Enzymes are employed in a diverse array of applications in industries and scientific research, ranging from the degradation of various natural substances to biosynthesis of different compounds (Wilke, 1999).

Molasses is an interesting raw material; it is rich in nutrients and minerals, cheap in price as well as abundant; hence a by-product in sugar industries. Molasses as nutrient medium can be used as a relatively inexpensive and economic alternative to synthetic medium for the production of industrial important enzymes (Johnvesly and Naik, 2001). This leads to the present study.

MATERIALS AND METHODS

The strains of *A. oryzae* and *B. subtilis* were obtained from University of Madras, Chennai and were cultured in Potato Dextrose Agar and Nutrient Agar respectively. Agro-industrial by product was tested as substrate: sugarcane molasses. The molasses was added until total sugars reached 10 g/L in the minimum medium and the other particular residues were used at 50 g/L. As nitrogen source, urea (300 g/L) was filtered (0.22 µm Micropore membrane) and added to the autoclaved medium in order to get 100 mM.

Effect of different concentrations of molasses on enzyme production using *A. oryzae* and *B. subtilis*

A. oryzae and *B. subtilis* were inoculated into production medium with different concentrations of molasses: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0%. The experiments were conducted for a period of 144 h for *A. oryzae* and 48 h for *B. subtilis*. Every 24 h of interval for *A. oryzae* and 4 h for *B. subtilis* cultures the samples were collected, centrifuged and used for the estimation of enzymes (amylase, protease and lipase) and protein.

Effect of different concentrations of additives on enzyme production from molasses using *A. oryzae* and *B. subtilis*

In addition to molasses and other carbon sources, two additives namely *A. sativa* and *C. arietinum* were amended separately at the concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 g at pH 7 in the medium; the experimental flasks were inoculated with tested cultures and incubated at 30±2°C for 144 h (*A. oryzae*) and 37±1°C for 48 h (*B. subtilis*). The samples of cultures were collected and centrifuged at regular interval; the supernatant was used for the estimation of enzymes (amylase, protease and lipase) and protein.

Analytical methods

The protein concentration was determined according to the method of Bradford (1976). Lipase activity was measured using various *p*-nitrophenyl esters as described by Hasan et al. (2006), with some modifications. One volume of a 10 mM solution of each substrate in 2-propanol was mixed with 9 volumes of 100 mM Tris-HCl buffer (pH 8). When long acyl chain *p*-nitrophenyl esters (C14-C18) were used, PVA 0.25% (w/v) was also incorporated into this buffer. This mixture was then pre-warmed at 40ºC in a water bath and immediately distributed (1 ml) into 1.5 ml cells. The reaction was started by adding 0.5 ml of enzyme solution at an appropriate dilution in 10 mM Tris-HCl (pH 8). The absorbance at 410 nm of the assay against a blank without any enzyme was continuously monitored for 2–5 min using a UV-vis spectrophotometer (ShimadzuUV-160A, Shimadzu Corporation, Kyoto, Japan). The reaction rate was calculated from the slope of the curve absorbance versus time, using a molar extinction coefficient of 12,750 cm^{-1} M^{-1} for *p*-nitrophenol. One enzyme unit was defined as the amount of protein releasing 1µmol of *p*-nitrophenol per minute under the above reaction conditions.

Amylase activity (Miller, 1959)

The activity of amylase was analyzed by incubating 0.3 mL enzyme with 0.5 mL soluble starch (1%, w/v) prepared in 0.05 M phosphate buffer, pH 6.5. After incubation at 90ºC for 10 min, the reaction was stopped and the released reducing sugars were analyzed colorimetrically by the addition of 1 mL of 3-5- dinitrosalicylic acid reagent. An enzyme unit is defined as the amount of enzyme releasing 1 mM of glucose from the substrate in 1 min at 90ºC.

Assay of protease activity

Protease activity was measured by the method of Johnvesly and Naik (2001) using casein as a substrate. Half milliliter of the diluted enzyme was mixed with 0.5 ml 100 mM Tris-HCl (pH 8.5) containing 1% casein, and incubated for 15 min at 500°C. The reaction was stopped by addition of 0.5 ml trichloroacetic acid (20%; w/v). The mixture was allowed to stand at room temperature for 15 min and then centrifuged at 10,000 rpm for 15 min to remove the precipitate. The acid soluble material (aromatic aminoacids) was determined spectrophotometrically at 280 nm. A standard curve was generated using solutions of 0-50 mg/l tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 mg of tyrosine per min under the experimental conditions used.

RESULTS AND DISCUSSION

In the present study, the concentration of molasses (0.1 – 1.0 %), used for enzyme biosynthesis by *A. oryzae* and *B. subtilis,* significantly influenced the extracellular lipase, amylase and protease secretion. In *A. oryzae*, the highest extracellular enzyme was at 96 h (72, 52, 65U/ ml respectively (Table 1). It was evident that the concentration of molasses (0.1 – 1.0%) used for enzyme production by *B. subtilis* significantly influenced the extracellular protease, amylase and lipase. The content respectively was 149, 113 and 89 U/ml with a highest extracellular enzyme at 36 h (Table 2).

Two different carbon sources of *A. sativa* and *C. arietinum* were tested for extracellular lipase, amylase and protease secretion by *A. oryzae* and *B. subtilis.* Among the different concentrations of *A. sativa,* 2.5 g/l allowed high enzyme activity in *A. oryzae* fermentation: 61; 83; 121 U/ml for lipase, amylase and protease

Table 1. Effects of molasses concentration on extracellular enzyme biosynthesis by Aspergillus oryzae in submerge fermentation.

Concentration of mollases (%)	Lipase (U/ml)			Amylase (U/ml)			Protease (U/ml)		
	48 h	96 h	144 h	48 h	96 h	144 h	48 h	96 h	144 h
0.1	15	29	22	12	29	23	22	48	29
0.2	16	31	22	12	31	23	24	52	33
0.3	16	33	26	14	34	28	26	56	35
0.4	18	36	30	16	36	30	26	58	39
0.5	19	39	30	18	38	30	30	60	41
0.6	19	41	31	18	41	32	32	62	43
0.7	21	44	32	21	44	32	32	62	43
0.8	22	46	40	23	46	40	36	68	45
0.9	23	50	40	26	50	42	38	71	45
1	25	52	42	28	52	44	40	74	45

Table 2. Effect of molasses concentration on extracellular enzyme biosynthesis by *Bacillus subtilis* in submerge fermentation.

Concentration of mollases (%)	Lipase (U/ml)			Amylase (U/ml)			Protease (U/ml)		
	24 h	36 h	48 h	24 h	36 h	48 h	24 h	36 h	48 h
0.1	55	90	65	50	68	60	58	98	68
0.2	40	55	40	50	80	60	68	118	88
0.3	48	62	48	52	82	70	70	120	84
0.4	48	70	58	50	88	68	78	122	94
0.5	52	80	68	60	92	82	80	138	100
0.6	56	90	62	70	110	90	96	150	130
0.7	60	92	78	60	105	84	80	120	90
0.8	52	80	68	70	95	80	75	120	90
0.9	50	75	60	60	80	70	65	105	95
1	48	70	60	50	90	75	65	105	75

Table 3. Effects of Avena sativa on extracellular enzyme biosynthesis by Aspergillus oryzae in submerge fermentation media.

Concentration of *A. sativa* (%)	Lipase (U/ml)			Amylase (U/ml)			Protease (U/ml)		
	48 h	96 h	144 h	48 h	96 h	144 h	48 h	96 h	144 h
0.5	24	52	30	15	49	30	28	58	32
1	25	58	31	18	52	32	30	62	34
1.5	29	62	33	20	56	32	34	64	34
2	36	78	45	26	62	40	39	80	50
2.5	32	68	42	25	58	39	36	76	50
3	29	59	34	22	54	32	29	62	40
3.5	22	52	32	20	50	32	27	52	34
4	22	44	32	16	44	30	24	49	30
4.5	22	46	26	14	42	24	20	42	30
5	18	38	27	14	32	21	16	39	30

respectively. The second substrate, *Cicer arietinum* (2.5 g/l) allowed biosynthesis of 71; 61, 80 U/ml lipase, amylase and protease respectively with highest extracellular enzymes at 96 h (Tables 3 and 4). With *the addition of B. subtilis* and *A. sativa* (3.5 g /L), protease (167 U/ml), lipase (113 U/ml) and amylase (150 U/ml) activity was higher. However, maximum enzyme secretion was obtained at the concentration of 3.0 g. The second additive, *Cicer arietinum (*2.5 g/l) allowed to obtain 149 U/ml of lipase, 121 U/ml of amylase and

Table 4. Effect of *Cicer arietinum* on extracellular enzyme biosynthesis by *Aspergillus oryzae* in submerge fermentation.

Concentration of *C. arietinum* (%)	Lipase (U/ml)			Amylase (U/ml)			Protease (U/ml)		
	48 h	96 h	144 h	48 h	96 h	144 h	48 h	96 h	144 h
0.5	18	38	28	19	40	34	22	60	46
1	20	42	30	21	54	38	30	62	52
1.5	22	44	34	26	56	38	36	80	60
2	30	56	50	30	64	42	40	84	62
2.5	34	60	50	42	82	62	44	88	70
3	38	66	62	46	86	59	46	100	72
3.5	32	64	58	32	62	50	48	119	102
4	30	56	42	29	58	40	46	98	80
4.5	28	52	40	26	52	38	44	82	64
5	26	48	40	20	44	36	38	79	56

Table 5. Effects of Avena sativa on extracellular enzyme biosynthesis by Bacillus subtilis in submerge fermentation.

Concentration of *A. sativa* (%)	Lipase (U/ml)			Amylase (U/ml)			Protease (U/ml)		
	24 h	36 h	48 h	24 h	36 h	48 h	24 h	36 h	48 h
0.5	51	70	50	50	71	59	98	144	130
1	53	80	58	48	79	59	64	116	90
1.5	53	82	69	50	88	64	64	120	98
2	58	88	70	58	88	71	80	130	96
2.5	60	97	82	81	131	95	90	140	120
3	70	106	80	90	150	130	96	152	132
3.5	71	113	91	81	131	95	96	164	152
4	67	97	79	75	117	83	70	150	140
4.5	63	90	67	75	130	95	70	140	95
5	50	88	71	50	88	71	70	119	80

Table 6. Effects of Cicer arietinum on extracellular enzyme biosynthesis by Bacillus subtilis in submerge fermentation.

Concentration of *C. arietinum* (%)	Lipase (U/ml)			Amylase (U/ml)			Protease (U/ml)		
	24 h	36 h	48 h	24 h	36 h	48 h	24 h	36 h	48 h
0.5	49	71	60	56	97	71	73	123	101
1	50	88	71	67	116	88	81	132	116
1.5	58	88	70	71	119	89	93	152	99
2	67	97	79	81	137	101	108	176	141
2.5	71	121	91	96	149	130	96	167	150
3	60	97	82	79	127	95	96	150	130
3.5	70	106	80	64	117	87	70	132	95
4	53	82	69	69	108	80	66	119	80
4.5	63	90	67	64	105	74	70	106	80
5	50	79	59	78	125	91	66	100	74

protease 176 U/ml. With the concentration of 2.0 g/l, highest extracellular enzyme activity was observed at 36 h (Tables 5 and 6). The addition of additives in molasses shows effective production of enzymes compared with without additive molasses fermentation.

The use of agro-industrial byproducts allows bio-processes development for the production of large quantities at viable cost enzymes. The growing demand for lower cost in industrial processes that are also highly specific and environmentally safe has stimulated the search for new enzymes. The use of agro-industrial residues in bioprocesses has enabled the production of enzymes employing alternative substrates at low cost as well as reducing environmental degradation caused by

the disposal of these residues (Elisashvili et al., 2008; Karp et al., 2013).

The sugar and ethanol industry produces different residues and many of them have great potential for bioprocesses application (Pandey et al., 2000). Sugarcane molasses is dark syrup obtained during sugar production from sugarcane or beets, resulting from the final stage of crystallization from which further recovery of sugar is no longer economically viable (Arakaki et al., 2011). Sugarcane molasses has an average of 50% total sugars in which sucrose predominates (Arakaki et al., 2011). The production of sugarcane in Brazil in 2012/2013 harvest reached 589 million tons, yielding from 23 to 35 tons of molasses (CONAB, 2013). Its composition and abundance makes this residue a potential substrate for the development of biotechnological processes, including the production of enzymes and other products (Miranda et al., 1999).

Conclusion

Sugarcane molasses is the best substrate for protease, amylase and probably lipase secretion by *Bacillus subtilis* and *Aspergillus orzyae* in submerged cultivation. Enzyme production is negatively affected by the increase in molasses concentration. The highest level of protease activity (74 and 150 U/ml) occurs in a cultivation medium containing of 1% and 0.6 to 0.7% of sugarcane molasses, respectively for *A. oryzae* and for *B, subtilis* process. In addition to molasses, the carbon sources *Avena sativa* (2%) and *Cicer arietinum* (3%) added medium enhance the enzyme producing activity of strain *A. oryzae.* Similarly, in addition to molasses, the carbon sources *Avena sativa* (2%) and *Cicer arietinum* (3%) added medium enhance the enzyme producing activity of strain *B. subtilis.* The results indicate the versatility of *B. subtilis* in production of significant levels of enzymes in submerged cultures based on abundant and inexpensive agro-industrial by products.

Conflict of interests

The authors did not declare any conflict of interest.

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Antimicrobial resistance in extended spectrum β-lactamases (ESBL)-producing *Escherichia coli* isolated from human urinary tract infections in Ndjamena, Chad

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The prevalence of antibiotic resistance among extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* has increased markedly in recent years. The purpose of this work was to investigate the occurrence and the antibiotic susceptibility of ESBL-producing *E. coli* in the urinary tract of the patients in Chad. Clinical strains of *E. coli* were isolated onto CLED agar and tested for ESBL production by using the double disk synergy test. Susceptibility to antibiotics was tested according to the guidelines of Clinical Laboratory Standards institute. Out of 283 cultures tested, 57 (20.14%) were positives for urinary tract infections of which 31 (54.5%) were identified as *E. coli* resistant to third generation cephalosporins. All these *E. coli* isolates expressed various level of resistance to antibiotics tested. Among these strains, 77.41 were detected to be ESBL-producers and 22.58% were non-producers. Moreover, resistance to the third generation cephalosporins was associated with significant cross resistance of 62, 38, 79, 70 and 95% to gentamicin, amikacin, nalidixic acid, ciprofloxacin and trimethoprim-sulfamethoxazole, respectively. However, cefoxitin and imipenem were found to still be efficient against the ESBL producers. These findings indicate that the trend was towards increased spread of ESBL-producing *E. coli* that could restrict the choice of antibiotic for the treatment of urinary tract infections in Chad.

Key words: *Escherichia coli*, resistance, antibiotic, extended-spectrum β-lactamase (ESBL), Chad.

INTRODUCTION

Escherichia coli is the primary pathogen of urinary tract infections (UTI), responsible for 75 – 95% of cases of morbidity in the community (Falagas et al., 2008). The emergence of extended-spectrum β-lactamase (ESBL)-producing *E. coli* restricted the choice of antibiotics for treatment of UTI, because β-lactamase resistance is often associated with cross resistance to other families of antibiotics (Messai et al., 2006). In Europe, according to the High Council of Public Health, ESBL-producing *E. coli* were 10% in Italy and Greece; 20% in Romania and

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Bulgaria; 28% in Turkey. In France, the rate of resistance was 75% to tobramycin, 35% to gentamicin, 25% to amikacin and 70% to ciprofloxacin (HCSP, 2010). In Africa, the resistance phenotype in *E. coli* has been reported in Nigeria, Cameroon, Benin, Algeria and Morocco (Gangoue et al., 2006; Ahoyo et al., 2007).

Whilst there is limited information concerning antimicrobial resistance in Africa, there are no data related to the subject in Chad. Therefore, this work constitutes the first attempts to evaluate antibiotic resistance.

MATERIALS AND METHODS

Study types and population

This prospective study was carried out from the 1st December 2012 to the 30th June 2013 at the "Hôpital de la Mere et de l'Enfant" of N'Djamena in Chad. It is a reference hospital for the treatment of the children from 0 - 14 years and women. This hospital has a capacity of 295 beds and 598 employees. In this study, the demographic variables noted were the age of patients and their provenance (inpatient or outpatient).

Specimen collection and isolation of *E. coli*

The urine samples from patients were collected in sterile disposable bottles and appropriately labelled. The specimens were transported immediately to the microbiology laboratory for bacteriological analysis. The samples were seeded onto Cystiene Lactose Electrolyte Deficient Agar and incubated at 35 - 37°C for 18 - 24 h. The colonies which grew on the CLED agar were suspected to be Gram-negative bacilli and were sub-cultured on Mueller Hinton agar for purification. Isolates were identified by Gram strains, indole production, Methyl Red, Voges Proskauer and citrate tests, and then confirmed by API 20 E identification system (bioMerieux).

Antibiotic susceptibility testing

Antimicrobial susceptibility testing of the isolated organisms was performed by the disk diffusion technique according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2011). The following antimicrobial agents were tested: amoxicillin/clavulanic acid (20/10 µg), cefoxitin (30 µg), cefotaxim (30 µg), ceftazidim (30 µg), imipenem (10 µg), aztreonam (30 µg), gentamicin (10 µg), amikacin (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg) and trimethoprim-sulfamethoxazole (1.25/23.75 µg). Data were reported as sensitive, intermediate or resistant. The antibiotic potency of the disks was standardized against the reference strains *E. coli* ATCC 25922.

Detection of ESBL production

Detection of ESBL production was screened on Muller-Hinton agar using a double-disc synergy test (DDST) according to the procedure of Jarlier et al. (1988). The plates were inoculated with *E. coli* strains as for standard disk diffusion test. Antibiotic disks containing aztreonam and expanded-spectrum cephalosporins were then placed 30 mm (center to center) from an amoxicillin/clavulanic acid disk prior to incubation. After overnight incubation at 35 - 37°C, the production of ESBL by the tested organism was detected by the presence of characteristic distortions of the inhibition zones, indicative of clavulanate potentiation of the activity of the test drug. Negative double-disk tests were repeated with a disk spacing of 20 mm (center to center).

Figure 1. Strain of *E. coli* showing positive DDST when swabbed on Mueller Hinton Agar and incubated with cefotaxim (CTX), ceftazidim (CAZ), ceftriaxone (CRO), aztreonam (ATM) applied 30 mm from the amoxicillin/clavulanic acid (AMC).

Statistical analysis

Laboratory results and data collected on the patients were performed using Microsoftt Excel (2010) and analyzed through Statistical Package for Social Sciences (SPSS) version19. Data Analysis of ESBL productions and resistances to antibiotics was made by Person chi-square for the comparison of two quantitative variables. The differences were considered significant when $p < 0.05$.

Ethical consideration

This study was authorized by the head office of hospital of Mother and Child of N'Djamena. The research was carried out on the samples received by the laboratory for clinical diagnoses. The results were given back to the doctors for the patients' treatment.

RESULTS

Prevalence of ESBL-producing strains

Figure 1 shows characteristic distortion of the inhibition zones obtained between third generation cephalosporins (CTX, CAZ and CRO), aztreonam (ATM) and amoxicillin /clavulanic acid (AMC) distant to 30 mm.

Out of 283 samples tested, only 57 cultures were positive for UTI infections. Among the 57 isolates, 31 (54.5%) were identified as *E. coli*, of which 24/31 isolates (77.41%) were ESBL producers and 22.58% ESBL non-producers. The variation of prevalence of ESBL-

Table 1. Number and rate of ESBL-producers and ESBL non-producers by age group.

Age of patient	Sample	*Escherichia coli* isolated		
		Isolates	ESBL $_{Producers}$ (%)	ESBL $_{Non\ producers}$ (%)
0 - 24	97	5	2 (8.33)	3 (42.85)
25 - 44	186	26	22 (91.66)	4 (57.14)
Total	283	31	24 (100)	7 (100)

Table 2. Resistance of ESBL producing *E. coli* to antibiotics tested.

Antibiotics	*E. coli* isolated from urines (n = 31)		
	ESBL $_{Non\ producer}$ (%) (n=7)	ESBL $_{Producer}$ (%)(n=24)	
	R (%)	R (%)	*P*
AMC	1(14)	14(58)	0.040
FOX	0(0)	1(4)	0.583
CRO/CTX	1(14)	24(100)	0.000
CAZ	1(14)	22(92)	0.000
ATM	0(0)	22(92)	0.000
IMP	0 (0)	0 (0)	-
CN	1(14)	15(62)	0.025
AK	0(0)	9(38)	0.054
NA	1(14)	19(79)	0.002
CIP	2(29)	17(70)	0.008
SXT	2(29)	21(95)	0.000

AMC (amoxicillin+clavulanic acid); FOX (cefoxitin); CRO (ceftriaxon); CTX (cefotaxim); CAZ (ceftazidim); ATM (Aztreonam); IPM (Imipénème); CN (Gentamicin) AK (amikacin); NA (nalidixic acid); CIP (ciprofloxacin); SXT (trimethoprim/sulfamethoxazole); R (%): Resistance (percentage), $P< 0.05$ was considered statistically significant. ESBL $_{Producer:}$ ESBL positive to DDST; ESBL $_{Non\ producer:}$ ESBL negative to DDST.

producers according to age is presented in Table 1. Of the 24 ESBL-producing *E. coli*, 2 (8.33%) were between the age range of 6-24 years and 22 (91.66%) 25 - 44 years. These results show that adults are often affected by ESBL-producing bacteria than children. ($p<0.05$)

Antibiotic susceptibility

The resistance to antimicrobials tested for ESBL non-producers and ESBL producers is presented in Table 2. The resistance rate of ESBL-producers was 58% to amoxicilline + clavulanic and 4% to cefoxitin. Moreover, all ESBL-producers were resistant to third-generation cephalosporins (CRO, CTX) (100%). None of the strains was practically resistant to Imipenem. The co-resistance to gentamicin, amikacin, nalidixic acid, ciprofloxacin, trimethoprim-sulfamethoxazole was 62, 38, 79, 70 and 95%, respectively.

DISCUSSION

In the present study, 31 out of 57 isolates were identified as *E. coli* of which 24 (77. 41) are ESBL-producers and 7 (22. 58) non-producers. Thus, the overall prevalence of ESBL producers was 8. 4% (24/283).

Observations of a high proportion of ESBL-producers among strains of *E. coli* analysed are disturbing because ESBL resistance is often associated with cross resistance to other families of antibiotic. However, these findings were in agreement with previously published results that show simultaneous resistance to both β-lactam and antibiotic of other groups.

Indeed, surveillance data showed that resistance in *E. coli* is consistently highest for antimicrobial agents that have been used for long time in human and veterinary medicine (NARMS, 2010). Moreover, *E. coli* is sometimes used as a sentinel for monitoring antimicrobial drug resistance in faecal bacteria because it is found more frequently in wide range of hosts, acquire resistance easily (Erb et al., 2007), and it is a reliable indicator of resistance in Salmonellae (Chijioke and Christian, 2013). It was also shown that *E. coli* strains can efficiently exchange genetic material with pathogen such as *Salmonella, Shigella, Yersina* and *Vibrio* species as well as pathogenic *E. coli*. This could explain the ease with which resistance develop in *E. coli*. The data generated in this study sound a warning because the indiscriminate use of antibiotics along with poor hygiene and infection

control are highly prevalent in Chad and others developing countries.

A recent study reported a prevalence of 1.3% of ESBL-producing *E. coli* in Morocco (Barguigua et al., 2011). A similar study conducted In Nigeria showed a prevalence of 26.4% ESBL-producers in Ebonyi State (Iroha et al., 2009) and 15% in Kano in North West Nigeria (Yusuf et al., 2013). It was 14.3% in Yaoundé (Cameroon).

As far as the other continents are concerned, the prevalence of ESBL-producing isolates of *E. coli* were 0.7% in Bosnia and Herzegovina (Uzunovic-Kamberovic et al., 2006), 9.2% in Korea, 10.3% in Arabia, 13.3% in Lebanon and 17% in Turkey (Ananthan and Subha, 2005). Other data have shown that ESBL-producing *E. coli* are found to be the highest, 60% in India (Hsueh et al., 2011) and 57.8% in Israel (Colodner et al., 2004), followed by Hong Kong (48%) and Singapore (33%) (Hsueh et al., 2011).

In vitro antimicrobial susceptibility revealed a rate of resistance of 100% to cephalosporins (CRO, CTX). This rate is much higher than the ones reported in Nigeria, where the rate of resistance in *E. coli* to ceftriaxon was 74.5%. However, our results were similar with the data obtained in Spain (Colodner et al., 2004). In other work, human *E. coli* isolates recovered in 1997 showed resistance to ceftiofur and ceftriazone. The same isolates were also resistant to other antimicrobial drugs. Moreover, studies showing decreased susceptibilities to ceftiofur and ceftriaxone showed carriage of *blacmy* allele that conferred resistance to cephalothin, ampicillin and amoxicillin/clavulanic acid in *Salmonella* (Shaohua et al., 2005).

The co-resistance to thrimetoprim-sulfamethoxazole was the most common co-resistance phenotype (95%) followed by resistance to Nalidic acid (79%), ciproflaxin (70%), gentamicin (62%), amoxicillin (58%) and amikacin (38%). However, cefoxitin and imipenem were the most effective antibiotics tested.

Co-resistance to different antibiotics within the same isolate, as detected in this study has also been reported in other countries. The ESBL producers were resistant to different antibiotics families including the β-lactams, fluoroquinolons, aminoglycosides and trimethoprim /sulfamethoxazole. The resistance to amino-glycosides was also significant with gentamicin (62%) and the amikacin (38%). Similar rate of resistant to gentamicin were reported in Nigeria, 80% in UNTN and 87% in ESUTH (Iroha et al., 2009). For the amikacin, 25% of resistances have been reported in Israel (Bishara et al., 2005).

As far as quinolone are concerned, high level resistance were observed with nalidixic acid (79%) and ciprofloxacin (70%). In Soudan, these rates were 72% to nalidixic acid and 58.4% to ciprofloxacin (Ibrahim et al., 2012). The rate of resistance to ciprofloxacin can be compared to the data obtained in Israel, Spain, London and Nigeria which were 72.05, 77, 91.3 and 80.9%, respectively (Aruna and Mobashshera, 2012; Iroha et al., 2009; Melzer and Petersen, 2012; Tamayo et al., 2007).

Trimethoprim-sulfamethoxazole resistance was among the highest in our study (95%). Similar rates have been reported in several studies: 91% in Nigeria, 88.3% in Soudan (Ibrahim et al., 2012) 81% in Pakistan (Ullah et al., 2009) and 82% in India (Supriya et al., 2004). In France, resistance to trimethoprim-sulfamethoxazole in *E. coli* varies from 50 to 80% (Goldstein, 2006). The combination of trimethoprime/sulfametoxazole is extensively used in Chad owing to its antimicrobial spectrum of activity and its low cost (Goldstein, 2006). In addition, ESBL-production is usually associated with resistance to non β-lactam antibiotic such as aminoglycosides, fluroquinolones and trimethoprim/sulfamethoxazole (Ibrahim et al., 2012). It is most likely that the selective pressure generated by overuse could explain the relatively high prevalence of resistance in *E. coli*.

ESBL-producing *E. coli* shows simultaneous resistance to both β-lactam and antibiotic of other group are defined as multidrug resistant strain (Chan-Tompkins, 2011). It was shown that resistant genes for β-lactams are often located in mobile genetic elements such as plasmids and integrons, whereby the horizontal transfer of these genes is possible not only in bacteria of the same species but also between bacteria of different species (Bush et al., 2008; Dominika et al., 2014). This characteristic location of genes responsible for resistance could explain the high prevalence of ESBL-producers among strains of *E. coli* observed in this work

Interestingly, our data indicated that infection with ESBL-producing *E. coli* was significantly higher in adults than in children. Similar results have been reported (Jahad et al., 2005). Other work using multivariate analysis (Johnson and Wichern, 2007) demonstrated that age over 60 years was found to be an independent risk factor for infection of ESBL-producing bacteria. The explanation behind these results is not clear, but it is likely that factors such as immunity status and host-microbe interactions need to be taken into account.

Due to the study design, our investigations have certain limitations because the work concerns only one of the five hospitals in N'djamena. Therefore, the data collected cannot be considered representative of N'djamena population. Also, patients' information was limited because we have no data for prior antimicrobial drug exposure. These could bring to the possibility of selection bias.

Conclusion

Despite these limitations, our study provides foundational information for resistance development in Chad. Indeed, our data demonstrated a high prevalence of ESBL-producing *E. coli* resistant to β-lactams, quinolones, aminoglycosides and sulfonamides. This is a clear

indication of a high trend increased multi-drug resistance in *E. coli* in Chad. Further characterization of these strains will help to throw light on the underlying molecular mechanisms of resistance. However, cefoxitin and imipenem were found to be the most effective antibiotics tested that can be used in treating infections caused by *E. coli* and other related bacteria in Chad. The future usefulness of these drugs will depend on the rational and judicious use of antimicrobial agents.

Conflict of interests

The authors did not declare any conflict of interest.

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Haemaglutination of *Shigella dysenteriae* subunit pili protein with anti-haemaglutination of *S. dysenteriae* subunit pili protein as a molecule adhesion in mouse enterocytes

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Shigellosis is a global human health problem. Unfortunately, there has not been any effective vaccine available worldwide. Molecule adhesion of bacteria can be used as a component of vaccine as it facilitates attachment to a cell surface structure in which the adhesion molecule is located in the pili. The purpose of this study was to clarify whether the protein subunit pili *Shigella dysenteriae* has a molecule adhesin. The purification of pili was done by isolating them using a pilus bacterium cutter. Purification of protein subunit pili resulting from SDS-PAGE was obtained by an electro-elution method. Adherent assays for mice enterocyte were used by conducting in a dose dependent manner and by doing an immuno-cytochemistry. The purified pili proteins with MW 49.8 kDa showed a haemagglutinin towards mouse erythrocytes. The pili proteins with MW 7.9 kDa showed an anti-haemaggutinin if added to a haemaggutinin pili proteins with MW 49.8 kDa and can prevent haemagglutination. Furthermore, pretreatment of the enterocytes purified with MW 49.8 and 7.9 kDa pili proteins, the adherence of *S. dysenteriae* to mouse enterocytes was inhibited. Immuno-cytochemistry showed that haemaggutinin protein with MW 49.8 kDa and anti-haemagglutinin protein with MW 7.9 kDa adhered to mouse erythrocyte. These results suggest that haemaggutinin protein with MW 49.8 kDa and anti-haemaggutinin protein with MW 7.9 kDa of *S. dysenteriae* pili proteins are adhesive proteins involved in the *S. dysenteriae* initial adherence mechanisms for the enterocytes.

Key words: *Shigella dysenteriae*, protein subunit pili, haemagglutinin, molecule adhesion.

INTRODUCTION

Diarrheal diseases remain a major cause of morbidity and mortality in all age groups in impoverished areas of South East Asia (Agtini et al., 2007; Kosek et al., 2003; Herwana et al., 2010). Shigellosis is a type of diarrhae

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due to *Shigella* spp. The annual number of Shigella episodes throughout the world was estimated to be 164.7 million, of which 163.2 million were in developing countries (with 1.1 million deaths) and 1.5 million in industrialized countries (Winickoff et al., 1999).

Shigellosis accounts for 70% of morbidity cases, 60% of which cause mortality mostly amongst children under 5 years of age. In Indonesia, diarrhea is the third leading cause of overall morbidity and the leading cause of infant mortality (Nazir et al., 1985). In a study conducted from 1997 to 1999, *Shigella* spp. was found to be the most frequent isolated organism from diarrhea patients in a community setting in the slums of Jakarta, the capital of Indonesia (Oyofo et al., 2002a, b).

In order to reduce the number of cases of diarrhea, it is critical to develop a vaccine for Shigellosis. Unfortunately, there has been no report on the development of adhesion molecule base of Shigellosis vaccine so far (Levine et al., 2007; Kweon et al., 2008). One of the molecule adhesins containing vaccine is a pertussis vaccine. The pertussis vaccine contains pertactin, the *Bordetella pertussis* adhesin molecule which has a molecular weight of 65 kDa (Poolman and Hallander, 2007).

Infection is initiated by ingestion of shigellae (usually via fecal-oral contamination) (Hale and Keusch, 1996). *Shigella* organisms can survive transit through the stomach since they are less susceptible to acid than other bacteria; for this reason, as few as 10 to 100 organisms can cause disease (Bennish, 1991). There are some major steps before it acquires its pathogenicity. The initial step is to attach to enterocyte (Todar, 2012). Then, the bacteria can enter the enterocyte or colonize on the suface of the enterocyte. During this step, the bacteria produces enterotoxin shiga toxin and verotoxin – which is also produced by *Escherichia coli* which causes diarrhea (Leelaporn et al., 2003).

The adherence of *Vibrio cholerae El Tor* into enterocyte uses fimbriae or pili (Ehara et al., 1987). This study aimed at clarifying whether pili *S. dysenteriae* has a role in the adhesion process into the enterocyte of mice.

MATERIALS AND METHODES

Subject

The bacteria used for this research is *S. dysenteriae* in which no process of typing was obtained from Surabaya Referral Laboratory, East Java Indonesia.

Culture conditions and isolation of pili protein

Our previous study with a slight modification was done for pili isolation (Sumarno et al., 2012). The pili were harvested and collected from cultures of bacteria that grew on every bottle of TCG medium that had been incubated. This medium contained 0.02% thioproline; 0.3% NaHCO 3, 0.1% mono sodium 1-glutamate, 1% bactotryptone; 0.2% yeast extract, 0.5% NaCl, 2% bacto agar and 1 mM β amino-ethyl ether-N,N,N'n',-tetra acid (EGTA). Centrifuged pellets were taken and resuspended with PBS pH 7.4. The bacterial suspension was then sheared by using pili bacteria cutter. The cutting was performed using the speed specifications for 6 cycles; respectively (from 1 - 6): 5,000 rpm for 30 s; 5,000 rpm for 1 min; 5000 rpm for 2 min; 10,000 rpm for 1 min; 10,000 rpm for 2 min; and lastly 10,000 rpm for 2 min. After each cycle, the sample was centrifuged with a speed of 6,000 rpm for 30 min at 4°C. The supernatants containing pili pieces were transferred into an Eppendorf. The pellets were then re-suspended with PBS pH 7.4 and continued to the next cycle of pili cutter. After the last cycle, all the supernatants-containing pili proteins were centrifuged at 12,000 rpm for 15 min at 4°C. The supernatants were transferred (containing fractions of pili) to Eppendorf and stored at -20°C.

Sodium dodecyl sulfat polyacrylamide electrophoresis (SDS-PAGE)

Obtaining the weight of molecules is mostly done by using SDS-PAGE (Laemli, 19770). Protein sample was heated at 100°C for 5 min in a buffer solution containing 5 mM Tris pH 6.8: 5% 2-mercapto ethanol, 2.5 w/v sodium dodecyl sulfate, 10% v/v glycerol tracking gel 4%. The applied voltage electric current is 120 mV. The color protein, coomassie brilliant blue was used along with sigma standard low range molecular marker. After the calculation of molecular weight of the proteins, multiplications for protein of interest were done in six gels SDS-PAGE product.

Pili protein purification

Purification of protein HA pilus refers to the method of electro-elusion (Agustina et al., 2012). Results of pili collection were run for electrophoresis by using SDS-PAGE. The result of gel electrophoresis gave us the characterization of pili proteins. Bands of interest were cut perpendicularly so that each piece contained one protein band. The cut bands were collected and inserted into a piece of membrane tape which was filled with an electrophoresis running buffer. The membrane was put in a horizontal electrophoresis apparatus, taking 90 min with 120 mV. Following this, the membrane tape was dialyzed with PBS pH 7.4 fluid buffer for 28 h with the replacement of the buffer 4 times in between.

The haemagglutination assay

Haemagglutination assay was done based on Hanne and Findkelstein (1982) method. Duplo dilution of the sample was made of several concentrations on microplate having 96 wells with V bottom hole where each well had the volume of 100 µl. Each well was added with a suspension of red blood of bulb-c 50 ml with concentrations of 0.5% volume shaken using a rotator plate for 1 min. Subsequently, the plate was placed at room temperature for 1 h. The titer was determined by observing the agglutination of red blood on the lowest dilution. The tested samples were the whole *S. dysenteriae* cell, and pili protein of interest that has been extracted through the first up to sixth cycle of pili cutter and purified. Red bloods were taken from healthy Balb/c mice.

Mouse enterocytes preparation

Balb/c enterocytes were prepared by the Weiser (1973) method. Briefly, the small intestine was excised from a mouse. The intestine was slit open and cleaned from any mucus and excreta with PBS containing 1.0 mM dithiothreitol (DTT) at 4°C. The intestinal tissue was placed in a solution (pH 7.3) containing 1.5 mM KCl, 9.6 mM NaCl, 27.0 mM sodium citrate, 8.0 mM KH_2PO_4 and 5.6 mM Na_2HPO_4 and incubated at 37°C for 15 min with gentle shaking

resulting in supernatant. The supernatant rich with tissue was taken, transferred into PBS containing 1.5 mM EDTA and 0.5 mM DTT and incubated for a further 15 min at 37°C with vigorous shaking. Following the process, the centrifugations at 1,500 rpm for 5 min took place and this process was repeated three or more times until the clean supernatant was obtained. Furthermore, the supernatant was removed, and the sediment was added with PBS.

The isolated enterocytes were collected by centrifugation at 1500 rpm for 5 min and suspended in PBS containing 1% bovine serum albumin (BSA) with concentration of approximately 10^6/ml. A number of enterocytes were counted with a hemocytometer. Enterocytes were kept at 4°C until they were used in the adherence assays.

Assay of adherence to mouse enterocytes

Strains of *S. dysenteriae* grown in BHI broth for 24 h at 37°C were harvested and suspended in PBS containing 1% BSA with concentration of approximately 10^8/ml. One hundred micro liter of the bacterial suspension was mixed with 100 ml of a suspension of 10^6 mouse enterocytes per ml in PBS containing 1% BSA. The mixture was allowed to incubate at 37°C for 30 min with gentle shaking, and then non-adherent bacteria were removed by repeating the washing with PBS containing 1% BSA. The enterocytes were collected by centrifugation at 1500 rpm for 2 min, suspended in 300 µl of PBS.

Twenty micro liters of the sample suspension was extracted and put on a glass slide to form a smear. Smear was stained by Gram and adhesion index was calculated by microscopic observation.

Assay of inhibition adherence to mouse enterocytes

Strains of *S. dysenteriae* grown in BHI broth for 24 h at 37°C were harvested and suspended in PBS containing 1% BSA with concentration of approximately 10^8/ml. 100 µl of the bacterial suspension was mixed with 100 ml of a suspension of 10^6 mouse enterocytes/ml and 100 µl of purified pili protein in PBS containing 1% BSA. The mixture was allowed to incubate at 37°C for 30 min with gentle shaking, and then non adherent bacteria were removed by repeated washing with PBS containing 1% BSA in the centrifugation process. The enterocytes were collected by centrifugation at 1,500 rpm for 2 min and suspended in 300 µl of PBS.

Twenty µl of the sample suspension was extracted and put into a glass slide to form a smear. Smear was stained by Gram and adhesion index was calculated by microscopic observation through dose-dependent interaction (Nagayama et al., 1995).

Gram staining

Staining was done to see the big picture and the description of enterocytes morphology and *S. dyesnteriae* adhesion on enterocyte cells. Slides were covered by using crystal violet for 20 s and rinsed with water. Gram's iodine was applied for 1 min and followed by washing with 95% ethyl alcohol down the slides. Before and after covering with safranin for 20 s, slides were rinsed by using water. Once the slides were dry, observation was done under a microscope of 1000x magnification.

Isolation of antibody anti protein HA

Protein HA of *S. dysenteriae* subunit pili MW 49.8 kDa was used as an antigen. Balb/c was immunized with the 100 µg antigen which was emulsified with Complete Freud's Ajuvant in 100 ul PBS through intra peritoneal route. Moreover, another similar antigen was emulsified with Incomplete Freud's Ajuvant which was given three times in every week as booster injection. The anti body produced was then isolated from the mouse blood serum (Harlow and Lane, 1988).

Immunocytochemistry to detect molecule adhesion

The concentration of erythrocytes was adjusted until the amount was sufficient to examine under a microscope. Eppendorf tube containing sufficient concentration of the erythrocytes was added with 500 µl sample of *S. dysentriae* subunit pili protein anti HA MW 7.9 kDa. Then Eppendorf tube was placed in a shaker water bath with the temperature of 37°C for one hour. To wash the protein which did not adhere to erythrocytes, the sample tube was centrifuged at 1000 rpm, temperature 4°C for 15 minwith PBS for three times. The final volume of Eppendorf was adjusted to 500 µl. 20 µl sample from Eppendorf was placed on glass to be dried in room temperature.

Methanol solution was used to fix the sample on the glass object twice. Every 5 min, the sample was washed with H_2O for 3 times. Then H_2O_2 3% was dropped on the surface of glass object and was incubated for 10 min in room temperature. After that, the sample was washed with PBS pH 7.4. To block nonspecific protein, the NGS 1% was added to the solution for one hour. Blocking solution was taken by using the filter paper**.**

The sample was given primary antibodies (serum rich of anti HA protein) and was incubated at room temperature over night. After incubation period has finished, the sample was cleaned up by PBS pH 7.4 every 5 min for three times. After that, a secondary antibody labeled alkali phosphatase was dropped on it and the mixture was incubated at room temperature for 60 min. The sample was washed with pH 7.4, three times every 5 min and then SA-HRP was dropped on it and incubated for 40 min. In the next washing process, the PBS pH 7.4 was used to wash the mixture every 5 min for three times. It was floated with DAB for 3 min, and washed again every 5 min with H_2O three times. Methylene green was used as the counter stain. It was then washed with tap water and after that it was dried at room temperature and was ready for examination (Sakanaka et al., 1988).

RESULTS

Identification of pili *S. dysenteriae* proteins

SDS-PAGE was used to identify the proteins in *S. dysenetriae* pili. The profile of the result showed that the cutting of pili subjected to SDS-PAGE was similar to our previous study (the data is not shown).

The result of profile of pili proteins from the 1st (Sp1) to 5th cycle is depicted in Figure 1. Using this result, molecular weight (MW) of the bands was calculated by using linear regression. Bands ranged from 7.9 to 117.7 kDa, the most intense bands weighed 7.9, 11.2, 27.3, 49.8, 85 and 117.7 kDa (Figure 2A). In relation to the production of proteins of interest (weighed at 7.9, 11.2, 27.3, 49.8 and 85 kDa), there was a consistency and complete separation seen throughout the SDS-PAGE. Figure 1B shows the result of isolation by doing electroelution of protein subunit pili with MW 49.8 (P2) and 7.9 (P5) kDa *S. dysenteriae*.

For the subsequent experiments, pili proteins from the

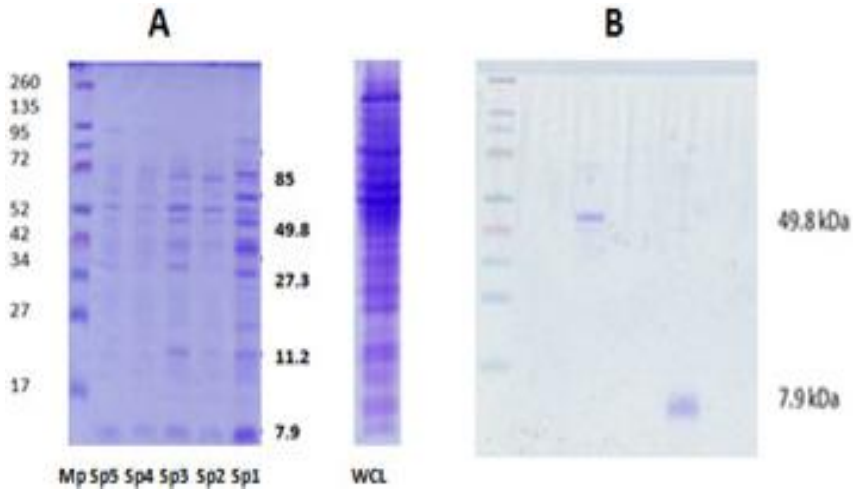

Figure 1. A: Profile of pili proteins from the 1st cycle (Sp1) to 5th used SDS-PAGE. B: The result of purification protein subunit pili with MW 49.8 and 7.9 kDa *S. dysenteriae.*

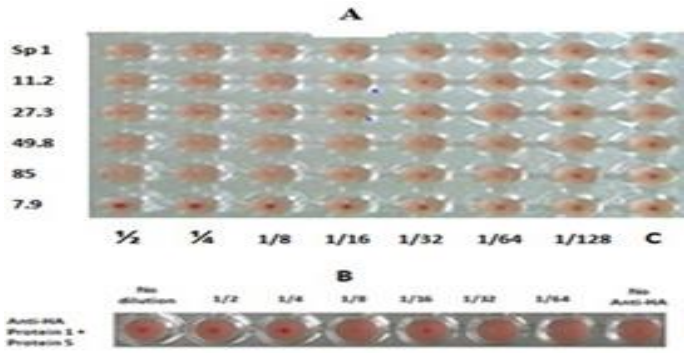

Figure 2. A: HA assay results of protein 1 and 5. B: Anti HA assay (anti-HA) for protein 5 by using protein 1.

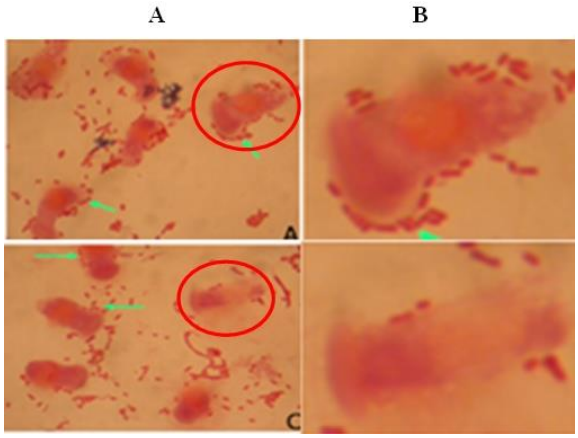

Figure 3. Attachment of *S. dysenteriae* in dose dependent manner of protein HA. 4A dose of protein 1 was 12.5 ug; 4B dose of protein 1 was 50 ug; → Attachment of *S. dysenteriae.*

1st cycle (Sp1) were used as the main source for HA. First HA assay was done by using the supernatant of pili protein from the first through the fifth cycles (Sp1 – Sp5) (data is not shown). The results showed that all of them were HA positive (able to bind to the erythrocytes). Isolation of protein subunit 85 (P1), 49.8 (P2), 27.3 (P3), 11.9 (P4) and 7.9 (P5) kDa was done by electro-elution and tested separately for their agglutination property. The interesting result observed was that P5 was not able to bind the erythrocytes, forming sediment at the bottom of the plate with dilution of 1 1/128. The sedimentation rate was faster than control (erythrocytes without protein pili). However, P1 was positive for HA, meaning that the protein was able to bind the erythrocytes, stopping it from forming sediments at the bottom of the plate.

To demonstrate directly that P1 and P5 have the capacity to adhere to mouse enterocytes, the inhibitory effects of the purified P1 and P5 prepared from pili fraction were examined by incorporating these preparations into the test of number bacterial attachment and amount of protein concentration. The pretreatment of mouse enterocytes with the purified P1 and P5 to mask the receptor involved in the adherence of bacteria resulted in an inhibition of further adherence of *S. dysenteriae* to mice enterocytes ($P<0.05$) (Figure 3). The ability found in P1 and P5 is adhesins therefore using the principle antigen antibody in a dose dependent test was performed.

Using P1 and P5 with concentrations of 100, 50, 25, 12.5 and 0 µg, we found that at 100 µg the adhesion index is significantly inhibited ($P<0.01$) as compared to 0 µg or control. As the concentration is decreased to 200 µg, the inhibition for both proteins further decreased though no significance was found as compared to 400 µg.

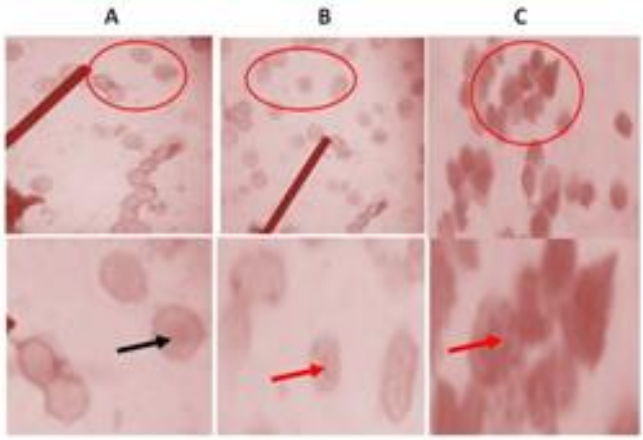

Figure 4. The attachment of P1 and P5 *S. dysentriae* on the surface of erythrocyte can be seen by using immunocytochemistry method with antibody of anti-protein P1. A: The erythrocyte was taken from C (control); B: The erythrocyte was taken from P5 (1/2 dilution), no agglutination; C The erythrocyte was taken from P1 (1/2 dilution), with agglutination; ⟶ no dots on the surface of erythrocytes (protein 7.9 kDa is not present); ⟶ dots on the surface of erythrocytes (protein 7.9 and 49.8 kDa is present).

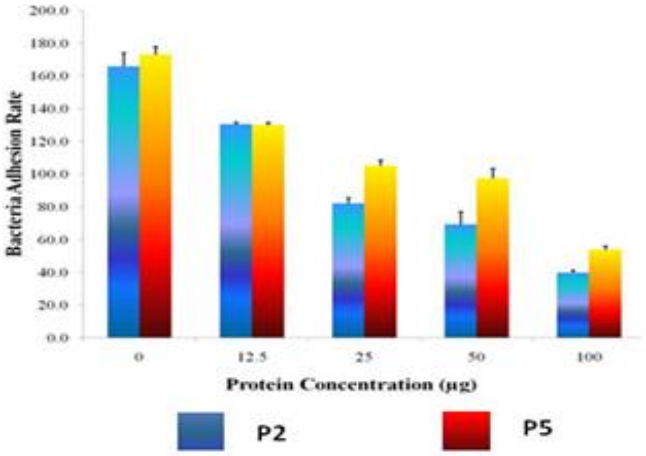

Figure 5. Inhibition of adherence of *S. dysenteriae* to mouse enterocytes by pre-treatment of the purified protein 1 and 5 ± SEM. At 100 µg, bacterial adherence rate significantly decreased as compared to 0 µg for both P2 and P5.

At 100 µg, the adhesion index rate decreased further reaching its maximum capability in inhibition and at this concentration, the difference was significant as compared to all other concentrations for both P1 and P5 (P<0.05).

Further decrease in bacterial concentration (50 µg) afterwards did not have any effect on the adhesion index, in fact the rate increased, leveling up gradually to the value of 0 µg adhesion rate for both P1 and P5. This rate was going up as the concentrations decreased until it reached its peak at 0 µg (Figure 5).

The adhesion rate at 100 µg for both proteins was significantly different as compared to other bacteria concentrations (P<0.01). At 50 µg, the adhesion rate increased and from here onwards as the concentration decreased the adhesion rate increased until it reached its peak at 0 µg or control.

Anti-HA assay was performed to display that P5 has the capacity to inhibit the adherence of protein 1 to mice erythrocyte. P2 that showed negative HA at dilution 1/64 was used in the entire well (HA titer 1/32). P2 with dilution 1/16 was used and were put on entire wells. After that, P5 at dilution of 1 up to 1/64 were added to entire wells. The result shown in Figure 2B and P5 has anti-HA (not able to bind to the enterocytes) with the titer 1/16 (no dot at the dilution 1/32).

To show that P5 and P1 *S. dysenteriae* were attached to erythrocyte was confirmed by immunocytochemistry as seen in Figure 4.

DISCUSSION

Bacteria are able to resist the cleansing action of solutes to colonize the epithelial tissues and infect the underlying tissues through attachment to mucous surfaces (Gibson, 1973). The tissue and cell specific adherence of bacteria are key aspects for the bacterium-host cell interaction. Association between bacteria and mucous surfaces does not occur randomly but specifically for each species (Beachey, 1981). The results from studies clearly confirm that the attachment of *Enterobacteriacae* strains with a given set up of adhesion varies with different target cells, that is, depending on the availability of receptors. For a given target cell, on the other hand, bacterial binding will occur only if the appropriate adhesion is present on the bacterial surface. Many of the adhesion involved in the adherence of the bacterium towards host cell enables the bacteria to agglutinate erythrocytes of different species (Beachey, 1981).

To the best of our knowledge, no study have ever looked specifically into the pili components that act as HA and adhesion. Previously, it was shown that *S. dysenteriae* of different strains can cause HA and some of the strains were unable to agglutinate the erythrocytes due to the growth condition that causes them to have almost no HA pili expression (Qadri et al., 1989). However, none has tried to find out the protein part of pili that acts as HA and anti HA adhesion of their protein profiling.

Prior to the profiling of the pili protein using SDS-PAGE, isolation of the protein pili is necessarily done using the pili bacterial cutter. Based on the profiling, we observed that the 1st to 5th cycle of pili cutter is enough to obtain almost all the pili on the bacterium (Figure 1A). The supernatant of the 1st cycle is used for subsequent test as it gives a strong and clear band and contains abundant amount of *S. dysenteriae* pili.

Many *Enterobacteriacae* can cause agglutination of erythrocytes, but previous investigations have not proven

Table 1. Average adherence rate of *S dysenteriae* after dose-dependent manner of pretreatment of P1 and P5.

Protein concentration (µg)	Protein	Average	Standard Deviation	Standard Error Mean
100	Protein1	39.8	5.3	1.7^{A}
	Protein5	54.0	6.5	2.1^{1}
50	Protein1	69.4	24.8	7.8^{B}
	Protein5	97.4	19.7	6.2^{2}
25	Protein1	82.3	10.9	3.4^{B}
	Protein5	105.1	11.2	3.5^{2}
12.5	Protein1	130.5	4.4	1.4^{C}
	Protein5	130.1	4.9	1.5^{3}
6.125	Protein1	166.0	25.4	8.0^{D}
	Protein5	173.0	15.2	4.8^{4}

Letter $^{A-D}$ and $^{1-4}$ indicate significant difference ($P<0.05$), whereas the same letter or number are no significant different.

which components of the *Shigella* bacteria are responsible. We used a strain of *S. dysenteriae* which causes HA in mice enterocyte cells. Pili were purified from these bacteria by shearing them from the bacteria. These pili are purified by electro-elution of SDS-PAGE (Figure 1B).

We tested the complete pili proteins (un-purified) for its agglutination properties and some of them did not have any agglutination properties (data is not shown). This is due to the presence of other proteins on the pili that can bind towards each enterocyte but without HA and can inhibit the HA proteins subunit pili to exert their effect (Figure 2A). However, when the band is purified individually (P1), it exhibits HA properties which by linear regression method has an MW of 49.8 kDa. This was the protein that caused the agglutination of the erythrocytes. The use of the purified pili confirmed that HA is caused by the P1 alone. Although the mode of such agglutination is unclear, it is likely that a specific recognition binding event occurs as found in lectin binding of sugar molecules since saccharides with only a limited range of configurations inhibit binding. Such saccharides are presumed to resemble or in some cases are identical to residues available for binding of pili on the mammalian cell membrane (Sharon, 1987). It is concluded that the purification of pili P1 by electro-elution that causes HA of erythrocytes by binding to such sugar molecules on the erythrocyte surface shouldbe clarified. Meanwhile P5, with MW of 7.9 kDa is a protein that enables a bigger and faster formation of erythrocyte sediments as compared to control (Figure 2A). This protein can attach to enterocyte and without agglutination, it is assumed that P5 indeed inhibits HA properties to P1 (competitive reaction) (Figure 2B).

The results of this study strongly suggest that the pili are responsible also for the attachment to epithelial cells of *S. dysenteriae* tested in this study. The ability of *S. dysenteriae* to adhere on mice enterocytes is correlated to the presence of pili on the bacteria. Therefore, a dose dependent manner of the adhesion assay was performed (Figure 3). At concentration of 100 µg, the protein 1 (results from electro-elution) pre-treated mouse enterocytes showed a significant decrease of bacterial adherence as compared to the control (0 µg) ($P<0.05$). As the concentration of protein 1 decreased we expected the adhesion rate of the bacterium would slowly increase until it reached its peak at 0 µg. At 50 µg, adhesion rate of the bacterium increased and at 25 µg, the rate leveled up gradually towards the 0 µg adhesion rate. As the concentration further decreased to 12.5 µg, the adhesion rate increased closely to the adhesion rate of those with 0 µg. Finally, at 0 µg, the rate reached its peak as the bacteria had their full adhesion towards the enterocytes. These results were also observed in P5 in dose dependent manner. At 100 µg, pre-treated enterocytes with P5 had its lowest bacterial adhesion rate which was significantly different as compared to 50, 25, 12.5 and 0 µg. Further decrease in the concentration of P5 pre-treated enterocytes did not lower the adhesion rate of the bacterium but rather increased it slowly until it reached its peak at 0 µg.

S. dysenteriae attachment towards mice enterocytes was significantly inhibited by P1 and P5 ($P<0.05$). This result strongly suggested that P1 and P5 are adhesions, while P5 is anti HA (no erythrocyte agglutination) and can inhibit agglutination of P1 subunit pili *S. dysenteriae* (Table 1 and Figure 5). However, this result alone is insufficient to claim that they are indeed adhesions.

P5 protein anti HA subunit pili with MW 7.9 kDa to protein HA *S. dysenteriae* is a molecule adhesion to erythrocyte improved by immunocytochemistry method (Figure 4).

Recently, Mitra et al. (2012) have found that hemagglutinating activity is directly correlated with colonization ability of *Shigellae* in suckling mouse model.

Maybe we can make clarification of the profile of *Shigella* sero group haemaglutinating activity protein subunit pili with MW of 49.8 kDa and anti- haemaglutination

protein subunit pili with MW of 7.9 kDa.

Accellular pertussis vaccines currently available has one or more different components pertussis toxin, filamentous haemagglutinin (FHA), 69 kDa protein (also know a pertactin/adhesion molecule), fimbrial-2 and fimbrial-3 antigens in different concentrations, and with different adsorption to different adjuvants (WHO, 2009).

Thus, the haemaggutinin protein with MW 49.8 kDa and anti-haemaggutinin protein with MW 7.9 kDa of pili *S. dysenteriae* proteins are adhesive proteins HA bacterial pili that can serves as component of Shigellosis vaccine and useful model system for the early determination of mechanism of bacterial pili attachment to cell membranes of the host cell.

Conclusion

Based on this study, it can be concluded that subunit pili proteins of *S. dysenteriae* have two kinds of protein. They are P1 with MW 49.8 kDa which has haemagglutinin capacity towards mice erythtocytes, and P5 with MW 7.9 kDa which has anti-haemagglutinin capacity towards P1 with MW 49.8 kDa, making both of them adhesion molecules in enterocyte. This finding, by far, suggests that these two proteins are prime candidates for vaccine development of *S. dysenteriae.* This research was approved by the ethical commitee of Medical Faculty, University of Brawijaya, Malang, Indonesia.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

In vitro antimicrobial activity of ethanolic seeds extract of *Nigella sativa* (Linn) in Sudan

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***Nigella sativa* Lin. (family- Ranunculaceae) is a widely used medicinal plant globally and popular in various Indigenous system of medicines. The seeds are used as astringent, stimulant, diuretics and anthelmintic traditionally. They are also useful for treating jaundice, intermittent fever, dyspepsia, paralysis, piles and skin disorders. The ethanolic extracts of *N. sativa* (seeds) were tested against four standard bacteria, that is, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and against two standard fungi species, that is,. *Aspergillus niger* and *Candida albicans* using the agar plate diffusion method. The ethanolic extracts of *N. sativa* (seeds) exhibited inhibitory effects against most of the tested organisms with the zone of inhibition ranging from 18 to 32 mm in length. The largest inhibition zone in the case of bacteria was obtained for against bacteria *E. coli* (32 mm) while in case of fungi highest inhibition was observed against *Apergillus niger* (25 mm). Thus, it can be concluded from the present study that *N. sativa* possess both antibacterial as well as antifungal activity.**

Key words: *In vitro*, antimicrobial activity, *Nigella sativa* (seeds), Sudan.

INTRODUCTION

Nigella sativa is a herbaceous plant found in the Middle East, Europe as well as the Western and Middle Asia. Its seeds have a great medicinal importance and have been reported to exhibit many pharmacological properties that

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Figure 1. Laboratory sample of *Nigella sativa* seeds.

include antiparasitic, antimicrobial anti-oxidant and anti-inflammatory activities (Ali and Blunden, 2003). Its oil is used as condiment, carminative, food preservative, analgesic and in the treatment of many ailments in different parts of world (Salem, 2005).

The seeds of *N. sativa* have been used traditionally for centuries in the Middle East, Northern Africa and South Asia for the treatment of various diseases as mentioned (Brutis and Bucar, 2000; Gilani et al., 2004). As such, *N. sativa* is a natural remedy against many diseases and the aromatic seeds are extensively used as spice, carminative and condiment traditionally, the seeds have been used as adiuretic, diasphoretic, stomachic and liver tonic. As a combination with other ingredients, they are used in diarrhoea (Mansour et al., 2002), indigestion, dyspepsia and sour belching; they also remove foul breath and watering from the mouth, the seeds of *N. sativa* are consumed with buttermilk to cure obstinate hiccups and are also useful in treating loss of appetite, vomiting and dropsy, in different combinations, the seeds have been used in obesity and dyspnoea as well, they have antibilious property and are administered internally in intermittent fevers (Usmanghani et al., 1997). The herbs of *N. sativa* has been regarded as a valuable remedy in hepatic and digestive disorders, constant inhalation of fried seeds relieves cold and catarrh. They have also been used in chronic headache and migraine (Evans, 1996). The decoction of seeds with some sweet oil forms a useful application or ointment in skin diseases, the seeds have been useful in mercury poisoning, sores and leprosy (Evans, 1996). Brayed in water, its application removes swelling from hands and feet, *N. sativa* is also used externally in leucoderma, alopecia, eczema, freckles and pimples (Usmanghani et al., 1997). The purpose of the present study was to evaluate the antimicrobial activity of *N. sativa* against few common bacterial and fungal species.

MATERIALS AND METHODS

The *N. sativa* seeds were collected from central Sudan (Khartoum) between January and February 2008. The plant was identified and authenticated by the taxonomists of Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI) (Figure 1). The *N. sativa* seeds were air-dried, in shade with good ventilation and then ground finely in a ml until their use for extracts preparation.

Preparation of crude extracts

Extraction was carried out from the seeds of *N. sativa* (Seeds) plant by using overnight maceration techniques (Harborne, 1984). About 50 g of round seeds material was macerated in 250 ml of ethanol for 3 h at room temperature. With occasional shaking for 24 h at room temperature, the supernatant was decanted and filtered through a filter paper. After filtration, the solvent was then removed under reduced pressure by rotary evaporator at 55°C. Each residue was weighed and the yield percentage was calculated and then stored at 4°C in tightly sealed glass vial ready for further use. The remaining extract which was not soluble was successively extracted by ethanol. Using the previous technique, extracts were kept in deep freezer for 48 h, and then induced in freeze dryer (Virtis, USA) until completely dried. The residue was weighed and the yield percentage was calculated. The extracts were kept in 4°C until the time of their use.

Test microorganisms

The ethanolic extract of *N. sativa* seeds were tested against four bacterial species: two Gram-positive bacteria viz., *Bacillus subtilis* (NCTC 8236) and *Staphylococcus aureus* (ATCC 25923), two Gram-negative bacterial strains *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). Also, the same was tested against two fungal strains viz, *Aspergillus niger* (ATCC 9763) and *Candida albicans* (ATCC 7596). The bacterial and fungal strains used in the study were obtained from the Department of Microbiology, Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI) and National Health Laboratory of Khartoum in Sudan.

The bacterial cultures were maintained on nutrient agar and incubated at 37°C for 18 h and then used in the antimicrobial test.

Table 1. The antimicrobial activity of *N. sativa* seeds against the standard bacterial and fungal.

Standard microorganisms	Concentration (mg/ml)			
	Mean diameter of growth inhibition zone (mm)			
	100	**50**	**25**	**12.5**
Tested bacteria used (M.D.I.Z. mm)				
Bacillus subtilis	20	20	19	18
Staphyococcus aureus	18	17	16	16
Escherichia coli	32	25	22	20
Pseudomonas aeruginosa	20	19	18	16
Tested fungi used (M.D.I.Z. mm)				
Apergillus niger	25	22	20	19
Candida albicans	21	20	19	18

Interpretation of results: MDIZ (mm) : >18 mm: sensitive, 14 to 18 mm: intermediate: <14 mm: resistant. (-): no inhibition.

In vitro testing of extracts for antimicrobial activity

Antibacterial testing: The cup-plate agar diffusion method as reported (Kavanagh, 1972) was used adopted with some minor modifications to assess the antibacterial activity of the prepared extracts. 1 ml of the standardized bacterial stock suspension (between 10^8 and 10^9 CFU/ml) was thoroughly mixed with 100 ml of molten sterile nutrient agar which was maintained at 45°C. 20 ml aliquots of the inoculated nutrient agar were distributed into sterile Petri-dish plates. The agar was left to set and in all of these plates, 4 cups (10 mm in diameter) were cut using a sterile cork borer (No. 4) and agar discs were removed. Each cup was filled with 0.1 ml sample of the ethanolic extracts using an automatic microlitre pipette, and thereafter the extracts were allowed to diffuse at room temperature for two hours. The plates were then incubated in an upright position at 37°C for 18 h. Two replicates were carried out for each extract against each of the test organisms. After incubation, the diameters of the resultant growth inhibition zones were measured and averaged. The mean values were tabulated.

Antifungal testing: The same method used for the antibacterial test was employed. However, the growth media used in case of fungi, was Sabouraud dextrose agar instead of nutrient agar. The inoculated medium was incubated at 25°C for two days for *C. albicans* and three days for *A. niger*.

RESULTS AND DISCUSSION

The seeds of *N. sativa* family (Ranunculaceae) were screened for antimicrobial activity against two Gram positive bacteria (*B. subtilis*, *S. aureus*), two gram negative bacteria (*E. coli*, *P. aeruginosa*) as well as two fungi (*A. niger* and *C. albicans*) using the cup plate agar diffusion method screened.

The extracts obtained from the seeds of *N. sativa* exerted a pronounced activity against all bacteria and fungi strains tested. This was indicated by diameter of growth inhibition zones that varied from 18 to 32 mm. This result was similar to an earlier report (Khalid et al., 2011). These authors found that methanolic and aqueous extracts of the seeds of *N. sativa* possessed antibacterial activity against *B. subtilis, Entereococcus faecalis, P. aeruginosa, S. aureus* and *Salmonella typhi*.

Previous study on *N. sativa* crude plant extracts and phytoconstituents also supports the fact that *N. sativa* is active against various pathogens. In the past, many researchers investigated the antimicrobial potential of *N. sativa*. They found that *N. sativa* ethanolic extracts was active against a tested standard microorganism and multidrug resistant strains of bacteria (Salman et al., 2005). Our results were in agreement with those reported by Ani et al. (2006), which indicated that *N. sativa* has a significant antibacterial potential against *B. subtilis* and *B. cereus*. Thus, *N. sativa* with an array of polyphenolic compounds, possess antibacterial activity (Ani et al., 2006). In 1975, the purified compound thymohydroquinone (THQ) from *N. sativa* oil (NSO) was found to possess a high antimicrobial effect against Gram positive microorganisms (El-Fatatry, 1975). In later studies, the seed extracts of *N. sativa* were found to inhibit the growth of *E. coli*, *B. subtilis* and *Streptococcus feacalis* (Saxena and Vyas, 1986).

The antimicrobial activity of *N. sativa* was further established against several species of pathogenic bacteria and yeast (Topozada et al., 1965; Hanafy and Hatem, 1991). The result of minimum inhibition concentration from Table 1 shows that 12.5 µg/ml was the lowest concentration at which all the tested micro-organisms were inhibited. A comparison of observation given in Tables 2 and 3, show that the seed extracts of *N. sativa* dissolved in dimethyl sulphoxide inhibited all bacteria higher than 40 µg/ml ampicillin and a lower concentration of gentamicin. The seed extracts of *N. sativa* inhibited *E. coli* at 40 µg/ml, which was similar to the result obtained for gentamicin. The seeds extracts of *N. sativa* inhibited *A. niger* with a higher than 20 µg/ml of Clotrimazole, and inhibited *C. albicans* at more than 50 µg/ml of Nystatin. It is clear from Table 1 that the ethanolic extract of *N. sativa*

Table 2. Antimicrobial activity of *N. sativa* seeds against the standard bacterial and fungal species.

Standard microorganisms	Mean diameter of growth inhibition zone (mm)
Tested bacteria used (M.D.I.Z. mm)	
Bacillus subtilis	20
Staphyococcus aureus	18
Escherichia coli	32
Pseudomonas aerugino	20
Tested fungi used (M.D.I.Z. mm)	
Apergillus niger	25
Candida albicans	21

Interpretation of results: MDIZ (mm) : >18 mm: sensitive, 14 to 18 mm: intermediate: <14 mm: resistant. (-): no inhibition.

Table 3. Antibacterial and antifungal activity of reference antibiotics against standard microorganisms.

Drugs	Concentrations (µg/ml)	Standard microorganisms used MDIZ* (mm)			
		Gram (+ve)		Gram (-ve)	
		Tested bacteria used (M.D.I.Z. mm)			
		Bacillus subtilis	***Staphyococcus aureus***	***Escherichia coli***	***Pseudomonas aeruginosa***
Ampicillin	40	15	25	-	16
	20	14	20	-	13
	10	13	18	-	12
	5	12	15	-	-
Gentamicin	40	29	35	32	23
	20	22	33	30	22
	10	20	30	17	21
	5	17	28	-	19
Tested fungi used (M.D.I.Z. mm)					
		Aspergillus niger		***Candida albicans***	
Clotrimazole	40	30		42	
	20	22		40	
	10	19		33	
	5	16		30	
Nystatin	50	28		17	
	25	26		14	
	12.5	23		-	

MDIZ (mm) = Mean diameter of growth inhibition zone in mm. Interpretation of results: MDIZ (mm); >18 mm: sensitive, 14 to 18 mm: intermediate, <14 mm: resistant. (-): no inhibition.

seeds show a high activity against all bacteria and fungi.

Conclusion

Ethanolic seeds extract of *N. sativa* produced antimicrobial activity against all organisms tested. This study observes that *N. sativa* has useful antimicrobial properties. Further investigations regarding the mode of action and other related pharmacological studies such as *in vivo* investigation, drug formulation and clinical trials are highly recommended.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Screening of *Trichoderma* species for virulence efficacy on seven most predominant phytopathogens

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***In vitro* studies on the efficacy of *Trichoderma* species against phytopathogens revealed the antagonistic potential of eight different species of *Trichoderma* isolated from the rhizosphere soils of varied locations of Uttar Pradesh and were evaluated *in vitro* against the most widely occurring soil inhabiting plant pathogens viz., *Fusarium oxysporum* f. sp. *ciceri*, *Alternaria solani*, *Phytopathora infestans, Pythium aphanidermatum, Sclerotium rolfsii, Bipolaris sorokiyana* and, *Rhizoctonia solani* and identify the most potential and effective strains *Trichoderma* with high antagonistic activity. Eight species of *Trichoderma* highly inhibited the growth of the seven test phytopathogens by producing volatile compounds showing variability in antagonistic potential of different *Trichoderma* spp. against the different pathogens tested. The test antagonists grow faster than the pathogen and produced inhibition zones. These antagonistic interactions influence the incidence and severity of the disease caused by the pathogen. The present communication describes the impact of different *Trichoderma spp*. on growth inhibition of plant pathogens under *in vitro* conditions. The data revealed that *Trichoderma reesei* (Tr(CSAU*)* showed the maximum inhibition percentage of mycelial growth was recorded as against *Scelerotium rolfsii* (69.14%) followed by *Bipolaris sorokiniana* (80.33%), *Alternaria brasiciae* (83.3%), while, in case of remaining plant pathogens such as *Pythium aphanidermatum*, *Fusarium oxysporum* f. sp. *ciceri*, *Trichoderma harzianum (Th.azad)* were reduced the highest radial mycelium growth of *Pythium aphanidermatum*, *Fusarium oxysporum* f. sp. *ciceri* (85 and 80%) respectively. Apart from *Trichoderma reesei* and *Trichoderma harzianum* (*Th.azad*), *Trichoderma viride* (01PP) also inhibited the mycelial growth of *Phytophthora infestans* (80.83%) and *Rhizoctonia solani (*70.42%*).*

Key words: Antagonism, *Trichoderma*, phytopathogens.

INTRODUCTION

Plants are a major source of food, fodder, medicines and many other useful products for humans. Diseases are the important biotic causes for low crop yield and poor quality seed. Pathogens being soil and seed borne, possess a great problem in disease management. Soil borne diseases are difficult to control and seed treatment with fungicides does not protect the crop for longer periods. Continuous use of the same fungicide against the same pathogen results in the development of fungicide resistant strains of the pathogen (Shanmugam and Varma, 1998; Kumar and Dubey, 2001; Mamgain et al., 2013). Moreover, chemical measures may establish imbalances in the

microbiological community for the activity of beneficial organisms which otherwise improve the crop health. The demand for alternative to chemical control of plant pathogens has become stronger owing to the concerns about the safety and environmental aspects of chemicals. However, biological control offers the chance to improve crop production within the existing resources, besides avoiding the problem of pesticide resistance (Dekker, 1976; Khan et al., 2014). The genus *Trichoderma* is common filamentous imperfect fungi (*Deuteromycetes*), the most common saprophyte in the rhizospehere and foun in almost all soils. Characterization for the antagonistic potential of *Trichoderma* spp. is the first step in utilizing the full potential of *Trichoderma* spp. for specific applications. *In vitro* screening of different pathogens is an effective and rapid method for identifying species with antagonistic potential. *Trichoderma*, a filamentous soil borne mycoparasitic fungus, has been shown to be effective against many soil borne plant pathogens (Papavizas, 1985; Pan et al., 2001; Jash and Pan, 2004) as they have more than one mechanism of action. Therefore, the study was conducted to evaluate the antagonistic activity of eight different *Trichoderma* species viz., *Trichoderma viride*, *T. harzianum*, *T. reesei*, *T. atroviride, T. asperellum, T. koningii, T. longibrachiatum* and *T. virens*, in inhibiting the growth of some most widely occurring soil inhabiting plant pathogens viz., *Fusarium oxysporum* f. sp. *ciceri*, *Alternaria solani*, *Pythium aphanidermatum, Phytopathora infastans*, *sclerotium rolfsii , bipolaris sorokiayana Rhizoctonia solani* and identify *Trichoderma* spp. with a high antagonistic potential. Biological control of plant pathogens by microorganisms has been considered a more natural and environmentally acceptable alternative to the existing chemical treatment methods. *Trichoderma* spp. is now the most common fungal biological control agents that have been comprehensively researched and deployed throughout the world. Several fungal cell wall degrading enzymes, amongst them chitinase and glucanase, which seem to play an important role in the antagonistic action of *Trichoderma* against a wide range of fungal plant pathogens. The present study aimed to find out the efficiency of *Trichoderma* spp. against some phytopathogens.

MATERIALS AND METHODS

Isolation and purification of Trichoderma species

The isolation of eight *Trichoderma* spp. from rhizospheric zone of soil through serial dilution plate techniques (Johnson and Curl, 1972) on modified *Trichoderma* Selective Medium (TSM) (Saha and Pan, 1997). The green coloured colonies were identified by slide culture technique and compared with taxonomic key of Rifai (1969) at genus and species level and deposit to ITCC division of plant pathology New Delhi for reconfirmation. After confirmation cultures of *Trichoderma* spp. were maintained on PDA slants and stored in the refrigerator at 4ºC for further studies.

Isolation of plant pathogens

The pathogens were isolated from disease plants showing symptoms of disease. These isolated pathogens were identified, purified and tested for pathogenicity (Tapwal et al., 2011). The hyperparasitic potential of eight *Trichoderma* species were screened *in vitro* against seven test plant pathogens viz., *Fusarium oxysporum* f. sp. *ciceri*, *Alternaria solani*, *Rhizoctonia solani, Pythium aphanidermatum, Phytopathora infastans*, *sclerotium rolfsii and bipolaris sorokiayana* by dual culture plate technique and production of volatile and non-volatile metabolite part of Petri plate with respective test pathogen on the upper lid of plate served as control. Three replicates were maintained for each treatment. The assembly was opened after 72 h and the observations were recorded by measuring colony diameter of the test pathogen (in mm) in each plate and that of the control plates.

Efficacy of *Trichoderma* spp. on growth of the pathogens by dual-culture plate method

For testing antagonism in dual culture method (Morton and Stroube, 1955), a mycelial disc (6 mm) was cut from the margins of actively growing region of seven day old cultures of *Trichoderma* spp. and inoculated at one end of the petriplates (1 cm away from the edge of the plate) with sterilized potato dextrose agar (PDA) medium and simultaneously at the opposite end of a mycelial disc (6 mm) of the test pathogens. The experiments were conducted with three replications/plates for each treatment, while control plates were inoculated only by tested fungus. Plates were then incubated at 27 ± 1°C. Observation were recorded after seven days of inoculation including area covered by the *Trichoderma* spp. (eight *Trichoderma* spp.) and the pathogen while percent of inhibition was calculated using the following formula (Vincent, 1947):

$$\text{Percent growth of inhibition} = \frac{\text{colony growth in control plate-colony growth in intersecting plates}}{\text{colony growth in control}} \times 100$$

RESULTS

Our results explain that significant success in biocontrol is achieved under *in vitro* conditions. It is evident from the data presented in Table 1 and showed in Figures 1, 2 and 3, that the *Trichoderma* spp. suppressed the radial growth of different phytopathogens significantly on potato dextrose agar medium in the dual culture. *Trichoderma* spp. isolated from the rhizosphere soils of different location of Uttar Pradesh identified and confirmed on the basis of morphological and physiological characterization and micrometry observations revealed that they belong to eight different species viz., *Trichoderma viride,*

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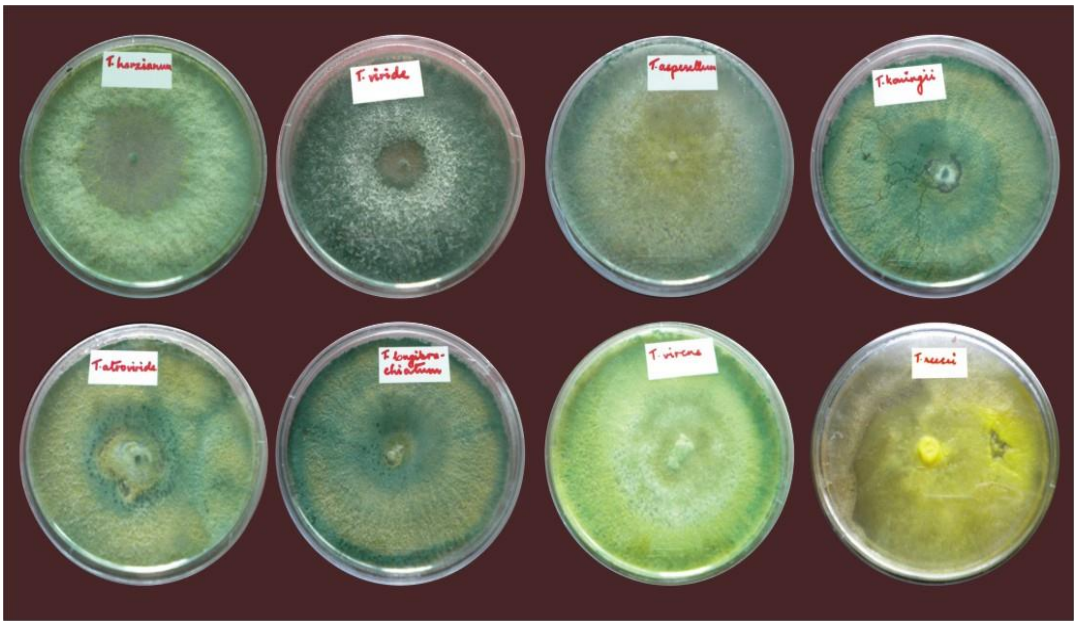

Figure 1. Eight *Trichoderma* spp. isolated from different locations of Uttar Pradesh.

Figure 2. Seven predominant plant pathogens.

T. harzianum, T. reesei, *T. atroviride, T. koningii*, *T. asperllum*, *T. virens, T. longibrachiatum* and *T. viride* was the most predominant species. The morphological and physiological characterization of these antagonistic species was accomplished on the basis of colony color, growth rate, texture, growth patterns, size of phialides and phialospores.

Eight *Trichoderma* spp. were tested against most

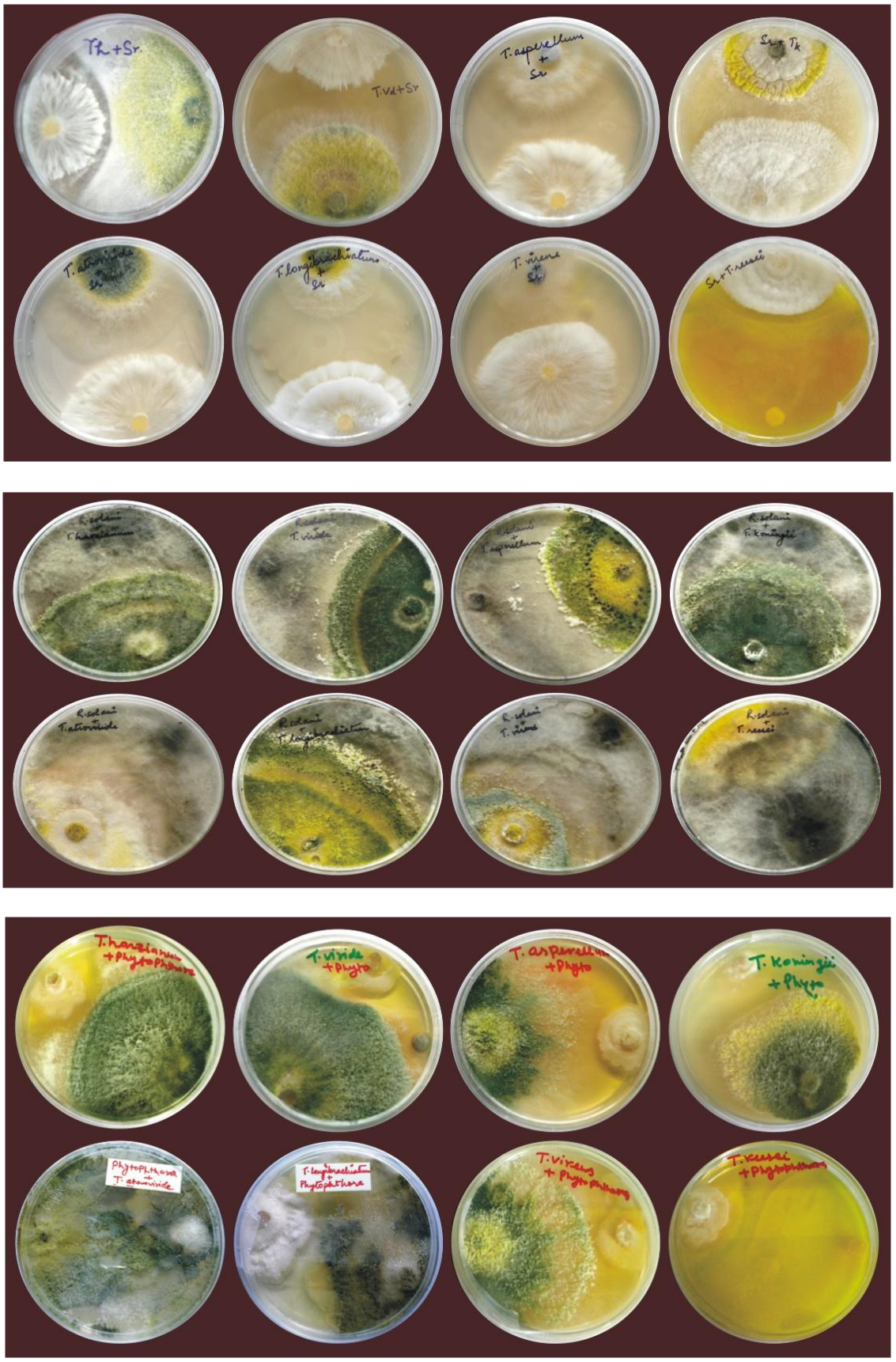

Figure 3. Confrontation test of *Trichoderma* spp. against notable plant pathogens.

Table 1. Submission of the gene sequences at NCBI database

Strain No.	Name of Bioagent	Strain code	ITCC Acc. No	NCBI GenBank Accession No.	NBAIM, Mau	Source	GPS Location
T1	*T. harzianum*	*Th* azad	6796	KC800922	TF-1271	CSA Kanpur Nagar	Latitude: 25˚ 8′ 34.821″ Longitude: 81˚ 59′ 2.979″
T2	*T. viride*	01PP	8315	JX119211	TF-1272	Hardoi	Latitude: 27˚ 23′ 40.729″ Longitude: 80˚ 7′ 47.751″
T3	*T. asperellum*	T_{asp}/CSAU	8940	KC800921	TF-1270	CSA Kanpur Nagar	Latitude: 25˚ 8′ 34.821″ Longitude: 81˚ 59′ 2.979″
T4	*T. koningii*	T_K (CSAU)	5201	KC800923	TF-1269	CSA Kanpur Nagar	Latitude: 26˚ 29′ 33.384″ Longitude: 80˚ 18′ 6.518″
T5	*T. atroviride*	71 L	7445	KC 008065	TF-1268	Hardoi	Latitude: 26˚ 29′ 28.323″ Longitude: 80˚ 18′ 26.361″
T6	*T. longibrachiatum*	21 PP	7437	JX978542	TF-1267	Kaushambi	Latitude: 26˚ 34′ 27.61″ Longitude: 79˚ 18′ 24.623″
T7	*T. virens*	T_{vi} (CSAU)	4177	KC800924	TF-1266	CSA Kanpur Nagar	Latitude: 25˚ 21′ 39.794″ Longitude: 81˚ 24′ 11.414″
T8	*T.reesei*	*T.r(CSAU)*	8372	KM999966	TF-1273	CSA farm	Latitude: 25˚ 21′ 39.794″ Longitude: 81˚ 24′ 11.414″

predominant seven phytopathogens in dual culture plates such as *Scelrotium rolfsii, Alterneria brasicae, Pythium aphanidermatum, Phytophthora infestans, Alterneria brasiciae, Bipolaris sorokiniana* and *Fusarium* oxysporum f. sp. cicero (Table 2). Among the eight *Trichoderma* spp. *Trichoderma reesei (Tr(CSAU)* shows that maximum inhibition percentage of mycelial growth of pathogen was recorded (69.14%) against *Scelerotium rolfsii* and minimum inhibition percentage was recorded (42.85%) by *Trichoderma atroviride* (71L)*,* Similarly *Trichoderma viride* (01PP) found that maximum inhibition percentage of mycelial growth of pathogen was recorded (70.42%) against *Rhizoctonia solani* and minimum inhibition percentage was recorded (56.25%) by *Trichoderma atroviride* (71L)*, Trichoderma harzianum (*Th. azad*)* revealed that maximum inhibition percentage of mycelial growth of pathogen was recorded (85.00%) against *Pythium aphanidermatum* and minimum inhibition percentage was observed (55.55%) by *Trichoderma reesei (Tr(CSAU), Trichoderma viride* (01PP) revealed that maximum inhibition percentage of mycelial growth of pathogen was recorded (80.83%) against *Phytophthora infestans* and minimum inhibition percentage was observed (66.25%) by *Trichoderma koningii (T_k(CSAU), Trichoderma reesei (*Tr(CSAU) revealed that maximum inhibition percentage of mycelial growth of pathogen was recorded (83.33%) against *Alterneria brasiciae* and minimum inhibition percentage was observed (70.37%) by *Trichoderma atroviride* (71L)*, Trichoderma asperellum (*T_{asp}/CSAU) revealed that maximum inhibition percentage of mycelial growth of pathogen was recorded (73.00%) against *Bipolaris sorokiniana* and minimum inhibition percentage was observed (65.00%) by *Trichoderma atroviride* (71L)*, Trichoderma harzianum (*Th. azad) revealed that maximum inhibition percentage of mycelial growth of pathogen was recorded (80.00%) against *Fusarium oxysporum* f.sp. *ciceri* and minimum inhibition percentage was observed (60.00%) by *Trichoderma reesei (Tr(CSAU).* From the above facts it was concluded that *Trichoderma harzianum* (Th. azad) shows the maximum inhibition against pytopathogens; *Pythium aphanidermatum* and *Fusarium oxysporum* f.sp. *ciceri,* bioagents *Trichoderma viride* (01PP) shows the maximum inhibition against *Rhizoctonia solani* and *Phytophthora infestans, Trichoderma atroviride* (71 L) shows the minimum inihibition against four pytopathogens namely; *Sclerotium rolfsii, Rhizoctonia solani, Alterneria brasiciae* and

Table 2. Antagonistic activity of *Trichoderma* spp. against seven *phytopathogens* by dual culture method.

Name of Bioagent	Culture No.	Source/ District	Id. No.	Average growth (mm)	% inhibition growth (mm)	Average growth (mm)	% inhibition growth (mm)	Average growth (mm)	% inhibition growth (mm)	Average growth (mm)	% inhibition growth (mm)	Average growth (mm)	% inhibition growth (mm)	Average growth (mm)	% inhibition growth (mm)	Average growth (mm)	% inhibition growth (mm)
				Sclerotium rolfsii		*Rhizoctonia solani* (Soyabean)		*Pythium aphanidermatum*		*Phytophthora infestans*		*Alterneria brasiciae*		*Bipolaris sorokiniana*		*Fusarium oxysporum f.sp. ciceri (CSAU)*	
T. harzianum	*Th* azad	CSA Kanpur Nagar	6796	34.68	50.48	25.00	68.75	13.33	85.00	21.60	72.92	16.66	81.48	26.00	65.50	45.00	80.00
T. viride	01PP	Hardoi	8315	35.00	50.00	23.66	70.42	19.00	78.88	15.33	80.83	20.00	77.70	23.00	71.25	54.00	76.00
T. asperellum	T_{asp}/CSAU	CSA Kanpur Nagar	8940	33.30	52.38	28.33	64.58	25.66	71.48	25.00	68.75	25.00	72.22	21.00	73.00	78.00	65.30
T. koningii	T_K (CSAU)	CSA Kanpur Nagar	5201	36.66	47.62	27.66	65.42	21.00	76.66	27.00	66.25	21.66	75.93	24.60	69.25	54.00	76.00
T. atroviride	71 L	Hardoi	7445	40.00	42.85	35.00	56.25	25.00	72.22	16.60	79.17	26.66	70.37	28.00	65.00	66.00	70.60
T. longibrachiatum	21 PP	Kaushambi	7437	33.33	52.38	28.00	65.00	15.66	82.60	20.0	75.00	20.50	77.22	27.60	65.50	75.00	66.60
T. virens	T_{vi} (CSAU)	CSA Kanpur Nagar	4177	33.33	52.38	31.00	61.25	17.33	80.74	23.00	71.25	18.33	70.63	23.30	70.80	60.00	73.30
T. reesei	Tr(CSAU)	CSA Kanpur Nagar	7284	21.66	69.14	26.66	66.66	40.00	55.55	15.00	80.33	15.00	83.33	22.00	72.50	90.00	60.00

Bipolaris sorokiniana and finally, *Trichoderma reesei (Tr(CSAU)* shows the maximum inhibition against *Sclerotium rolfsii, Alterneria brasiciae* and minimum inhibition against *Pythium aphanidermatum, Fusarium oxysporum f.sp. cicero,* respectively.

Experimental design and statistical analysis

Statistical analysis was performed following completely randomized block design (CRBD) with three replicates in each treatment.

DISCUSSION

In the study it may be concluded that among the eight different species of *Trichoderma* exhibited different growth inhibition percentage against the tested most predominant phytopathogens with variability in the antagonistic potential. *Trichoderma reesei, Trichoderma harzianum* (*T.azad*), and also *Trichoderma viride* (01PP) showed high antagonistic potential against tested phytopathogens. Plant pathogenic fungi are a widespread problem and the use of chemicals is hardly successful (Anand and Jayarama, 2009). However, the high cost associated with the use of chemical (fungicides) to control disease caused by soil borne fungi is a limiting factor in the profitability of crop production, in this case biological control could be the best alternative. *Trichoderma* is the most commonly used fungal biological control agent and have long been known as effective antagonists against plant pathogenic fungi (Chet et al., 1981; Papavizas, 1985). Some of the species of *Trichoderma* included in the present study significantly inhibited several pathogens (Dubey, 2003).

All the *Trichoderma* spp. restricted the growth of

all the seven test phytopathogens in their own way. Thus, it is well known that all species isolated from different samples of soil are not equally antagonistic to phyto-pathogen and searching of effective and potential species to locally suit the purpose is important.

The results reported, suggests that the species of *T. harzianum, T. viride, T. reesei* and *T. atroviride* were more capable of influencing the growth of tested seven pathogens in dual culture. Similarly, isolates of different *Trichoderma* spp. to control soil borne phytopathogens have been reported to differ in their effectiveness (Rama and Krishna, 2000; Anand and Jayarama, 2009; Singh et al., 2013; Kumar et al., 2014). This result is a pioneer information that particular isolate from a particular location can be employed in bulk for treatment of disease incidence.

Conclusion

In our study it was concluded that *Trichoderma harzianum* (Th. azad) show the maximum inhibition against pytopathogens; *Pythium aphanidermatum* and *Fusarium oxysporum* f.sp. *ciceri,* bioagents *Trichoderma viride* (01PP) shows the maximum inhibition against *Rhizoctonia solani* and *Phytophthora infestans* while *Trichoderma atroviride* (71 L) shows the minimum inhibition against four pytopathogens namely; *Sclerotium rolfsii, Rhizoctonia solani, Alterneria brasiciae* and *Bipolaris sorokiniana.* Finally, *Trichoderma reesei (Tr(CSAU)* shows the maximum inihibiton against *Sclerotium rolfsii, Alterneria brasiciae* and minimum inhibition against *Pythium aphanidermatum, Fusarium oxysporum f.sp. cicero,* respectively.

Conflict of interests

The authors did not declare any conflict of interest.

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Molecular characterization and *in vitro* evaluation of endophytic bacteria against major pathogens of rice

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Thirty one isolates of endophytic bacteria were collected from different plant sources together with *Bacillus subtilis* var*. amyloliquefaciens* (FZB24) that was obtained from Novozymes South Asia Pvt. Ltd. to screen against major pathogens of rice. All the isolates were characterized on the basis of biochemical and phenotypic analysis. Therefore, following these tests, it can be concluded that 31 isolates exhibited differences and they were subjected to partial 16S-rDNA gene sequencing using polymerase chain reaction for phylogenetic analysis. The molecular characterization through amplification of 16S rDNA fragment to an amplicon size of 546 bp confirmed that the thirty one isolates were *Bacillus*. The PCR analysis showed that all the 31 isolates were found to have the genes for iturin A, 30 isolates for surfactin, 27 isolates for bacillomycin D and one isolate was found to have gene for zwittermicin A. The biosynthetic gene for the production of ACC deaminase was also identified among 11 endophytic *Bacillus* isolates. The random amplified polymorphic DNA (RAPD) analysis showed more similarity among the isolates isolated from the same ecosystem as compared to the isolates collected from different ecosystems. Among the endophytic bacterial isolates tested against the major pathogens of rice *viz*., *Pyricularia grisea, Rhizoctonia solani* and *Xanthomonas oryzae*pv. *oryzae in vitro*, *Bacillus subtilis* var. *amyloliquefaciens* (FZB24) was found to be effective in inhibiting growth of all the three pathogens and it was also found to promote the growth of rice seedling by registering significantly higher vigour index in roll towel method.

Key words: Endophyte, *Bacillus*, antagonistic activity, antibiotic gene, random amplified polymorphic DNA (RAPD).

INTRODUCTION

Plants are constantly involved in interactions with a wide range of bacteria. These plant-associated bacteria colonize the rhizosphere (rhizobacteria), the phyllosphere (epiphytes) and inside the plant tissues (endophytes). Endophytes are sheltered from environmental stresses and microbial competition by the host plant and they

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seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots and seeds of various plant species (Kobayashi and Palumbo, 2000). Several bacterial endophytes have been shown to support plant growth and increase nutrient uptake by providing phytohormones (Kang et al., 2007) and biologically fixing nitrogen (Jha and Kumar, 2007). Endophytic bacteria cannot only promote plant growth and act as biocontrol agents, but also produce antibiotics to control plant diseases and reduce disease severity (Senthilkumar et al., 2007). Plants have latent defense mechanism against pathogens, which can be systemically activated upon exposure of plants to stress or infection by pathogens (Baker et al., 1997). This phenomenon is called induced systemic resistance (Tuzun and Kuc, 1991). The classical inducers include pathogens, plant growth promoting rhizobacteria (PGPR), plant growth promoting endophytic bacteria (PGPE), chemicals and plant products. Some endophytes offer increased resistance to pathogens thus making them ideal candidates for biological control (Madhaiyan et al., 2004).

Polymerase chain reaction (PCR) based detection is a favoured approach as it is accurate, rapid and sensitive. The small subunit 16s rDNA sequence has shown to be useful for the detection of bacteria (Stead et al., 1997). Many *Bacillus* species are capable of producing a wide variety of secondary metabolites that are diverse in structure and function. The production of metabolites with antimicrobial activity is one determinant of their ability to control plant diseases (Silo-suh et al., 1994). Antibiotics from Iturin family, *viz*., fengycin with limited antibacterial activity, show strong antifungal and haemolytic activities which is specific against filamentous fungi (Nishikiori et al., 1986). Surfactin shows antiviral and antimycoplasma activities (Vollenbroich et al., 1997). Zwittermicin A has a broad spectrum activity against certain Gram-negative and eukaryotic microorganisms (Silo-suh et al., 1998). With this background, endophytic bacteria were evaluated *in vitro* against the major rice pathogens viz., *Pyricularia grisea, Rhizoctonia solani* and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). And also, the antibiotic genes in their genome for the synthesis of antibiotics were detected. Variability among the endonphytic bacteria were studied using RAPD analysis.

MATERIALS AND METHODS

Isolation of endophytes

Source plants from different ecosysytem were manually uprooted and brought to the laboratory. Root, stem and leaf sections (2-3 cm long) were made using a sterile scalpel. The root samples were taken just below the soil line for younger plants and 5-10 cm below the soil line for older plants. Stem samples were first weighed and surface sterilized with hydrogen peroxide (20%) for 10 min. and rinsed four times with 0.02 M potassium phosphate buffer (pH 7.0). Root samples were surface disinfected with sodium hypochlorite (1.05%) and washed in four changes of 0.02 M phosphate buffer solution. Measured quantity of 0.1 ml aliquot from the final buffer wash was removed and transferred into 9.9 ml tryptic soya broth to serve as sterile check. Samples were discarded, if growth was detected in the sterile check within 48 h. Selected samples were triturated in 9.9 ml of buffer in sterile pestle and mortar. The triturate was serially diluted in potassium phosphate buffer solution and plated on Tryptic Soya Agar (TSA). Representatives of colony morphology were transferred to fresh TSA plated as pure cultures (McInroy and Kloepper, 1995).

Preparation of bacterial inoculum

Endophytic bacteria were grown on King's B (KB) with constant shaking at 100 g for 48 h at room temperature (28±2°C). Bacterial cells were harvested by centrifugation at 12,000 g for 15 min and bacterial cells were resuspended in phosphate buffer (PB) (0.01 M, pH 7.0). The concentration was adjusted to approximately 10^8 cfu ml^{-1} (OD595 = 0.3) with a spectrophotometer and used as bacterial inoculum (Thompson, 1996).

Seed bacterization

Rice seeds (cv. ADT 39) were surface-sterilized with 2% sodium hypochlorite for 30 se, rinsed in sterile distilled water and dried overnight under a sterile air stream. Endophytic bacterial strains, inoculated into their respective broths and bacterial suspension was prepared as mentioned above. The required quantity of seeds was soaked in bacterial suspension containing 3×10^8 bacteria ml^{-1} for 2 h and dried under shade.

Plant-growth promotion

The plant-growth promoting activity of the bacterial endophytic strains was assessed on the basis of seedling vigour index as determined by the standard roll towel method (ISTA, 1999). Twenty seeds were kept on presoaked germination paper. The seeds were held in position with another presoaked germination paper strip on top of them and gently pressed. The polythene sheet along with the seeds was then rolled and incubated in a growth chamber for 14 days. Three replications were carried out for each treatment. The root and shoot length of individual seedlings was measured and seed germination percentage calculated. The vigour index was calculated using the formula of Baki and Anderson (1973):

Vigour index = germination (%) × seedling length (shoot length + root length)

Antagonism of endophytic bacterial strains against *P. grisea*

Endophytic bacterial strains were tested for their antagonistic activity against mycelial growth of *P. grisea* and *R. solani* by following the dual culture technique (Dennis and Webster, 1971). Mycelialdisc (8 mm diameter) of seven days old culture of pathogens were placed at one side of the Petri plate containing PDA medium at 10 mm away from the periphery. Bacterial cultures were streaked onto the medium exactly opposite to the mycelial disc 10 mm away from the periphery. The plates were incubated at room temperature (28±2°C) for 10 days. Efficiency of the antagonistic organisms against the sheath blight pathogen was assessed based on the inhibition zone observed.

Antagonism of endophytic bacterial strains against *Xoo*

Cell suspension of *Xoo* was prepared in the sterile distilled water to

Table 1. Sequences of oligonucleotide primers.

Antibiotic gene	Primer	Sequence	Amplicon size (bp)
Iturin A	ITUD1F	5'GATGCGATCTCCTTGGATGT3'	647
	ITUD1R	5'ATCGTCATGTGCTGCTTGAG3'	
Surfactin	SUR3F	5'ACAGTATGGAGGCATGGTC3'	441
	SUR3R	5'TTCCGCCACTTTTTCAGTTT3'	
Zwittermicin A	ZWITF2	5'TTGGGAGAATATACAGCTCT3'	779
	ZWITR1	5'GACCTTTTGAAATGGGCGTA3'	
Bacillomycin D	BACC1F	5'GAAGGACACGGCAGAGAGTC3'	875
	BACC1R	5'CGCTGATGACTGTTCATGCT3'	
ACC deaminase	ACCD F	5'ATGAACCTGCAACGATTC3'	1000
	ACCD R	5'TCAGCCGTCTC GGAAGAT3'	

Table 2. Sequences of RAPD primers used to study the genetic variability among isolates of endophytic *Bacillus*.

S/N	Primer	Sequence
1	OPA 01	5'CAGGCCCTTC3'
2	OPA 08	5'GTGACGTAGG3'
3	OPB 11	5'GTAGACCCGT3'
4	OPB15	5'GGAGGGTGTT3'
5	OPG 5	5'CTGAGACGGA3'
6	OPG 11	5'TGCCCGTCGT3'
7	OPG 16	5'AGCGTCCTCC3'
8	P7	5'GATAGCTCGCTG3'
9	CAG	5'CAGCAGCAGCAGCAG3'
10	GACA	5'GACAGACAGACAGACA3'

a concentration of 10^7 cfu/ml. 1 ml of the bacterial cell suspension (*Xoo*) was mixed with 19 ml of nutrient agar (NA) medium and poured onto the sterile Petri dishes. After solidification, sterile paper discs (6mm diameter) were placed on the surface of the medium at 1 cm away from the side of the Petri dish and 5 µl of the endophytic bacterial culture in NA broth of 4h old was applied to each disc. The plates were incubated at 37±2°C and the inhibition of bacterial growth was measured 48 h after the treatment (Salah et al., 2010).

Statistical analysis

The present experimental data were analyzed using analysis of variance (ANOVA) by Agres Statistical Software Package Version 3.01 (Agres, 1994).

Characterization of endophytes

Isolation of genomic DNA of endophytes

The genomic DNA from each isolates of endophytes were isolated using the standard protocol of cetyltrimethyl ammonium bromide (CTAB) method proposed by Knapp and Chandlee (1996) with slight modifications (Melody, 1997) from actively grown culture. The genomic DNA was checked by agarose gel electrophoresis and stored at -20°C for further use.

Amplification of 16S rDNA gene was carried out by polymerase chain reaction using an Eppendorf Master cycler, German. Reaction volume of 25 µl, was prepared and mixed in the PCR tubes. Polymerase chain amplification of endophytic bacteria was done by using primers specific BCF 1 (5'CGGGAGGCAGCAGTAGGGAAT3'); and BCR2 (5'CTCCCCAGGCGGAGTGCTTAAT3'). These primers were used to get an amplicon of 546 bp size (Cano et al., 1994). The thermo cycling conditions consisted of a hold of 2 min at 95°C, 40 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C and a final extension of 5 min at 72°C. Amplified fragments of DNA were fractionated on a 1% w/v agarose gel during 100 min at constant voltage of 80 V in 0.5×TAE (Tris-Acetat EDTA). A 10-kb reference marker (campany and country) was used to allow standardization. Following staining with ethidium bromide (10 µg ml^{-1}), the gel was visualized using gel doct (company) under UV light to confirm the expected size of the product. Also PCR reactions were carried out using the methodology established by Ramarathnam et al. (2007) with the antibiotic specific primers and ACC deaminase specific primer (Sheehy et al., 1991) (Table 1).

RAPD-PCR analysis

In total, ten primers were used for RAPD analysis (Table 2). All the RAPD primers were purchased from Operon (Operon Biotechnologies, Cologne, Germany) and used as single primers. Amplification was performed in a 20 ml reaction volume consisting of 5 mM each dNTPs, 20 pmol of primer, 0.5 U of Taq DNA polymerase (Bangalore GeneiPvt Ltd, Banglore, India) and 50 ng of template. The PCR was performed, using Eppendorf – Master Cycler ep gradient S (Eppendorf, A G, Hamburg, Germany), with an initial denaturation step for 5 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 37°C and 2 min at 72°C, with a final extension for 10 min at 72°C. Following amplification, 10 ml of each PCR product was separated by electrophoresis in 2% (w/v) agarose gel in Tris-acetate-EDTA (TAE) buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0). A 1Kb ladder was used as a size standard. To visualize DNA, gels were stained with ethidium bromide (0.1 mg/l) and then photographed under transmitted ultraviolet light, using an AlphaImager 2000 (Alpha Innotech, San Leandro, CA, USA). All RAPD analyses were repeated at least three times for each primer.

Data analysis

The amplified fragments of each isolate were scored as 1 (present) or 0 (absent). Co-migrating bands were considered homologous characters. Faint bands and bands showing variable levels of intensity were not considered for scoring. A similarity matrix was constructed, using Jaccard's coefficient, and the resulting similarity data were used to construct a dendrogram, using UPGMA and the NTSYS-pc software version 2.02 developed by Rohlf (1990).

Table 3. Biochemical characters of collected endophytic bacterial isolates from different plant sources.

S/N	Isolates	Place	Source	Biochemical tests			Tentatively identified as
				Gram staining	KOH	Growth in 7% NaCl	
1	EPB 1	Coimbatore	Rice leaf	+	-	+	*Bacillus* sp.
2	EPB 2	Coimbatore	Rice leaf	+	-	+	*Bacillus* sp.
3	EPB 3	Coimbatore	*Trianthima* Leaf	+	-	+	*Bacillus* sp.
4	EPB 4	Coimbatore	*Trianthima* Leaf	+	-	+	*Bacillus* sp.
5	EPB 5	Coimbatore	*Trianthima* Leaf	+	-	+	*Bacillus* sp.
6	EPB 6	Bavanisagar	*Acalypha* leaf	+	-	+	*Bacillus* sp.
7	EPB 7	Mettur	Greengram leaf	+	-	+	*Bacillus* sp.
8	EPB 8	Bavanisagar	*Aloe* leaf	+	-	+	*Bacillus* sp.
9	EPB 9	Bavanisagar	Nerinji leaf	+	-	+	*Bacillus* sp.
10	EPB 10	Bavanisagar	Nerinji leaf	+	-	+	*Bacillus* sp.
11	EPB 11	Bavanisagar	*Cactus* leaf	+	-	+	*Bacillus* sp.
12	EPB 12	Coimbatore	Noni leaf	+	-	+	*Bacillus* sp.
13	EPB 13	Bavanisagar	*Opuntia* leaf	+	-	+	*Bacillus* sp.
14	EPB 14	Bavanisagar	*Agave* leaf	+	-	+	*Bacillus* sp.
15	FZB 24	Taegro, Novozymes South Asia Pvt. Ltd.		+	-	+	*Bacillus* sp.
16	EPB 15	Coimbatore	Cotton leaf	+	-	+	*Bacillus* sp.
17	EPB 16	Coimbatore	Cotton leaf	+	-	+	*Bacillus* sp.
18	EPB 17	Coimbatore	Cotton leaf	+	-	+	*Bacillus* sp.
19	EPB 18	Mettur	Redgram leaf	+	-	+	*Bacillus* sp.
20	EPB 19	Mettur	Redgram leaf	+	-	+	*Bacillus* sp.
21	EPC 5	Coimbatore	Cotton root	+	-	+	*Bacillus* sp.
22	EPC 8	Veppankulam	Cotton root	+	-	+	*Bacillus* sp.
23	EPCO 16	Aliyar	Coconut root	+	-	+	*Bacillus* sp.
24	EPCO 26	Aliyar	Coconut root	+	-	+	*Bacillus* sp.
25	EPCO 29	Thangachimadam	Coconut root	+	-	+	*Bacillus* sp.
26	EPCO 30	Thangachimadam	Coconut root	+	-	+	*Bacillus* sp.
27	EPCO 74	Aliyar	Coconut root	+	-	+	*Bacillus* sp.
28	EPCO 78	Aliyar	Coconut root	+	-	+	*Bacillus* sp.
29	EPCO 81	Aliyar	Coconut root	+	-	+	*Bacillus* sp.
30	EPCO 95	Veppankulam	Coconut root	+	-	+	*Bacillus* sp.
31	EPCO 96	Veppankulam	Coconut root	+	-	+	*Bacillus* sp.

RESULTS AND DISCUSSION

In the present study, 31 native endophytic bacteria were isolated from different range of plant sources such as rice, *Trianthema*, *Agave*, *Opuntia*, *Aloe*, greengram, *Tribulus*, cotton, redgram and coconut (Table 3). Also, endophytic bacteria appear to originate from seeds, vegetative planting material, rhizosphere soil and the phylloplane. The source of endophytic bacterial colonization is diverse and bacteria enter seeds and vegetative planting material from the surrounding environment such as rhizosphere and phyllosphere. They are found in numerous plant species with most being members of common soil bacterial genera such as *Pseudomonas, Bacillus*, *Paenibacillus*, *Azospirillum, Gluconacetobacter* and *Herbaspirillum* (Nogueira et al., 2001). Many PGPE strains have been isolated from internal tissues of different crops and tested against the plant diseases by several workers (Reiter et al., 2002; Sabaratnam and Beattie, 2003).

Endophytes *viz.,* EPB 18, EPB 11, EPCO 74, FZB24 and EPB 10 registered vigour index of 3343, 3225, 3127, 3035 and 3023 respectively as compared to untreated control that registered only 1168. This shows that endophytic bacteria were found to increase the vigour index of the rice seedlings *in vitro* as compared to the control (Table 4). The mechanisms by which plant growth is improved may be similar to those exhibited by rhizosphere microorganisms and include the production of phytohormones, promotion through enhanced availability of

Table 4. Growth promoting activity of bacterial endophytes on rice seedlings *in vitro.*

SN	Isolate	Root length (cm)*	Shoot length (cm)*	Germination (%)*	Vigour index
1	EPB 1	20.81	7.95	96	2761
2	EPB 2	19.58	7.93	94	2586
3	EPB 3	20.90	7.20	100	2810
4	EPB 4	20.20	7.70	100	2790
5	EPB 5	21.70	7.50	100	2920
6	EPB 6	21.30	9.33	98	3002
7	EPB 7	21.70	8.00	100	2970
8	EPB 8	18.54	8.21	98	2621
9	EPB 9	19.51	8.52	100	2803
10	EPB 10	22.06	8.17	100	3023
11	EPB 11	23.54	8.71	100	3225
12	EPB 12	19.09	8.49	84	2317
13	EPB 13	20.20	8.39	100	2859
14	EPB 14	19.11	8.12	100	2723
15	FZB 24	21.90	8.45	100	3035
16	EPB 15	20.95	8.06	82	2379
17	EPB 16	15.99	8.31	100	2430
18	EPB 17	20.44	8.00	100	2844
19	EPB 18	23.88	9.55	100	3343
20	EPB 19	19.50	8.60	100	2810
21	EPC 5	19.35	8.66	100	2801
22	EPC 8	19.39	7.90	100	2729
23	EPCO 16	20.15	8.39	100	2854
24	EPCO 26	18.87	8.24	96	2603
25	EPCO 29	15.60	6.41	100	2201
26	EPCO 30	12.91	6.87	86	1701
27	EPCO 74	22.63	8.64	100	3127
28	EPCO 78	16.63	8.64	94	2375
29	EPCO 81	20.99	8.48	100	2947
30	EPCO 95	19.51	9.74	100	2925
31	EPCO 96	12.74	8.13	98	2045
32	Control	8.70	5.90	80	1168
SED		1.60	0.47		112.19
CD (0.05)		3.19	0.94		224.28
CD (0.01)		4.25	1.24		298.18
CV		10.13	7.02		5.04

*Mean of three replications.

nutrients, reduction of ethylene levels, production of antibiotics, induced systemic resistance and out competition of pathogens (Holland, 1997). Several reports have indicated that bacterial endophytes promoted the growth and health of crop plants (Sturz et al., 2000).

In this study, endophytic *Bacillus* isolates *viz.,* FZB24, EPB 13, EPCO 95, EPB 8, EPB 11, EPCO 16, EPCO 26 and EPCO 96 EPCO 78, EPC 5, EPB 7, EPB 9, EPB 10, EPB 4, EPB 3, EPB 17, EPCO 29 and EPCO 81 were found to show more than 35% inhibition over control against rice blast pathogen *Pyricularia grisea in vitro* (Table 5).

The *Bacillus* isolates *viz.,* EPB 13, EPB 18, EPB 14, EPB 8, EPB 17, EPB 3, EPB 19, EPB 9, EPB 16, FZB24, EPB 6 and EPB 4 were found to have an inhibition of more than 35% over control against sheath blight pathogen, *R. solani in vitro*. Similarly, the endophytic isolates of *Bacillus viz*., FZB24, EPB 9, EPB 10, EPCO 29 and EPCO 78 significantly inhibited the growth of *X. oryzae* pv. *oryzaein vitro* by registering a inhibition zone of 20.0 mm diameter (Table 5) over the control. These results are in line with the findings of Bhuvaneswari (2005). She found that endophytic *Bacillus* strains *viz.,*

EPBC 68 and EPBC 73 recorded a inhibition zone of 8.3 and 9.7 mm, respectively, significantly inhibited the growth of *X. axonopodis* pv. *malvacearum* in cotton *in vitro* over the control. Sessitsch et al. (2004) screened 35 endophytic isolates, out of which seven isolates showed antagonistic activity against bacterial pathogens *viz. Streptomyces scabies* (43%) and *Xanthomonas campestris* (29%). Endophytic bacterial strain, EPCO 16 from cotton plants effectively inhibited the mycelial growth of *R. solani in vitro* (Rajendran, 2003).

Ting et al. (2003) identified three endophytic bacterial isolates *viz., P. aeruginosa*, *Serratia marcescens* and *Burkholderia glumae* from wild banana plants showing antagonistic activity against *F. oxysporum.* f.sp*. cubense*. Endophytic bacterial strain EPC 5 showed maximum mycelial inhibition of *Ganoderma lucidum* (Rajendran, 2006). Antagonistic strain of *Pseudomonas putida* (B0) isolated from sub-alpine exhibited antifungal activity against phytopathogenic fungi in Petri dish assays and produced chitinase, β-1,3-glucanase, salicylic acid, siderophore and hydrogen cyanide (Pandey et al., 2006). This inhibition process observed *in vitro* was reported to be the secretion of secondary metabolites and antibacterial agents released (Sessitsch et al., 2004). *Bacillus* species have special characteristics that make them good candidates as biological control agents. *Bacillus amyloliquefaciens* isolates produced surfactin, iturin, bacillomycine and azalomycin F, while *B*. *subtilis* isolates mostly synthesize surfactin and arthrobactin. Also surfactin, amphomycin, arthrobactin and valinomycin were found in culture extracts of *B*. *pumilus*isolates. The antagonistic activity found for the metabolites of *Bacillus* spp. associated with the synergistic effect is caused by the combination of antibiotics (Asaka and Shoda, 1996).

Endophytic bacterial strains *viz., Aureobacterium saperdae* and *B. pumilus* showed higher antifungal activity against *Fusarium* wilt in cotton (Chen et al., 1995). Reiter et al. (2002) isolated an endophytic *Clavibacter michiganensis*strain from potato with biocontrol activities against *Erwinia carotovora*. Wulff et al. (2002) reported the antagonistic activity of *B subtilis* and *B. amyloliquefaciens* against black rot of cabbage *in vivo* and the metabolic profiles produced *viz.,* surfactin, iturin, bacillomycin and azalomycin F were responsible for the inhibition of *Xanthomonas campestris* pv. *campestris*. Bhowmik et al. (2002) reported that seed bacterization with endophyte, Endo PR8 was found to be most effective in reducing the cotyledonary infection by *Xam*. With these evidences, it is predicted that production of antibiotics and secondary metabolites by endophytic *Bacillus* isolates might have played a major role in inhibiting the growth of *P. grisea, R. solani*and *X. oryzae*pv*. oryzae invitro*.

PCR based detection of microorganisms is a reliable approach as it is accurate, rapid and sensitive. In the present study, PCR amplification has confirmed that endophytic bacterial strains which were tentatively identified as the *Bacillus* spp. with the phenotypic and biochemical characterization were *Bacillus* spp. (Figure 1), using *Bacillus* genus specific primers which amplified a fragment of approximately 546 bp corresponding to the region of the 16S-23S rRNA intervening sequence for *Bacillus* sp. Similarly, Zinniel et al. (2002) identified six endophytes with the most promising levels of colonization in a range of host plants based on 16S rRNA gene sequence, commercial fatty acid and carbon utilization analyses. Also, Rajendran (2006) have identified that two endophytic isolates EPC5 and EPC 8 were isolated from coconut root to be *Bacillus* sp. with the 16S rDNA gene sequence analysis using gene specific primer.

The primary mechanism of biocontrol agents is the production of antibiotics. *Bacillus* spp. used to produce many antibiotics such as iturin, surfactin, bacillomycin, zwittermicin, fengycin (Athukorala et al., 2009), azalomycin F (Wulff et al., 2002), amphomycin, arthrobactin and valinomycin. In the present study, the presence of antibiotics producing gene is identified by the PCR analysis using gene specific primer. Almost all the isolates are found to contain the iturin and surfactin producing genes (Figure 2a and b). This clearly indicates that these two antibiotics are common to all *Bacillus* spp. This result supports earlier findings in which these two antibiotics were detected from a wide array of *Bacillus* spp. including *B. subtilis*, *B. amyloliquefaciens, B. licheniformis, Bacillus mycoides, B. cereus* and *Bacillus thuringensis* (Athukorala et al., 2009; Thaniyavarn et al. 2003; Ramarathnam, 2007). This implies that surfactin and iturinare among the most common lipopeptide antibiotics produced by *Bacillus* spp. The *Bacillus* strains which had less inhibiting effect *viz*., EPB 15 and EPCO30 were also found to contain the surfactin genes. This confirms the earlier findings of Hofemeister et al. (2004) that the surfactins help bacteria to form biofilms rather than defense functions.

In the present study, one isolate (EPCO 16) has produced zwittermicin (Figure 2c). A very low percentage of bacteria have the ability to produce this antibiotics. Athukorala et al. (2009) reported that among the twenty one isolates, only two isolates were found to produce zwittermicin A. Twenty seven isolates of *Bacillus* sp. produced bacillomycin (Figure 2d). Ramarathnam et al. (2007) reported that bacillomycin D was detected in *B. subtilis* and *B. amyloliquefaciens* respectively. Athukorala et al. (2009) also reported a very low percentage of bacillomycin D, fengycin and zwittermicin A producing bacteria among the different isolates.

There are few points of interest that relate to agricultural uses of PGPE containing 1-aminocyclopropane-1-carboxylate deaminase (ACCD) gene other than biological agent. It has been shown that some PGPE strains are able to counteract flooding problems by reducing the negative effect of irrigation of crops with highly saline water. This is reflected in lowering the plant ethylene levels elevated by salt stress by means of ACCD

Table 5. Effect of bacterial endophytic isolates against major pathogens of rice.

S/N	Isolates	*P. grisea*		*R. solani*		*Xoo*
		Radial growth of (mm)*	% inhibition over control	Radial growth of (mm)*	% inhibition over control*	Inhibition zone (mm)*
1	EPB 1	73.0	18.89	68.0	24.44	15.0
2	EPB 2	69.0	23.33	65.0	27.78	15.0
3	EPB 3	56.0	37.78	55.0	38.89	14.0
4	EPB 4	55.0	38.89	58.0	35.56	12.0
5	EPB 5	73.0	18.89	66.0	26.67	16.2
6	EPB 6	59.0	34.44	58.0	35.56	19.0
7	EPB 7	53.0	41.11	62.0	31.11	19.0
8	EPB 8	47.0	47.78	54.0	40.00	15.6
9	EPB 9	53.0	41.11	57.0	36.67	20.0
10	EPB 10	54.0	40.00	59.0	34.44	20.0
11	EPB 11	49.0	45.56	60.0	33.33	15.0
12	EPB 12	71.0	21.11	81.0	10.00	10.6
13	EPB 13	44.0	51.11	46.0	48.89	17.0
14	EPB 14	53.0	41.11	52.0	42.22	19.0
15	FZB 24	43.0	52.22	58.0	35.56	20.0
16	EPB 15	90.0	0.00	90.0	0.00	0.0
17	EPB 16	62.0	31.11	57.0	36.67	15.0
18	EPB 17	57.0	36.67	54.0	40.00	17.0
19	EPB 18	75.0	16.67	50.0	44.44	14.0
20	EPB 19	70.0	22.22	57.0	36.67	14.2
21	EPC 5	50.0	44.44	72.0	20.00	14.0
22	EPC 8	59.0	34.44	66.0	26.67	17.8
23	EPCO 16	49.0	45.56	65.0	27.78	19.0
24	EPCO 26	49.0	45.56	72.0	20.00	11.0
25	EPCO 29	57.0	36.67	67.0	25.56	20.0
26	EPCO 30	90.0	0.00	90.0	0.00	4.0
27	EPCO 74	68.0	24.44	90.0	0.00	11.0
28	EPCO 78	50.0	44.44	75.0	16.67	20.0
29	EPCO 81	57.0	36.67	74.0	17.78	2.0
30	EPCO 95	46.0	48.89	73.0	18.89	14.6
31	EPCO 96	49.0	45.56	67.0	25.56	7.8
32	Control	90.0	-	90.0	-	0
SED		2.49		2.16		0.38
CD (0.05)		4.97		4.32		0.75
CD (0.01)		6.61		5.74		1.00
CV		5.16		4.06		3.18

*Mean of three replications.

containing PGPE (Mayak et al., 2004a).

In the present study, ACC deaminase gene was amplified from the 11 isolates in the PCR with the gene specific primers (Figure 2e). Similar results were obtained by Babalola et al. (2003). *P. putida* GR12-12 contains the gene for ACCD, which inhibits ethylene synthesis which is a product of stress. This mechanism is more effective on dicotyledonous plants that are more susceptible to the effects of ethylene especially under stress conditions such as flooding (Grichko and Glick, 2001) drought (Mayak et al., 2004b) and phytopathogens (Wang et al., 2000). Thus, endophytic *Bacillus* strains that possess ACCD activity have the selective advantage over other bacteria during biotic and abiotic stresses.

A total of 31 isolates of endophytic *Bacillus* were tested for their genetic variability by RAPD analysis, using 10

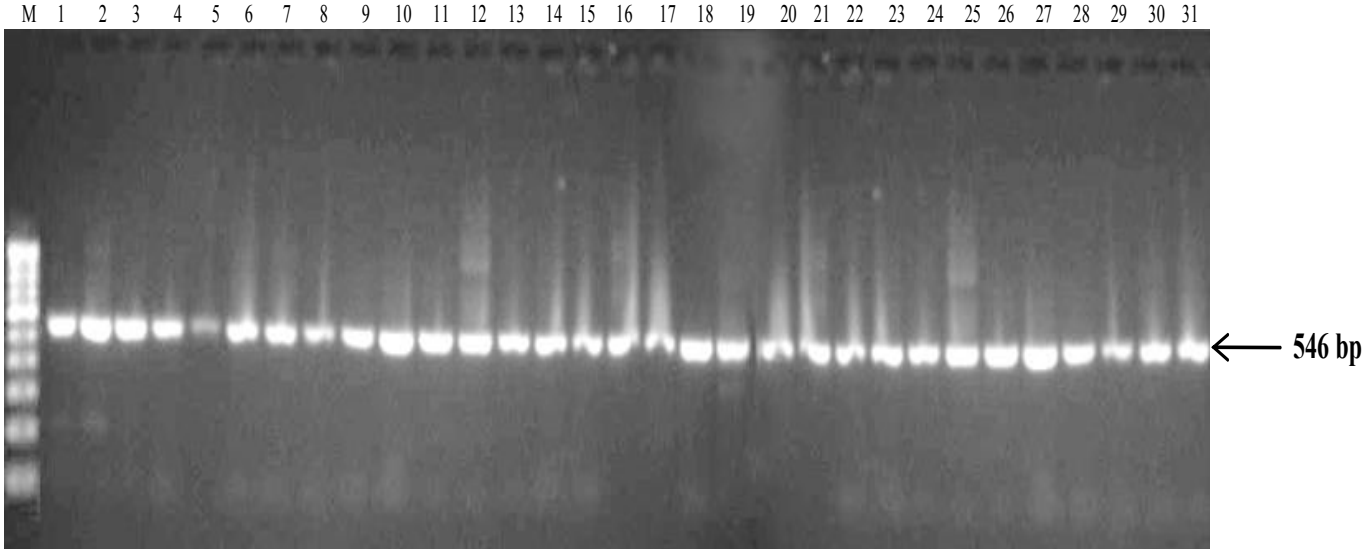
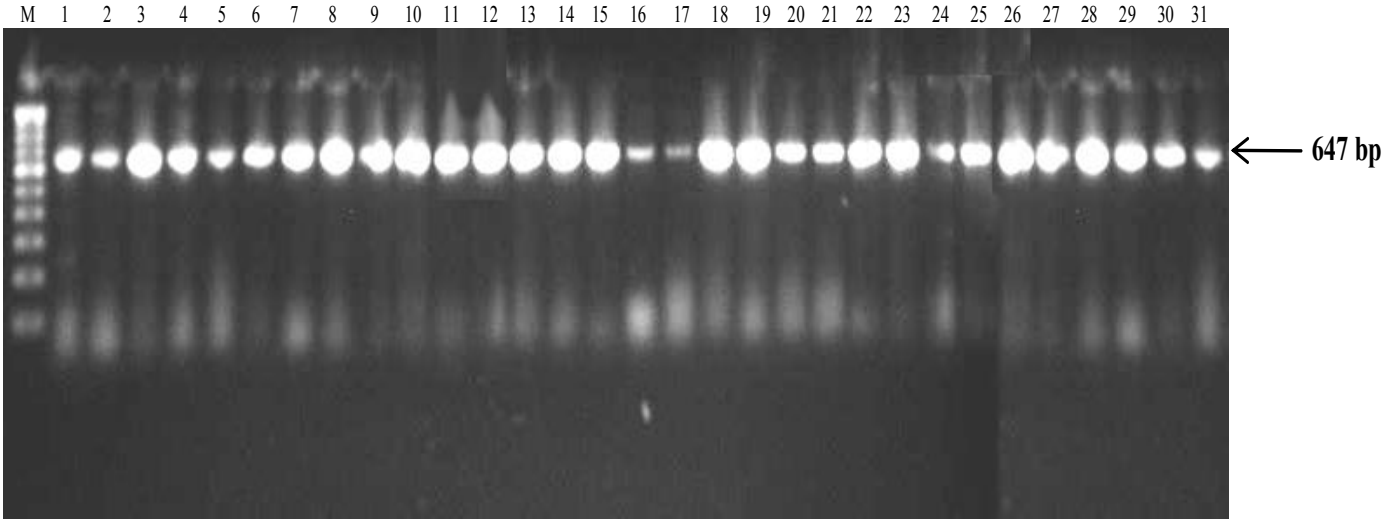


Figure 1. Detection of *Bacillus* species specific loci in the endophytic *Bacillus* strains using specific primers.



M. 100bp marker	1. EPB 1	2. EPB 2	3. EPB 3	4. EPB 4	5. EPB 5	6. EPB 6	7. EPB 7	8. EPB 8	9. EPB 9	10. EPB 10
11. EPB 11	12. EPB 12	13. EPB 13	14. EPB 14	15. FZB 24	16. EPB 15	17. EPB 16	18. EPB 17	19. EPB 18	20. EPB 19	21. EPC 5
22. EPC 8	23. EPCO 16	24. EPCO 26	25. EPCO 29	26. EPCO 30	27. EPCO 74	28. EPCO 78	29. EPCO 81	30. EPCO 95	31. EPCO 96	

Figure 2a. PCR amplification of antibiotic biosynthetic gene of iturin A from endophytic *Bacillus* isolates.

random primers. Of these, 7 random primers *viz.,* OPA01, OPG 5, OPG 11, OPG 16, CAG and GACA produce easily scorable and consistent banding patterns, which were used for RAPD analysis of thirty one isolates. The number of bands generated by each primer varied from 2 to 6. The amplified products ranged from 150 to 4500 bp, although majority was below 1.5 kb. The RAPD profiles produced with the primers OPA-01, OPG-5, CAG and GACA are shown in Figure 3. Analysis of the genetic coefficient matrix (Table 6), derived from the scores of RAPD profile, showed that minimum and maximum percent similarities among the endophytic *Bacillus*

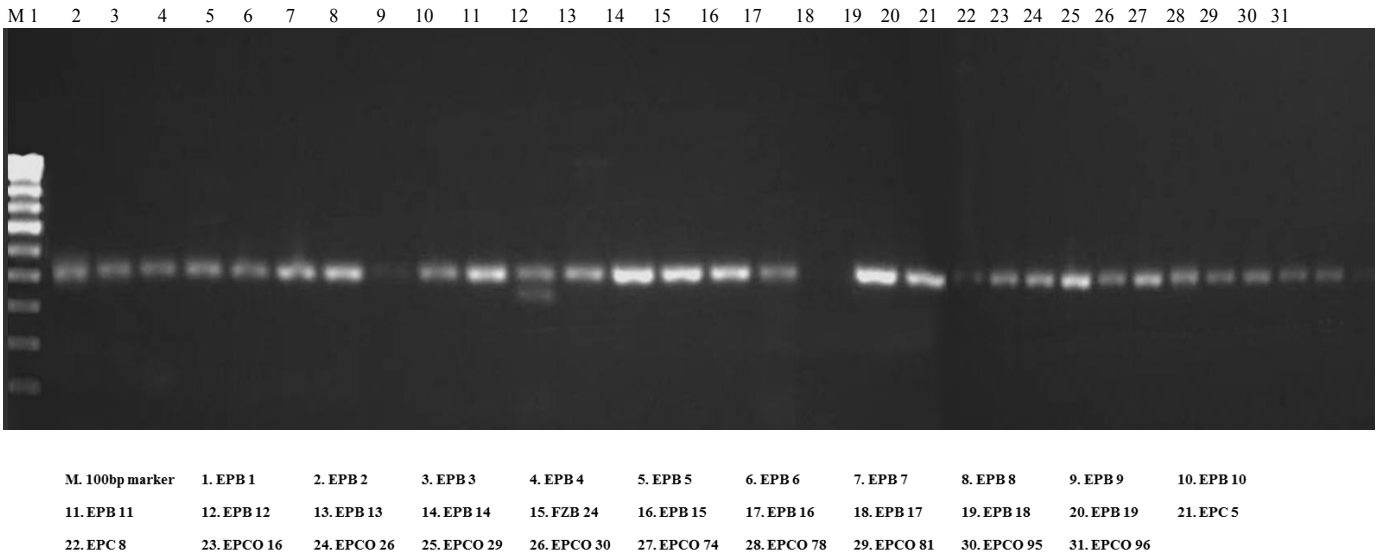


Figure 2b. PCR amplification of antibiotic biosynthetic gene of surfactin from endophytic *Bacillus* isolates.

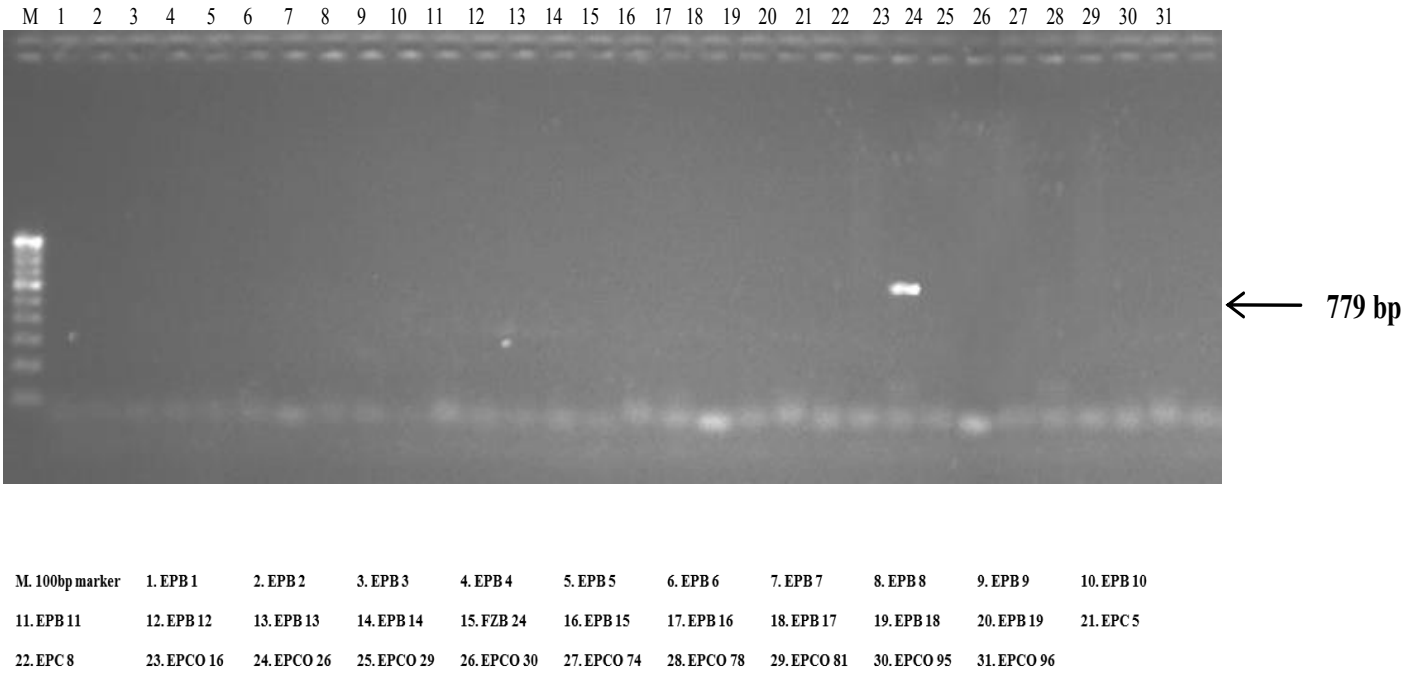


Figure 2c. PCR amplification of antibiotic biosynthetic gene of zwitermicin A from endophytic *Bacillus* isolates.

isolates were in the range of 7 to 73%, respectively (Table 4). Cluster analysis, using UPGMA, clearly separated the isolates into 2 clusters (I and II) confirming some level of genetic diversity among the isolates of endophytic *Bacillus* (Figure 4). Cluster I consisted of 12 isolates and cluster II consisted of 19 isolates. Interestingly, most of the coconut isolates which were isolated from the roots were clustered under the group 1. All the remaining isolates were clustered under group 2. The isolates which were isolated from the same plants

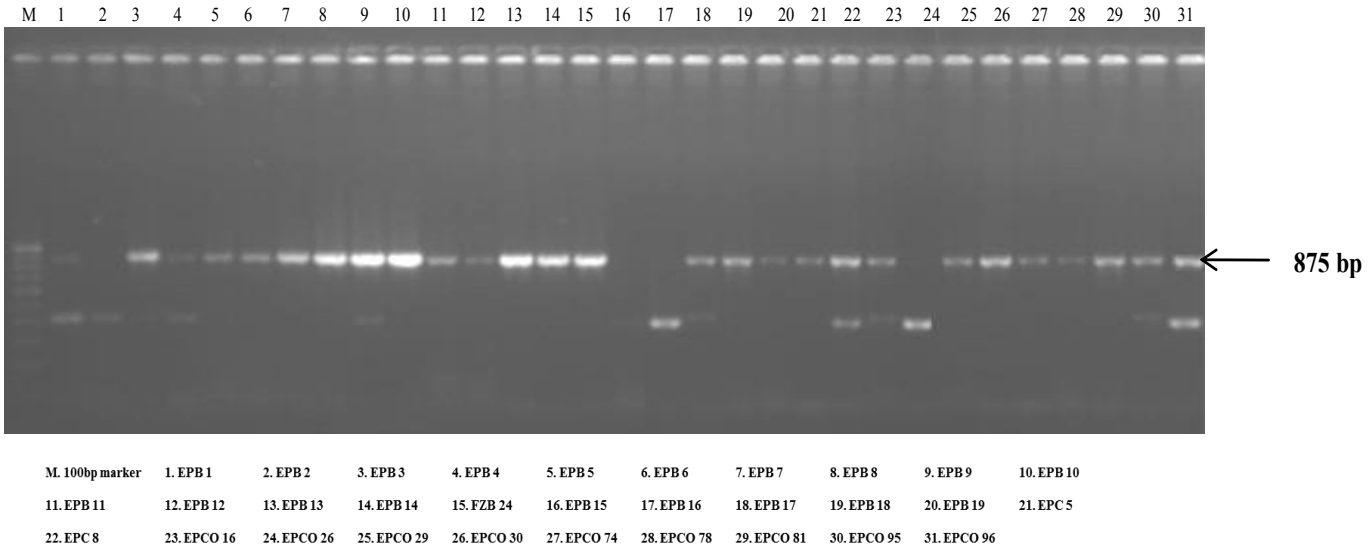


Figure 2d. PCR amplification of antibiotic biosynthetic gene of bacillomycin D from endophytic *Bacillus* isolates.

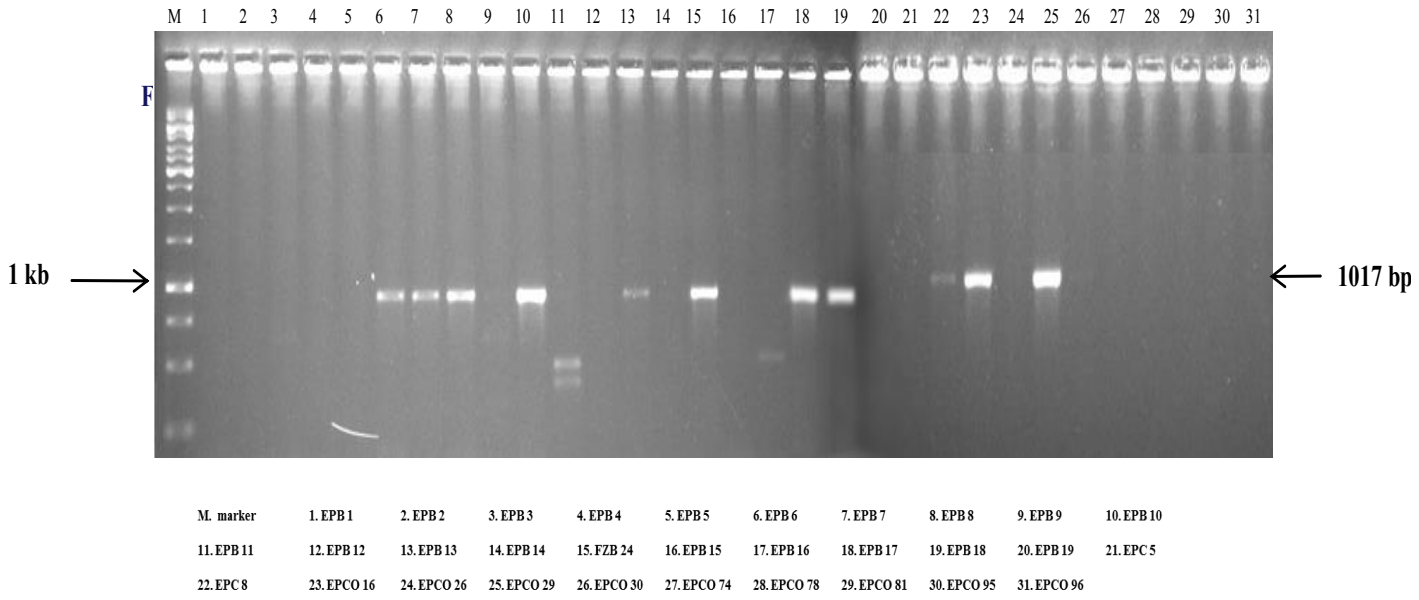


Figure 2e. Detection ACC deaminase biosynthetic gene in the endophytic *Bacillus* isolates using gene specific primer.

were also closer and they formed single clusters. Saveetha (2009) reported that typing of fluorescent pseudomonads using RAPD-PCR indicated more similarity between the DAPG producing strains than the non-producers. Also, Radjacommare (2004) had characterized several *Pseudomonas fluorescens* isolates from rice and vanilla ecosystems based on carbon source utilization, protein profiling, RAPD and ARDRA with Alu I and Hae III analysis.

Conclusion

Among all endophytic bacterial isolates tested against the major pathogens of rice *viz., P. grisea, R. solani*and *X. oryzae*pv. *oryzae in vitro*, endophytic *Bacillus* strains viz., FZB 24, EPB 13, EPB 8, EPB 9, EPB 7, EPB 10 and EPB 17 were found to be effective in inhibiting growth of all the three pathogens and it was also found to promote the growth of rice seedling *in vitro*. Also, it was found to

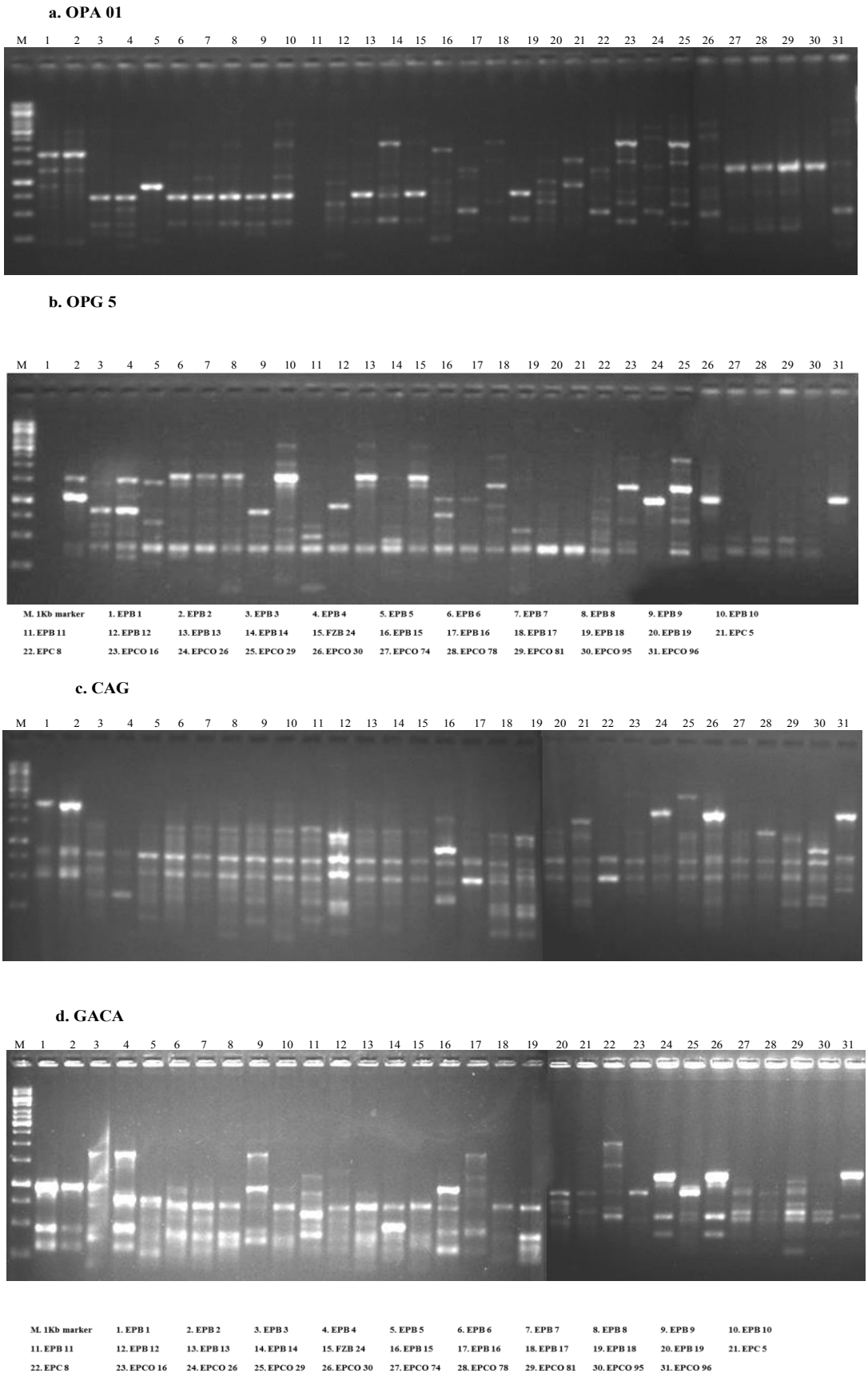


Figure 3. Random amplified polymorphic DNA analysis of endophytic *Bacillus* isolates with random primers.

Table 6. Similarity matrix for the endophytic *Bacillus* isolates generated through the RAPD primers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	1.00																														
2	0.63	1.00																													
3	0.37	0.30	1.00																												
4	0.27	0.27	0.53	1.00																											
5	0.42	0.38	0.30	0.27	1.00																										
6	0.27	0.24	0.38	0.33	0.38	1.00																									
7	0.24	0.21	0.39	0.28	0.31	0.69	1.00																								
8	0.24	0.21	0.38	0.28	0.34	0.72	0.81	1.00																							
9	0.25	0.22	0.48	0.32	0.32	0.50	0.62	0.66	1.00																						
10	0.22	0.19	0.33	0.25	0.22	0.51	0.49	0.51	0.43	1.00																					
11	0.29	0.26	0.33	0.27	0.32	0.41	0.46	0.49	0.43	0.30	1.00																				
12	0.21	0.21	0.33	0.27	0.24	0.34	0.38	0.38	0.36	0.33	0.30	1.00																			
13	0.21	0.21	0.33	0.30	0.27	0.62	0.59	0.57	0.43	0.52	0.39	0.43	1.00																		
14	0.18	0.21	0.30	0.30	0.24	0.62	0.59	0.62	0.43	0.45	0.36	0.54	0.67	1.00																	
15	0.18	0.18	0.33	0.38	0.20	0.51	0.53	0.56	0.42	0.48	0.35	0.36	0.56	0.56	1.00																
16	0.30	0.27	0.27	0.35	0.30	0.41	0.38	0.38	0.36	0.31	0.43	0.30	0.40	0.43	0.42	1.00															
17	0.21	0.19	0.28	0.31	0.19	0.29	0.29	0.29	0.33	0.26	0.37	0.28	0.31	0.34	0.33	0.40	1.00														
18	0.18	0.18	0.21	0.22	0.21	0.35	0.36	0.39	0.30	0.34	0.33	0.31	0.38	0.45	0.40	0.34	0.35	1.00													
19	0.25	0.19	0.29	0.29	0.32	0.36	0.41	0.39	0.38	0.27	0.34	0.41	0.41	0.41	0.34	0.41	0.30	0.29	1.00												
20	0.22	0.22	0.26	0.30	0.33	0.34	0.39	0.34	0.36	0.30	0.36	0.33	0.33	0.33	0.29	0.37	0.34	0.38	0.52	1.00											
21	0.21	0.21	0.22	0.28	0.28	0.36	0.33	0.32	0.31	0.23	0.38	0.26	0.32	0.32	0.31	0.38	0.39	0.36	0.37	0.53	1.00										
22	0.21	0.21	0.19	0.20	0.18	0.20	0.23	0.23	0.24	0.18	0.24	0.17	0.20	0.20	0.28	0.26	0.39	0.26	0.27	0.35	0.37	1.00									
23	0.25	0.25	0.26	0.23	0.22	0.33	0.31	0.33	0.31	0.30	0.25	0.29	0.36	0.39	0.38	0.23	0.24	0.41	0.24	0.25	0.31	0.24	1.00								
24	0.30	0.30	0.24	0.22	0.17	0.16	0.19	0.19	0.23	0.15	0.26	0.22	0.19	0.19	0.21	0.22	0.31	0.24	0.17	0.26	0.22	0.32	0.29	1.00							
25	0.21	0.24	0.17	0.20	0.18	0.20	0.17	0.20	0.20	0.19	0.16	0.18	0.22	0.22	0.27	0.15	0.16	0.31	0.18	0.21	0.23	0.20	0.56	0.19	1.00						
26	0.32	0.32	0.18	0.15	0.21	0.16	0.16	0.16	0.17	0.15	0.21	0.17	0.20	0.14	0.14	0.14	0.20	0.19	0.21	0.21	0.19	0.26	0.24	0.61	0.20	1.00					
27	0.20	0.20	0.18	0.17	0.24	0.18	0.15	0.15	0.16	0.17	0.17	0.16	0.13	0.13	0.13	0.13	0.13	0.18	0.14	0.24	0.23	0.23	0.19	0.21	0.19	0.32	1.00				
28	0.22	0.22	0.16	0.18	0.26	0.20	0.21	0.20	0.21	0.21	0.22	0.21	0.18	0.18	0.20	0.24	0.18	0.24	0.22	0.34	0.28	0.28	0.18	0.19	0.21	0.29	0.63	1.00			
29	0.16	0.16	0.18	0.22	0.23	0.18	0.18	0.18	0.22	0.20	0.20	0.19	0.16	0.16	0.18	0.21	0.16	0.21	0.20	0.26	0.22	0.22	0.19	0.18	0.21	0.25	0.52	0.73	1.00		
30	0.18	0.18	0.23	0.21	0.18	0.24	0.28	0.27	0.25	0.21	0.19	0.21	0.21	0.21	0.26	0.24	0.18	0.24	0.15	0.22	0.21	0.21	0.25	0.23	0.21	0.29	0.48	0.62	0.65	1.00	
31	0.18	0.18	0.16	0.08	0.08	0.08	0.11	0.11	0.11	0.09	0.16	0.12	0.07	0.09	0.09	0.15	0.12	0.11	0.13	0.11	0.14	0.14	0.14	0.19	0.15	0.20	0.15	0.17	0.19	0.17	1.00

1, EPB 1; 2, EPB 2; 3. EPB 3; 4, EPB 4; 5, EPB 5; 6, EPB 6; 7, EPB 7; 8, EPB 8; 9, EPB 9; 10, EPB 10; 11, EPB 11; 12, EPB 12; 13, EPB 13; 14, EPB 14; 15, FZB 24; 16. EPB 15 17; EPB 16; 18, EPB 17; 19, EPB 18; 20, EPB 19; 21, EPC 5; 22, EPC 8; 23, EPCO 16; 24, EPCO 26; 25, EPCO 29; 26, EPCO 30; 27, EPCO 74; 28, EPCO 78; 29, EPCO 81; 30, EPCO 95; 31. EPCO 96.

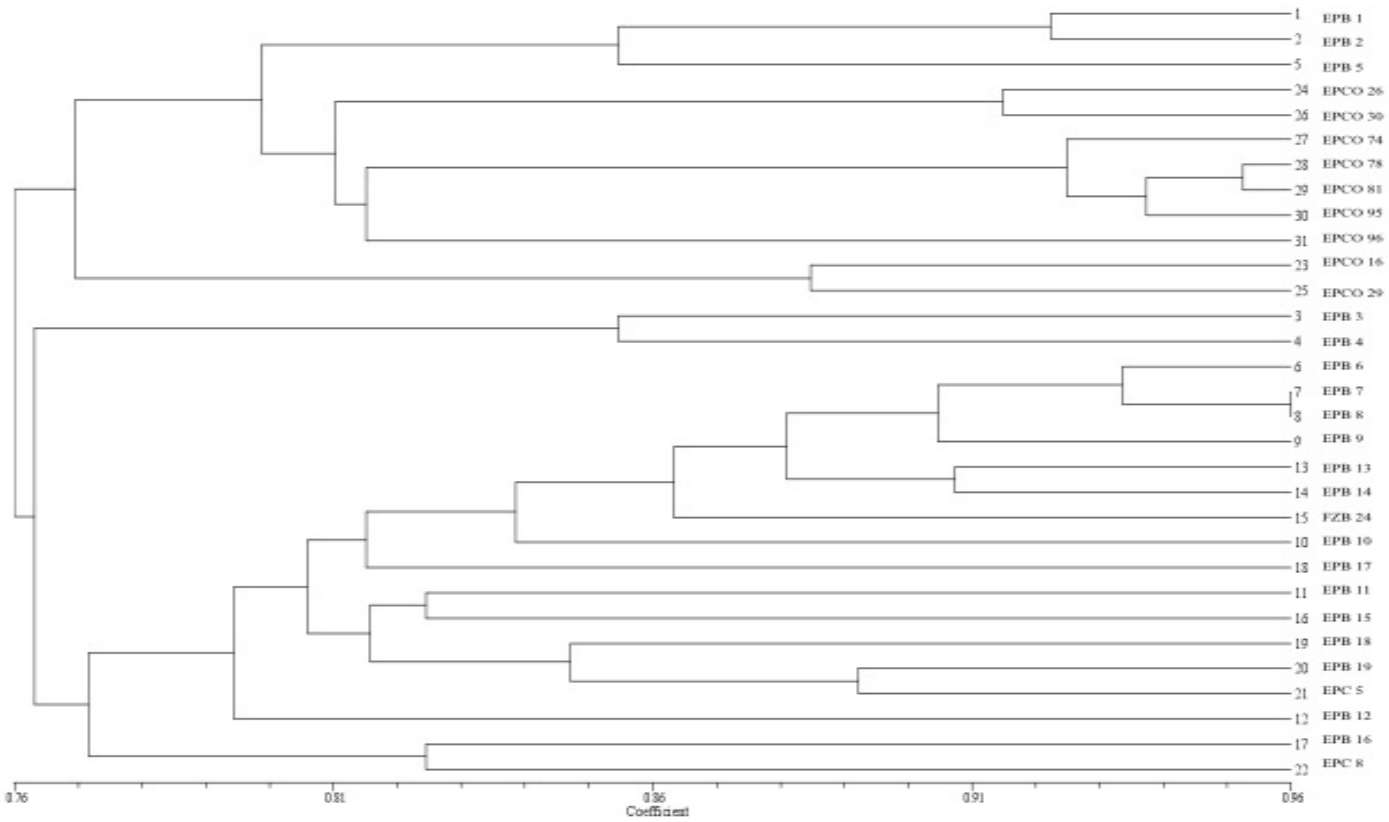


Figure 4. Dendrogram showing the molecular variability of the endophytic *Bacillus* isolates.

harbor antibiotics biosynthetic gene such as iturin, surfactin, bacillomycin and ACCD. So these strains of *Bacillus* sp. can be exploited under glasshouse and field conditions for the management of major rice diseases.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Isolation of potential phototrophic purple non-sulphur bacteria in paddy and their effects on paddy seedlings in hydroponic culture

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In order to promote the growth of photosynthetic bacteria (PB) use as biofertilizers in paddy fields, this study conducted during 2011 to 2012 at Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad, Karnataka, India. Twenty phototrophic purple non-sulphur bacterial (PPNSB) isolates were isolated from paddy rhizosphere soils of Northern Karnataka. Isolates were tested by morphologically, biochemically and physiologically according in to Bergey's manual. All the isolates belong to PPNSB *Rhodobacter* sp. *In vitro* nitrogen fixation was estimated by percent nitrogen content in cells which were grown on nitrogen free culture broth. Maximum percent nitrogen content was recorded in culture broth inoculated with isolate NKPRSR -1 (2.50%). Based on percent nitrogen content, the best five efficient isolates were selected for hydroponic culture to see the effect on paddy seedlings growth under laboratory condition. In hydroponics Complete Randomized Design was used to accommodate 13 treatments with 5 replication, with the combination of nitrogen source and without nitrogen sources. Results revealed that treatment having isolate *Rhodobacter* sp. NKPRSR 1+ Nitrogen source show maximum plant height (26.67 cm), root length (13.17 cm), dry weight (16.50 mg/ plant) and percent nitrogen content (0.99%) after 30 days of sowing.

Key words: Isolation, purple nonsulphur bacteria, paddy, hydroponics, rhodobacter, rhizosphere.

INTRODUCTION

Purple phototrophic non-sulfur bacteria (PPNSB) constitute a diverse group among the anoxygenic phototrophic bacteria (APB) with a versatile metabolism (Imhoff, 1995) with potential for various biotechnological applications (Sasikala and Ramana, 1995a, b). In nature, they occur in aquatic as well as terrestrial environments where light of sufficient quantity and quality is available and partial anaerobic conditions prevail (Drews and Imhoff, 1991).

Rice (*Oryza sativa* L.) is a major world crop and more than half of the world's population is dependent on it. Rice crop needs more nitrogenous fertilizer, these chemical nitrogenous fertilizers are the most costly input for the production of rice crop and also they are not ecofriendly. Different ways of reducing nitrogenous fertilizer use in rice cultivation were sorted, while maintaining or enhancing crop output is desirable from both economic and environmental perspectives (Roger and Ladha, 1992). Increasing biological nitrogen fixation (BNF) associated with rice plants is an attractive approach to raise crop yield and reduce nitrogen fertilizer requirements.

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The most-studied microbial groups responsible for BNF in flooded rice fields are free-living cyanobacteria, heterotrophic bacteria and symbiotic cyanobacteria associated with the fern Azolla (Choudhury and Kennedy, 2004). In contrast, little attention has been devoted to the N_2-fixing phototrophic purple non-sulfur bacteria (PPNSB). Anoxygenic phototrophic bacteria are the major groups of microorganisms existing in paddy soils and contribute significantly to soil fertility (Habte and Alexander, 1980). Elbadry et al. (1999) have demonstrated beneficial effect of *Rhodobacter capsulatus* on rice variety in hydroponic cultures.

Kobayashi and Haque (1971) suggested a possible growth-promoting role of phototrophic purple non-sulphur bacteria (PPNSB) in rice fields. Maudinas et al. (1981) showed that in the absence of chemical nitrogen (Ammonium chloride), but in the presence of the diazotrophs *Azotobacter vinelandii* and *Rhodopseudomonas capsulatus* (now *Rhodobacter capsulatus*) in a liquid medium, rice plants can benefit from germination up to ear stage. Eldin and Elbanna (2011) also showed field evidence for the potential of *Rhodobacter capsulatus* as biofertilizer for flooded rice. So far, not much work has been carried out in India on PPNSB biofertilizer, therefore, this work is first of its kind aimed to study the effect of potential PPNSB isolates on rice seedling development in laboratory condition.

MATERIALS AND METHODS

Isolation

The paddy rhizosphere soil at tillering stage was collected from different rice growing districts of North Karnataka. For isolation enrichment culture technique followed as described by Archana et al. (2004) paraffin wax-overlay of pour plate, modified Biebl and Pfennig's (1981) (BP) medium of the following composition was used for plating the soil samples and for culturing the isolates (g $^{-}$1); KH_2PO_4: 0.5; $MgSO_4$, $7H_2O$: 0.2; NaCl: 0.4; NH_4Cl: 1.2; $CaCl_2$, $2H_2O$: 0.05; Yeast Extract: 0.3; Organic acids: 3.0 (Malate, Succinate and Pyruvate-1.0 g each); Ferric-Citrate (0.1% w/v) : 5 ml; Trace salt solution [(mg^{-} 1): HCl (25% v/v)-1 ml; $ZnCl_2$ 70; $MnCl_2$, $4H_2O$ 100; H_3BO_3 60; $CoCl_2$, $6H_2O$ 200; $CuCl_2$, H_2O 20; $NiCl_2$, $6H_2O$ 20; $NaMoO_4$, $2H_2O$ 40]: 1 ml; Agar:20 pH of the medium: 6.8 to 7.0 before autoclaving. In this study, in order to isolate nitrogen fixing isolates from the samples nitrogen compound was vomited from the media composition.

Approximately a gram of paddy soil was suspended in 10 ml saline water (0.7% NaCl w/v), mixed by overtaxing and used for subsequent tenfold dilutions in the saline water. Five hundred microlitres of the dilutions, used as inoculums, were pour plated with 20 ml of Biebl and Pfennig's (1981) agar medium (40 to 45°C) and the medium was allowed to solidify. The plates were then overlayed with molten paraffin wax (55 to 60°C; solidifies immediately on pouring over the agar). The plates were rotated gently in a circular motion while pouring the wax in order to spread it evenly over the agar surface (keep the plates open for a period of 10 min after pouring the paraffin wax in order to radiate the heat of the wax before closing the lid) and incubated at a temperature of 30 ± 2°C with the agar side of the plate exposed to a light intensity of 2400 lux. Following the development of colonies, the overlying paraffin wax was gently removed with a scalpel, colonies embedded in the agar sectioned out as blocks, transferred aseptically into 15 to 125 mm screw cap tubes fully filled with modified Biebl and Pfennig's (1981) liquid medium and the tubes incubated under a light intensity of 2400 lux at a temperature of 30 ± 2°C for 3 to 5 days. The cultured isolate was purified by repeated streaking on modified Biebl and Pfennig's (1981) agar slant prepared in 25 to 150 mm test tube. The pure colony obtained was transferred aseptically into a screw cap tube completely filled with Biebl and Pfennig's broth.

Physiological and biochemical characters tested according to Bergey's manual of determinative bacteriology after confirming the isolates belongs to PPNSB group (Imhoff and Truper, 1989). Cells grown in nitrogen free medium after 10 days of incubation were subjected for quantitative estimation of the amount of nitrogen fixed in the broth culture by Microkjeldahl method of Bremner and Mulvaney (1982). To the 10 ml of broth culture, 5 ml of concentrated H_2SO_4 and 200 mg catalyst mixture (potassium sulphate, copper sulphate and selenium in the ratio of 10:1:0.1) were added and allowed for digestion in block digester for 2 h to get clear digest. The clear digest was cooled and diluted with distilled water up to 10 ml. This was distilled in a distillation unit after addition of 20 ml of 40% sodium hydroxide solution to make the digest alkaline, in a Parnas-Wayner type distillation unit. The evolved ammonia was absorbed in four percent boric acid with mixed indicator and finally titrated with 0.05 N H_2SO_4 for colour change from green to red. From the volume of acid consumed, total nitrogen content was calculated.

Hydroponics study

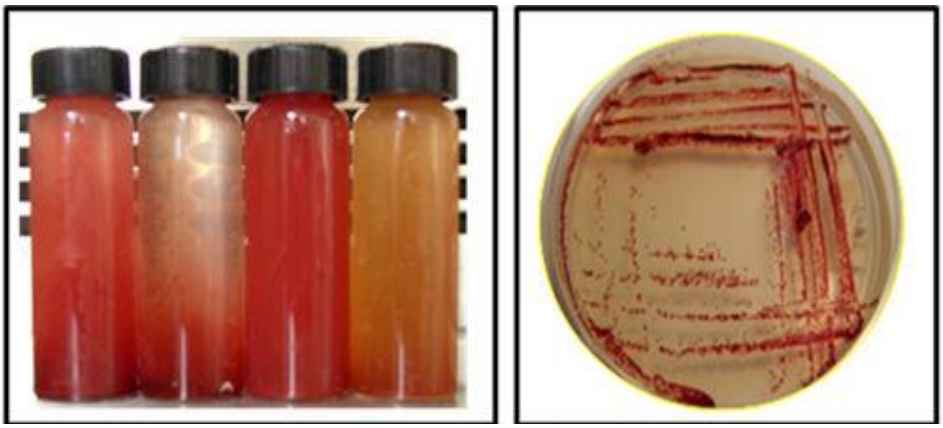
Isolated PPNSB isolates used for seeing the effect on rice seedling procedure was used for hydroponic culture according to Elbadry et al. (1999).

Rice seeds

Rice (*Oryza sativa* L.) seeds of Ahilasha variety brought from Aerial Rice Research Station (ARS) Mugad, Dharwad, Karnataka, India.

Microorganism

Five strains of the phototrophic purple non-sulfur bacterium *Rhodobacter* sp. isolates were used in the inoculation experiments. Bacterial inoculums were prepared immediately before inoculation. *Rhodobacter* sp. based on total nitrogen estimation selected five best isolates and reference strain *Rhodobacter capsulatus* KU002 was brought from Hydrabad Department of Biochemistry, MG University, Nalgonda. All the selected isolates were grown photosynthetically on Biebl and Pfennig's medium (1981). The cells were collected at the exponential growth phase by centrifugation at 10,000 rpm for 10 min and the cell pellets were washed twice with sterilized distilled water. The cells were resuspended in a medium having the same composition as the growth medium except that of NH_4Cl .The cultures were grown in completely filled glass bottles of 1000 ml capacity and incubated by degassing and using nitrogen. The logarithmic growth phase cells were centrifuged and the cell pellets were washed twice with sterilized distilled water and then resuspended in phosphate buffer 0.05 M, pH = 7. Here after these starved bacterial cells will be referred to as inoculums. The inoculums contained about 3.3 - 10^8 cells/ml.



(a) (b)

Figure 1. Appearance of pure culture of the isolates *Rhodobacter* sp. NKPRSR-1 (a) Cell suspension (b) Colony.

Rice nutrient solution (Heulin et al., 1987)

Solution A. ($g^{-}L$): $ZnSO_4 7H_2O$, 0.42; $MnSO_4 H_2O$, 1.30; $Na_2MoO_4 2H_2O$, 0.75; H_3BO_3, 2.8; $CuSO_4 7H_2O$, 0.026; $CoSO_4 7H_2O$, 0.07.

Solution B. ($g^{-}L$): $MgSO_4$, $7H_2O$, 2.00; $CaCl_2$, $2H_2O$, 2.00; $FeSO_4 7H_2O$, 0.44; EDTA, 0.40; Solution A, 20 ml. Solution C. ($g^{-}L$): K_2HPO_4, 90; KH_2PO_4, 60. Final nutrient solution: Solution B, 50 ml; Solution C, 15 ml; distilled water, 1000 ml. For N-treatments, the final nutrient solution was supplemented with 40 ppm nitrogen as NH_4Cl.

Seedling growth unit

The seedling growth unit used in the laboratory inoculation experiment is made of two parts. The upper part (A) is a plastic cup of 7 cm diameter and 9.2 cm height with a pored bottom. This part supports the germinating seeds and the aerial parts of the rice seedling. The lower part (B) is a 650 ml plastic bottle, with a mouth larger than the base of the plastic cup. At the start of the experiment, the two parts of the unit were sterilized separately using acetone and 70% ethanol. Thereafter, the plastic cup was tightly placed over the mouth of the plastic bottles, so that the solution level reached the rice plants continuously. The germinated rice seeds were transferred aseptically to the upper part, one seed for each hole.

Plant experiment

In this experiment, Abhilasha rice variety was used. Rice seeds of approximately similar size were surface sterilized with a 0.1% mercuric chloride solution for 1 min; followed by washing thoroughly with several changes of sterile distilled water (Yoshida et al., 1976) and allowed to germinate on nutrient agar in Petri dishes for 3 days at 30°C in the dark. Contaminant-free uniformly germinated seeds were aseptically transferred to sterilized growth assemblies containing 600 ml of the sterile nutrient solution with or without nitrogen. Nutrient solutions inoculated immediately with 60 ml prepared inoculums to yield approximately 3.3 - 10^8 cells/ml. The final volume of the solutions in the growth assembly was sufficient to cover the rice seeds.

The experiment comprised 13 treatments

First set

(1) Nitrogen-free nutrient solution + isolates 1, 2, 3, 4, 5 and ref. strain

Second set

(2) With nitrogen nutrient solution + isolates 1, 2, 3, 4, 5 and ref. strain

Uninoculated control

(3) Only nutrient solution with nitrogen

Five replicate assemblies were used and for each, treatment design used was Completely Rondomized Design (CRD). The plants were grown in a cabinet under natural light conditions at 29°C at day and 20°C at night. Measurements of the growth parameters of rice seedlings were recorded on 15^{th} and 30^{th} days after sowing. At the end of the 30^{th} day experimental period, the plants were removed gently from the assemblies and washed in distilled water, the shoot and root portions were separated and the growth measurements were taken. All the plants in each assembly were taken together for measurements of dry weight (DW) and all the plants in each treatment were pooled for nitrogen determination by the Micro Kjeldohol technique. Statistical analysis data obtained from the different treatments were subjected to analysis of variance. Multiple mean comparisons were made by Duncan Multiple Range Tests using MSTAT- C software.

RESULTS AND DISCUSSION

In this study, totally 20 isolates were isolated and characterized according to their morphology, pigmentation and photo assimilated substrates. The cells of these isolates were rod to ovoid shaped all of the strains were Gram negative and motile. Red cell suspensions were formed by reddish brown colour colonies (Figure 1).

Table 1. Morphological, biochemical and physiological characteristics of the isolated purple non-sulphur bacteria in paddy PPNSB strains 1 to 20.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16	17	18	19	20
G. stain	G. N	G. N	G. N	G.N	G.N	G.N	G.N	G.N	G. N	G. N	G. N	G.N	G.N	G.N	G.N	G.N	G.N	G.N	G.N
Cell shape	rod	rod	rod	oval	rod	rod	rod	oval	rod	rod	rod	oval	rod	oval	oval	rod	rod	oval	oval
Motility	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Colour	Brownish	red	brown	red	brown	brown	brown	brown	Purplish	brown	red	red	red	red	red	brown	red	red	red
Slime production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nacl. Rec.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH	7.0	7.5	7.0	8.0	7.5	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	6.5	6.5	7.0	7.5	7.5	7.5
Temperature	30	29	30	30	30	30	28	29	30	30	28	30	30	30	30	30	30	30	30
Growth on N free media	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Vitamins rec.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carbon source																			
Formate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acetate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Propionate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tartarate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Malate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fumarate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Monnitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pyruvate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucinate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Absorption spectral(nm)	865	865	850	860	850	860	855	860	850	865	860	850	850	865	850	860	865	850	850

All the isolates grew well on neutral pH (6.8 to 7.5), optimum temperature (28 to 30°C) and isolates required both NaCl (0.2 to 0.4) and vitamins (Biotin). The photoheterotrophic growth of the isolates was tested on different organic carbon sources (Table 1). All the isolates inability to assimilate tartarate, citrate, glutamate, glycerol, mannitol and mannose they were shown luxuriant growth in rest of the carbon sources which were used in the study. Morphologically, one important characteristic of the PNSAB is the reddish pigmentation which indicated the presence of thephotosynthetic pigments (carotenoids and bacteriochlorophyll. The photosynthetic pigment present in PPNSAB could be bacteriochlorophyll *a*, *b* or both. The isolated bacteria in this study showed the characteristic reddish bloom under partial anoxy-photosynthetic conditions and the presence of only bacterichlotophyll *a* was observed (850 to 865 nm), (Braun and Scherz, 1990). Results were confirmed with Chen et al. (2003)

Table 2. Total percent nitrogen content in N free medium grown cells of PPNSB isolates *Rhodobacter sp.* isolated from paddy soils.

S. no	Isolates	Per cent nitrogen (%)
1	NKPRSR1	2.50[a]
2	NKPRSR2	2.33[ab]
3	NKPRSR3	1.93[bc]
4	NKPRSR4	2.00[abc]
5	NKPRSR5	1.75[c]
6	NKPRSR6	0.35[d]
7	NKPRSR7	0.39[d]
8	NKPRSR8	0.32[d]
9	NKPRSR9	0.30[d]
10	NKPRSR10	0.30[d]
11	NKPRSR11	0.29[d]
12	NKPRSR12	0.23[d]
13	NKPRSR13	0.32[d]
14	NKPRSR14	0.34[d]
15	NKPRSR15	0.26[d]
16	NKPRSR16	0.26[d]
17	NKPRSR17	0.28[d]
18	NKPRSR18	0.16[d]
19	NKPRSR19	0.12[d]
20	NKPRSR20	0.28[d]
21	Ref. *R. capsulatus* KU005	1.43[abc]
	CD@0.01	0.48

who isolated and characterized purple non-sulphur bacteria in swine manure waste and Soto-Feliciano et al. (2010) Isolated and characterized the purple non-sulfur anoxyphototropic bacteria after confirmation of characteristics of the isolates, tentatively grouped to phototrophic purple non-sulphur bacteria *Rhodobacter* sp*.* and for different isolates we coded NKPRSR (North Karnataka Paddy Rhizosphere Soil Rhodobacter).

All the PPNSB isolates were subjected to grow on nitrogen free medium and *in vitro* nitrogen fixation was estimated by estimating total per cent nitrogen content results presented in Table 2. All the isolates were able to grow on Nitrogen free BP medium and Highest per cent Nitrogen was recorded in PPNSB *Rhodobacter* sp. coded NKPRSR-1(2.25 %) and NKPRSR- 2 (2.33 %). Based nitrogen estimation five efficient strains selected for hydroponic culture NKPRSR- 1(2.25 %), NKPRSR- 2 (2.33 %l), NKPRSR- 3(1.95 %), NKPRSR- 4 (2.00 %) and NKPRSR 5 (1.75 %). Effects of PPNSB *Rhodobacter* sp*.* inoculation on rice seedling development of rice variety Abhilasha in hydroponic culture results presented in Table 3. Maximum shoot, root length, dry weight and per cent nitrogen content in plant were recorded in the treatment having both nitrogen source and efficient *Rhodobacter* sp*.* isolate NKPRSR- 1. Root length of rice seedlings inoculated with NKPRSR-1 (12.83 cm) (I±N) were significantly longer as compared to the treatment having only inorganic nitrogen source (7.17 cm)) at the end of the 30th day experimental period. Our results similar with the Elderly and Elbanna (1999) findings on rice varieties in hydroponics inoculated with *Rhodobacter capsulatus*. Murt and Ladha (1988) showed that *Azospirillum* inoculation of rice under hydroponic conditions significantly increased the root length of rice in agreement with our results. Apart from nitrogen fixation findings several research article on PPNSB group bacterium *Rhodobacter* sp. able to produce growth hormone production like indole acetic acid (Mujahid et al, 2011; Rajasekhar et al, 1998) and cytokinine (Serdyuk et al, 1993). In the present work, it was observed that elongation of root and increased numbers of new roots were initiated from the crown. Tien et al (1979) found similar effects of *A. brasilense* and plant hormones on lateral root production and main root elongation by pearl millet. In hydroponics lower jar consists of nutrient solution and isolated organism PPNSB bacterium *Rhodobacter* sp. is enable to grow in absence of chemical nitrogen and also presence of chemical nitrogen. Environment provided in the jar was not fully aerobic; it was partially anaerobic meaning microaerophillic condition because lower jar mouth was completely closed with alumilium foil and para filim. So, PPNSB bacterium

Table 3. Effect of *Rhodobacter capsulatus* strains inoculation and nitrogen fertilization on shoot height, root length, dry weight and N% content of rice grown in hydroponic culture.

Treatment	15 DAS (cm)	30 DAS (cm)	30 DAS Root length(cm)	Dry wt. (mg/plant)	% N content
Isolate NKPRSR1	13.50^{ab}	26.67^{ab}	12.50^{ab}	14.17^{ac}	0.99^{a}
Isolate NKPRSR2	12.17^{bcd}	24.03^{ab}	11.50^{bc}	13.17^{bgde}	0.83^{abc}
Isolate NKPRSR3	11.00^{def}	22.50^{cdef}	9.83^{def}	12.17^{def}	0.72^{bc}
Isolate NKPRSR4	10.33^{ef}	21.60^{ef}	9.17^{de}	11.00^{fg}	0.58^{cd}
Isolate NKPRSR5	9.83^{f}	21.17^{f}	8.50^{f}	10.17^{g}	0.42^{d}
Rhodobacter capsulatus KUOO5	13.33^{abc}	25.17^{abc}	10.00^{de}	13.50^{bcd}	0.86^{ab}
Only nitrogen	14.00^{a}	25.00^{abcd}	7.17^{g}	12.17^{def}	0.88^{ab}
Isolate NKPRSR1+N	13.67^{a}	27.67^{a}	13.17^{a}	16.50^{a}	0.99^{abc}
Isolate NKPRSR2+ N	11.83^{cde}	24.50^{bcde}	12.83^{a}	14.67^{b}	0.75^{bc}
Isolate NKPRSR3+ N	11.50^{def}	22.00^{def}	10.00^{de}	12.50^{def}	0.72^{bc}
Isolate NKPRSR4+ N	11.17^{def}	21.00^{f}	9.50^{def}	12.00^{def}	0.67^{bc}
Isolate NKPRSR5+ N	11.75^{cde}	21.00^{f}	9.17^{ef}	11.50^{efg}	0.73^{bc}
Rhodobacter capsulatus KU002+ N	13.83^{a}	21.50^{f}	10.83^{cd}	11.67^{fgg}	0.90^{ab}
CD @0.01%	1.14	2.78	1.27	1.58	0.17

N- Nitrogen

can grow luxuriantly with the provided condition, this was confirmed from earlier findings Elbanna et al (1999) in soilless culture. Inoculation in the presence of nitrogen were considerably maximum than those found in the nitrogen-free treatments and uninoculated (only nitrogen source) treatment. Results also confirmed with the Elbanna et al. (1999) findings in hydroponic culture. Nitrogenase regulation was different in this organism compare to other nitrogen fixing microorganisms (Masepohl et al, 2002). Results clearly indicate that inoculation with PPNSB *Rhodobacter* sp. with the inorganic nitrogen source, gave good plant growth performance in terms of improved seedling growth, as indicated by increase in dry weight (DW) of the aerial part and shoot height compared to the uncirculated once. The pronounced increase in seedling, dry weight, nitrogen percentage in roots and shoots with observed results from the study PPNSB *Rhodobacter* sp. isolates could produce more ammonium even with the presence of inorganic nitrogen source and the rice plants could benefit from the inoculated Isolates; *Rhodobacter* sp. can also produce growth hormones which were having plant growth promotion activity. Based on others findings confirmation with this paper result it can be concluded that inoculation gives benefits to rice seedling growth that almost commensurate with the addition of 40 ppm of nitrogen (ammonium chloride). This result is in harmony with the findings of others (Lee et al, 1977; Maudinas et al, 1981; Elderly and Elbanna 1999; Ramchander et al, 2012). With all these supportive findings and obtained results, PPNSB group of bacterium definitely have plant growth promoting activity.

The promotion of satisfactory seedling development is an important stage in crop development, essential for achieving optimal populations, and in turn, maximum yield. Therefore, the results obtained have been highly encouraging and provide good grounds for conducting further trials in pot and field experiment to bring potential PPNSB isolates as potential biofertilizer for paddy ecosystem.

Conclusion

Phototrophic purple non-sulfur bacterium *Rhodobacter* sp. isolates benefits rice seedling (*Oryza sativa* L.) in terms of growth and development under laboratory conditions. To the knowledge of this paper, this is the first reported evidence in India to study the potentialities PPNSB isolates in hydroponic culture study. Through optimizing rice cultivation and bacterial inoculation, it is possible to obtain the promotion of satisfactory seedling development as an important stage in crop development, essential for achieving optimal populations, and in turn, maximally yields an increase in grain yield and a decrease in chemical nitrogenous fertilizer requirement by inoculation with PPNSB isolates. This bacterium has great potential for the development use as a biofertilizer in rice cultivation to sustain soil fertility and reduce application of chemical fertilizer in future days.

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Full Length Research Paper

Biology and artificial inoculation of *Ustilaginoidea virens* (Cooke) Takahashi in rice

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False smut of rice (*Oryza sativa* L.) caused by *Ustilaginoidea virens* (Cooke) Takahashi (teleomorph *Villosiclava virens*) is one of the most common fungal diseases of rice in China. The objectives of the research were: 1) to study the biology of *Ustilaginoidea virens,* 2) to develop suitable media for inoculum production of *U. virens,* and 3) to develop an artificial inoculation technique for the study of false smut resistance in rice. Seventy diseased rice samples were collected from seven districts of Guizhou province, China during 2007-2008 and 138 single-spore isolates of *U. virens* were isolated. Among two inoculation methods applied at 3 rice growth stages of rice variety Gangxiang 707 using spore suspension of *U. virens* isolate 2008-33-1, conidia injection at late booting stage produced the highest false smut incidence of 50.43%, while conidia spraying during the same stage gave only 34.75% disease incidence. Pathogenicity test of selected eight *U. virens* isolates on rice variety Zhongyou 177 using the conidia injection at late booting stage, showed that the isolates were different in their aggressiveness in which isolate 2008-11-1 gave the highest virulence score of 9 with the disease incidence of 81.66%, while isolate 2007-48-1 gave the lowest score of 5 with only 15.57% disease incidence. These eight *U. virens* isolates were further tested on six rice varieties using the same inoculation technique. Most of the rice varieties were either susceptible or moderately susceptible except Fengyouxiangzhan that was highly susceptible to *U. virens* isolate 2007 -79-1 and Nongfengyou 256 was moderately resistant. When the evaluation was done across all the *U. virens* isolates, Gangyou 827, Jixiangyou 830 and Fengyouxiangzhan were among the most susceptible varieties with disease incidence of 55.06, 54.79 and 53.50%, respectively, while Nongfengyou 256 varieties showed the lowest disease severity as 23.85%. For the pathogen strain, 2008-33-1 was the most aggressive giving 58.09% disease incidence while the fungal pathogen strain 2008-2-2 gave the lowest of 28.17%.

Key words: False smut, rice, colony morphology, fungal characterization, *Ustilaginoidea virens.*

INTRODUCTION

The rice false smut, caused by *Ustilaginoidea virens,* is a worldwide disease and is a minor rice disease throughout major rice-growing countries in the world before 1970's (Deng, 1989; Yaegashi et al., 1989; Sugha et al., 1992). It has been found in many countries, such as China, India, Japan, Italy, Australia, Philippines, Brazil and Mayanma (Ou, 1972; Dodan and Singh, 1996). With the change of weather condition, large application of nitrogen

fertilizer and large-scale planting of hybrid rice, the rice false smut has become more and more serious. It has already changed from a minor disease to a major disease in all rice growing areas in China, and many rice-growing countries in Asia since 1970. In 1982, the disease had spread more than 666,000 hectares in Hunan, China. The epidemic area had increased from 200,000 to 330,000 hectares from 1984-1996 at Liaoning, China. In 1993, the disease was reported to increase from 60,000 to 100,000 hectares in Yunnan, China (Liao and Li, 1994), and respect as 13.7% of the total rice production. The disease incidence was 10-30%, but in some serious fields it could be as high as 50-60%. Up to 39 false smut balls could be found on each rice plant. The rice yield was reduced 5-30% as a result of false smut infection in Guizhou. The false smut balls have toxin including ergot alkaloid toxin that can cause rumination stopping in cows, suppress the tubulin of mammals and cause necroses of liver, kidney, and bladder tissues in mice (Dhindsa et al., 1991; Nakamura and Izumiyama, 1992; Chib et al., 1992; Iwasaki, 1992; Yukiko et al., 1994; Nakamura et al., 1994; Li et al., 1995; Sinha et al., 2003). Therefore, the rice false smut not only threatens rice production in yield and quality, but also produces toxins that are dangerous to the health of human and livestocks.

Lu et al. (1996) reported artificial culture conditions of temperature, carbon source, and pH value. However, there have been few reports on optimal sporulation culture conditions and component of culture media, single spore isolation and conservation methods. There have been some studies on disease resistance using artificial inoculations (Zhang et al., 2003), but there were different results of disease incidence and lower reproducibility. At present there have not been an established artificial inoculation technique and evaluation criteria for disease resistance to rice false smut in China.

Selecting and using resistant varieties are the most cost-effective measures to control plant diseases. In recent years, some investigations have been done in Guizhou. Results show that the disease incidence of rice false smut was significantly different among rice varieties. For example, in 2008, the disease incidence and disease index of various rice varieties were 1.07 to 39.19% and 0.32 to 16.77%, respectively. The differences observed among the varieties were 36.6 times and 52.4 times. In order to effectively control rice false smut by using resistant varieties, the study of biological characteristics leading to the successful artificial inoculation is most important. Therefore, this study aims to examine the biology of *U. virens* and to develop suitable medium for inoculum production of *U. virens* and moreover, to develop an artificial inoculation techniques suitable for the study of false smut resistance in rice plant.

MATERIALS AND METHODS

Sample collection and isolation of *Ustilaginoidea virens*

Samples of the rice false smut balls were collected from 7 districts (Zunyi, Guiyang, Tongren, Qiandongnan, Qiannan, Anshun and Xingyi) in Guizhou in 2007-2008. To isolate the causal agent, the diseased samples were washed thoroughly under distilled water and dried under a lamina flow in the laboratory. Subsequently, the smut balls from each location were separately surface sterilized with 75% ethanol (EtOH) for 25 s. The excess traces of EtOH on the balls were removed by washing 2 times in sterile distilled water and then transferred aseptically into a flask containing sterile distilled water and 10 glass beads. The cultured flasks were then incubated and shaked at 28±2°C in the dark for 2 min after that 2 ml of the culture suspension was spread on surface of potato sucrose agar (PSA: potato 200 g, sucrose 20 g, agar 17 g, distilled water 1000 ml) (Liu et al., 2009). The PSA plates were incubated at 28±2°C for few hours and were examined frequently under a microscope for germinating single spores which were then marked with ink on the plate surface. These single spores were aseptically transferred with a sterile cork borer to fresh PSA and incubated at 28±2°C for 10 days. The pure single spores were then transferred into PSA slants and maintained for the future experiments. To maintain the culture, the fungus that was isolated from respective geographical region and maintained as a separate isolate, was sub-cultured on PSA slants and allowed to grow at 28±2°C for two weeks. Subsequently, the slants were preserved in a refrigerator and renewed once every two months.

Pathogenicity test of the *Ustilaginoidea virens* isolates

To confirm and prove pathogenicity of the eight *U. virens* isolates, spore suspension of each isolate at 1×10^6 spore/ml were inoculated into rice cultivar Zhongyou 177 at the late booting stage using the conidia injection technique. In another set, instead of spore suspension, only sterile distilled water was injected to serve as a negative control. The experiment was conducted as factorial in CRD with 4 replications. Observations were made at regular intervals for symptom development within 3 days. The virulence level of each isolate was evaluated based on the percentage of disease incidence classified into different scores as indicated in Table 1.

Study on suitable inoculation techniques

Inoculum preparation

The randomly selected *U. virens* isolates were used in this experiment. Details of the isolates are as shown in Table 2. Spore suspensions of the respective fungal isolates were prepared at approximately 1×10^6 conidia/ml. For the injecting inoculation, tween 80 was added into the spore suspension prior to use. For the spraying inoculation, 0.5% gelatin was added prior to use to prevent spore desiccation.

Rice plant preparation

Seeds of Gangxiang 707 were soaked in warm water at 60 °C for

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Table 1. Scoring criteria for categorizing virulence level of *Ustilaginoidea virens.*

Scoring	Disease incidence (%)	Smut ball density (No/panicle)
0	≤1%	0
1	＞1 but≤5%	1
3	＞5 but≤10%	＞1 but≤5
5	＞10 but≤25%	＞5 but≤10
7	＞25 but≤50%	＞11 but≤15
9	＞50%	＞16

Table 2. *Ustilaginoidea virens* single spore isolates used in the experiment.

Isolate	Origin (District)
2008-1-3	Zunyi
2007-79-1	Zunyi
2008-2-2	Guiyang
2007-11-1	Guiyang
2007-48-1	Tongren
2007-6-1	Qiandongnan
2007-66-1	Qiandongnan
2008-11-1	Qiannan
2008-33-1	Anshun
2008-36-1	Xinyi

1 h to kill seed-borne pathogens and left overnight to absorb water. After that, the seeds were pre-germinated at 30°C for 36 h before planting in a small field plot. At 25 days after planting, seedlings were transplanted into cement plots, 90×80 cm in size, in the greenhouse, 25 seedlings per plot.

Artificial inoculation of rice

The spore suspension of *U. virens* isolate 2008-33-1 (1×10^6 spore/ml) was used for inoculating Gangxiang 707. Two different artificial inoculation methods, conidia suspension spraying and conidia injection were investigated at tillering, booting and late booting stages. For conidia spraying, the suspension was sprayed on leaf surface until run–off by an air compressor. For conidia injection, 1 ml of the suspension was injected into the upper part of leaf sheath covering the developing panicle of each test plant using a syringe. In another set, instead of spore suspension only sterile distilled water was sprayed or injected to serve as a negative control. The experiment was conducted as factorial in CRD with four replications. Subsequently, the inoculated rice plants were kept in moist condition and observations were made at regular intervals for symptom development within 3 days after inoculation.

Reaction of rice varieties to *Ustilaginoidea virens*

Eight rice varieties of different resistant levels and from different areas of origin (Table 3) were used. The plants were prepared as described above. The inoculation was done by conidia injection technique at late booting stage (5~7 days before flowering). Selected eight virulent fungal isolates, including 2008-1-3, 2008-2-2, 2007-11-1, 2007-6-1, 2008-33-1, 2008-11-1, 2008-36-1 and 2007-48-1, were prepared in sterile distilled water as spore suspensions.

The experiment was conducted as factorial in CRD with four replications. Observations were made at regular intervals for symptom development within 3 days. Number of infected rice plants and grains were counted at 40~50 days after the inoculation (maturity stage). The disease incidence, disease index and density of rice smut balls were calculated and evaluated as follows:

$$\text{Disease incidence (\%)} = \frac{\text{Total infected rice panicles}}{\text{Total inoculated rice plants}} \text{x } 100$$

The rice false smut incidence and density of smut ball were classified into six scoring scales based on Zhang et al. (2006) (Table 1). The disease index was calculated according to the amount of rice smut balls of each score and corresponding value of scale (Table 1) as follows:

$$\text{Disease index} = \frac{\sum(\text{infected panicles of each rating} \times \text{rating value})}{\text{Total panicles} \times \text{The highest rating value}} \times 100$$

The comprehensive evaluation index (CEI) was calculated as follows:

$$\text{CEI} = [(\text{score of disease incidence} \times 60) + (\text{score of smut ball density} \times 40)]/100$$

(Ministry of Agriculture of P. R. China, 2006). Subsequently, the CEI was used for the resistance evaluation of the rice varieties using the criteria in Table 4.

Statistical analysis

Treatment effects on most experiments were analyzed using

Table 3. Rice varieties and their observed reaction to *U. virens* employed in the study.

Rice variety	Area of origin	Reaction to false smut
Gangxiang 707	Sichuan	S
Zhongyou 177	Sichuan	R
Gangyou 827	Sichuan	S
Heyou 6	Sichuan	R
Suaiyoulianhe 2	Guizhou	R
Jinxiangyou 830	Guizhou	S
Fengyouxiangzhan	Jiangshu	S
Nongfengyou 256	Anhui	R

R=resistant; S=susceptible.

Table 4. Scoring of the comprehensive evaluation index (CEI) of reaction to rice varieties of false smut disease.

Scoring	CEI	Resistant level
0	0	Highly resistant (HR)
1	≤1	Resistant (R)
3	＞1 but≤3	Moderately resistant (MR)
5	＞3 but≤5	Moderately susceptible (MS)
7	＞5 but≤7	Susceptible (S)
9	＞7	Highly susceptible (HS)

ANOVA by the SPSS program. In some experiments, the data were arcsine transformed before the analysis. Duncan's multiple range test (DMRT) at p≤0.05 was used to separate treatment means.

RESULTS

Sample collection and single spore isolation of *Ustilaginoidea virens*

To obtain *U. virens* in the test, 70 rice diseased panicle samples (Figure 1) were collected in Guizhou province during 2007- 2008. From the samples, 138 single spore isolates were isolated from different regions (Table 5). There were 10 samples and 16 single spore isolates from Zunyi, 13 and 26 from Guiyang, 6 and 14 from Tongren, 11 and 24 from Qiandongnan, 10 and 21 from Qiannan, 8 and 23 from Anshun, and 8 and 14 from Xinyi, respectively.

Pathogenicity test of the *Ustilaginoidea virens* isolates

The results are shown in Table 6. Most of the *U. virens* isolates selected for the test could infect the rice variety Zhongyou 177, indicating that they all were pathogenic fungi. Among of them, the isolate 2008-11-1 revealed the highest rice panicle disease incidence with 81.66% which is the maximum according to the grading of disease incidence at score 9.

Study on suitable inoculation technique

In the experiment, 40 rice panicles were used per replication. The average disease incidences of spraying inoculation were 0.00, 21.18, and 34.75% at tillering, early booting and late booting stages respectively. The disease incidence was much higher when rice plants were inoculated by conidia injection (Figure 2), which had 0.00, 38.50 and 50.43% at tillering, early booting and late booting stages respectively (Table 7). Result from combined analysis showed clearly that conidia injection was the suitable inoculation technique in this experiment (Table 8) and it should be done at the late booting stage (Table 9).

Reaction of rice varieties to *Ustilaginoidea virens* isolates

The results were as shown in Table 10. Most of the rice varieties were susceptible or moderately susceptible to the *U. virens* isolates except variety Fengyouxiangzhan which was highly susceptible to the 2007-79-1 isolate while variety Nongfengyou 256 showed moderately resistant reaction to the same isolate. Combined analysis of all factors contributed to the rice panicle disease reaction was presented in Tables 11, 12, and 13. Among the six rice varieties tested, Gangyou 827 appeared to have the highest disease incidence (55.06%) while Nongfengyou 256 seem to have the lowest (23.85%) (Table 11). Differences in virulence among the *U. virens* isolates were also observed in that isolate; 2008-33-1 showed the highest disease incidence (58.09%) while isolate 2008-2-2 gave the lowest incidence (28.17%) (Table 12). When the overall disease reactions were analyzed by combining all disease parameters, it appeared that Nongfengyou 256 performed the best by being moderately resistant to 12.5% of the *U. virens*

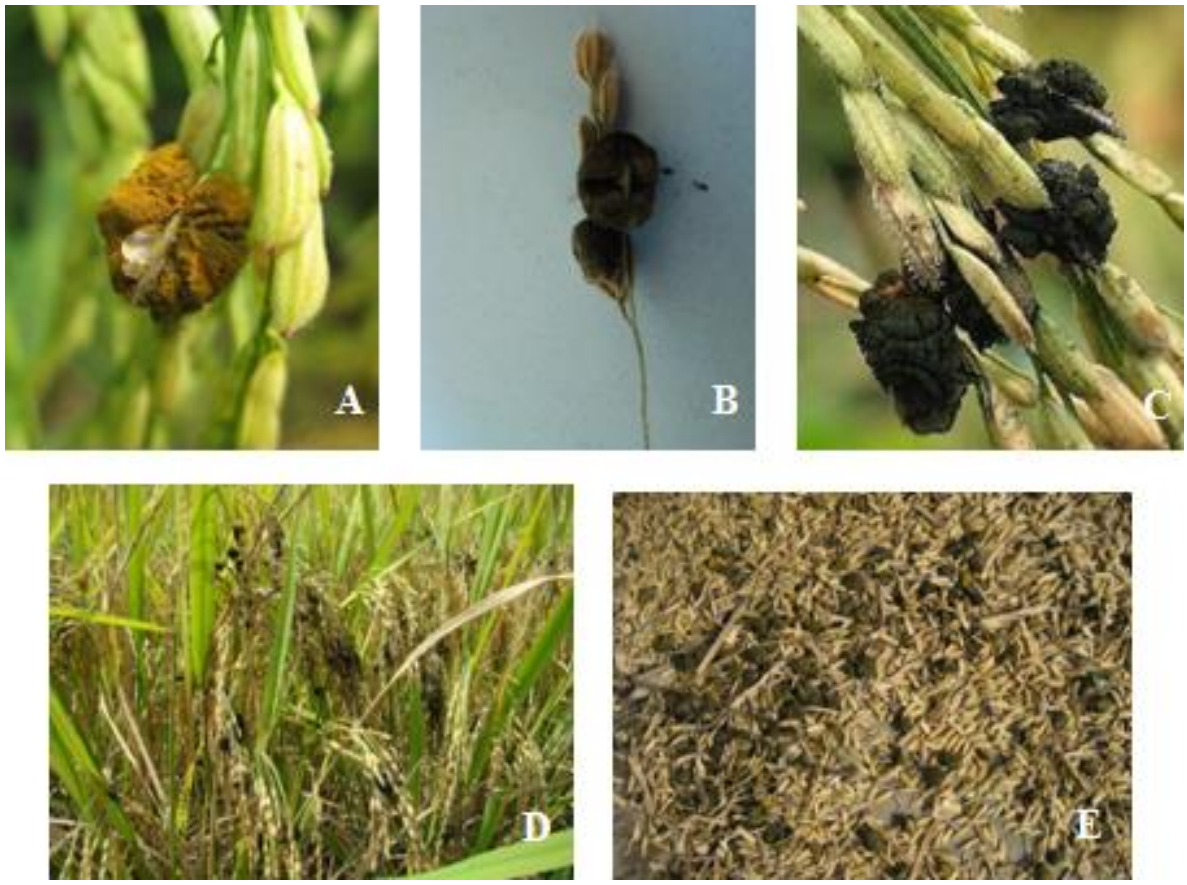

Figure 1. Symptoms of false smut on rice panicles. (A) orange stage; (B) brown stage; (C) green black stage; (D) field symptoms; (E) smut balls on harvested grains.

Table 5. Single spore isolates of *U. virens* collected from various districts of Guizhou Province, China.

District	Year of collection	Number of sample	Number of single spore isolate
Zunyi	2007-2008	10	16
Guiyang	2007-2008	13	26
Tongren	2008	6	13
Qiandongnan (Southeast)	2008	11	24
Qian nan (South)	2008	10	21
Anshun	2007-2008	12	23
Xinyi	2008	8	15
Total	—	70	138

isolates and only moderately susceptible to the left 87.5% (Table 13).

DISCUSSION

Seventy diseased samples were collected from seven districts of Guizhou province. 138 single-spore isolates of *U. virens* were successfully isolated. Among the two inoculation methods applied at three rice growth stages, conidia injection at late booting appeared to be the most effective giving the smut incidence of 50.43% while conidia spraying at the same growth stage gave only 34.75% incidence. Both methods at early booting gave

Table 6. The virulence of *U. virens* isolates on Zhongyou 177 rice cultivar.

U. virens isolate	Disease incidence (%) [1]	Virulence score
2008-1-3	33.05^{d}	7
2008-2-2	33.72^{cd}	7
2007-11-1	41.85^{c}	7
2007-6-1	51.26^{b}	9
2008-33-1	58.76^{b}	9
2008-11-1	81.66^{a}	9
2008-36-1	23.97^{e}	5
2007-48-1	15.57^{f}	5
CK	0.00^{g}	
F-test	**	
CV(%)	8.98	

[1]The data were arcsine transformed before analysis. Means in the same column followed by different letter are significantly different at P≤0.05 by DMRT technique.

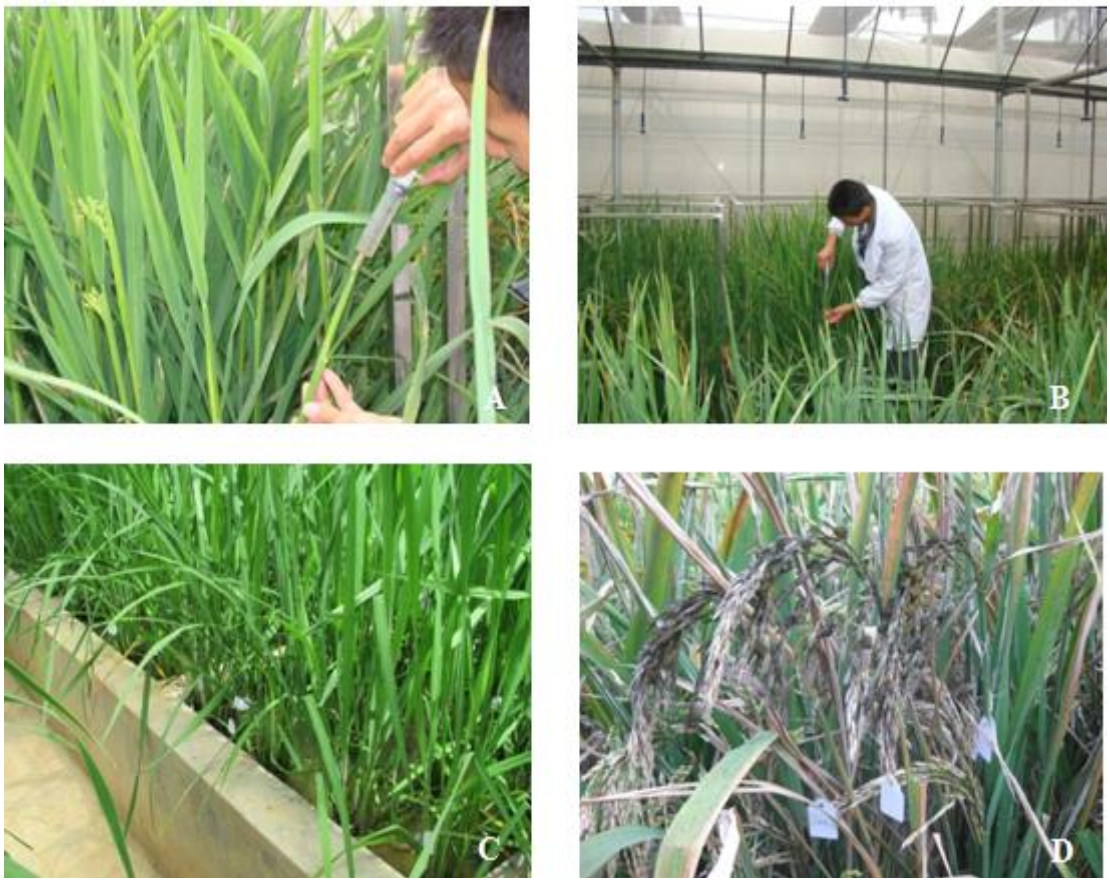

Figure 2. Artificial inoculation and symptom of rice false smut (A) and conidia injection inoculation (B), labeling of the inoculated plants (C), symptoms of rice false smut developed from artificial inoculation (D).

lower smut incidence, while applying inoculum at tillering failed to cause any infection. This finding indicates that growth stage is an important factor contributing to the success or failure of the inoculation. Similar finding has been reported by Wang et al. (1996) who found that the smut incidence was higher when rice plants were injected than when they were sprayed with conidia suspension and the critical susceptible stage was at booting.

Table 7. False smut incidence on Gangxiang 707 rice variety inoculated by 2 different methods at 3 growth stages of rice.

Inoculation method	Inoculation stage	Disease incidence (%) [1]
Spraying inoculation	Tillering stage	0.00^{e}
	Early booting stage	21.18^{d}
	Late booting stage	34.75^{c}
	Blank control	0.00^{e}
Injecting inoculation	Tillering stage	0.00^{e}
	Early booting stage	38.50^{b}
	Late booting stage	50.43^{a}
	Blank control	0.00^{e}
F-test		**
CV (%)		12.86

[1]The data were arcsine transformed before the analysis. Means in the same column followed by different letters are significantly different at P≤0.05 by DMRT.

Table 8. Combined analysis of false smut incidence on Gangxiang 707 inoculated by 2 different methods at 3 growing stages of rice.

Inoculation method	Disease incidence (%) [1]
Spraying	13.98^{b}
Injection	22.23^{a}
F-test	**
CV(%)	8.12

[1] The data were arcsine transformed before the analysis. Means in the same column followed by different letters are significantly different at P≤0.05 by DMRT.

Table 9. Combined analysis of false smut incidence on Gangxiang 707 inoculated at 3 growth stages of rice by different methods.

Inoculation stage	Disease incidence (%) [1]
Tillering	0.00^{c}
Early booting	29.84^{b}
Late booting	42.59^{a}
Blank control	0.00^{c}
F-test	**
CV(%)	8.12

[1] The data were arcsine transformed before the analysis. Means in the same column followed by different letters are significantly different at P≤0.05 by DMRT.

When the pathogenicity was tested among eight selected *U. virens* isolates on Zhongyou 177 rice cultivar, the 2008-11-1 isolate showed the highest virulence score of 9 with the disease incidence of 81.66% while the 2007-48-1 isolate performed the lowest score of only 5 with the disease incidence of 15.57%. Such results reflected the diversity in virulence among the *U. virens* isolates. The 2008-11-1 isolate was collected from Qiannan which was the major planting area for hybrid rice with excessive use of nitrogen fertilizer, a very conducive condition for *U. virens* epidemic (personal observation, 2011). Smut incidence of 15.55% has been commonly observed on rice grown in Qiannan. Crop monoculture and excessive use of nitrogen have been known to cause epidemic of many rice diseases (Ou, 1972; Long et al., 2000). The existing of aggressive race in Qiannan should be made known so that the farmers would be more cautious in selecting rice cultivars and applying nitrogen fertilizer.

The eight selected *U. virens* isolates were further tested on six rice varieties using the conidia injection at late booting stage. Most of the rice varieties tested showed different resistant level from those that had been observed in the field prior to the experiment. Most varieties were either susceptible or moderately susceptible except Fengyouxiangzhan that was highly susceptible to *U. virens* isolate 2007-79-1 and Nongfengyou 256 was moderately resistant to the same *U. virens* isolate. The different reaction observed in the field could have come from the existing of different *U. virens* races in different locations, the phenomenon that has been noted earlier. After combined analysis, such diversity could be seen again in that among the 8 *U. virens* isolates tested, 2008-33-1 isolate was found to be most aggressive giving disease incidence as high as 58.09% while 2008-2-2 isolate gave only 28.17%. All cultivars tested, Gangxiang 827, Jixiangyou 830 and Fengyouxiangzhan had highs smut incidence of 55.06, 54.79 and 53.50%, respectively while Nongfengyou 256 had the lowest of only 23.85%. This cultivar appeared to perform best being moderately resistant to 12.5% of the

Table 10. Reaction of rice varieties to infection by *U. virens* isolates evaluated as smut ball density scoring (SDS), disease incidence (DI), disease incidence score (DIS), comprehensive evaluation index (CEI) and CEI scores (CEIS).

Rice variety	*U. virenes* isolates	SDS (ball/panicle)	DI(%) [1]	DIS	CEI	CEIS	Reaction
	2008-2-2	1	34.72^{d}	7	4.6	5	MS
	2008-33-1	3	80.78^{a}	9	6.6	7	S
	2007-11-1	3	55.44^{c}	9	6.6	7	S
	2007-6-1	3	69.53^{ab}	9	6.6	7	S
Gangyou 827	2008-1-3	3	56.95^{c}	9	6.6	7	S
	2008-36-1	1	42.05^{d}	7	4.6	5	MS
	2007-66-1	3	60.27^{bc}	9	6.6	7	S
	2007-79-1	1	40.77^{d}	7	4.6	5	MS
F-test			**				
CV(%)			18.49				
	2008-2-2	3	39.11^{d}	7	5.4	7	S
	2008-33-1	3	74.14^{a}	9	6.6	7	S
	2007-11-1	3	53.78^{c}	9	6.6	7	S
	2007-6-1	3	74.14^{ab}	9	6.6	7	S
Jinxiangyou 830	2008-1-3	3	57.17^{c}	9	6.6	7	S
	2008-36-1	1	40.67^{d}	7	4.6	5	MS
	2007-66-1	3	60.11^{bc}	9	6.6	7	S
	2007-79-1	1	39.23^{d}	7	4.6	5	MS
F-test			**				
CV(%)			13.04				
	2008-2-2	1	14.94^{c}	5	3.4	5	MS
	2008-33-1	3	42.12^{a}	7	5.4	7	S
	2007-11-1	1	36.22^{a}b	7	4.6	5	MS
	2007-6-1	1	42.05^{a}	7	4.6	5	MS
Heyou 6	2008-1-3	1	32.90^{ab}	7	4.6	5	MS
	2008-36-1	1	26.57^{b}	5	3.4	5	MS
	2007-66-1	1	29.89^{ab}	5	3.4	5	MS
	2007-79-1	1	31.55^{ab}	7	4.6	5	MS
F-test			**				
CV(%)			30.05				

Table 10. contd.

Rice variety	*U. virenes* isolates	SDS (ball/panicle)	DI(%) [1]	DIS	CEI	CEIS	Reaction
	2008-2-2	1	29.89^{ab}	5	3.4	5	MS
	2008-33-1	3	37.66^{ab}	7	5.4	7	S
	2007-11-1	1	37.73^{ab}	7	4.6	5	MS
Suaiyou	2007-6-1	3	40.67^{a}	7	5.4	7	S
lianhe 2	2008-1-3	1	31.39^{ab}	7	4.6	5	MS
	2008-36-1	1	26.57^{bc}	5	3.4	5	MS
	2007-66-1	1	17.89^{c}	5	3.4	5	MS
	2007-79-1	1	17.89^{c}	5	3.4	5	MS

Table 10. contd

F-test			**				
CV(%)			27.91				
	2008-2-2	1	22.1[d]	5	3.4	5	MS
	2008-33-1	3	80.78[a]	9	6.6	7	S
	2007-11-1	3	49.33[c]	9	6.6	7	S
	2007-6-1	3	61.77[b]	9	6.6	7	S
Fengyou xiangzhan	2008-1-3	3	49.33[c]	9	6.6	7	S
	2008-36-1	1	42.05[c]	7	4.6	5	MS
	2007-66-1	3	60.27[b]	9	6.6	7	S
	2007-79-1	5	62.31[b]	9	7.4	9	HS
F-test			**				
CV(%)			14.24				
	2008-2-2	1	28.23[ab]	5	3.4	5	MS
	2008-33-1	1	33.06[a]	7	4.6	5	MS
	2007-11-1	1	24.53[ab]	5	3.4	5	MS
Nongfengyou	2007-6-1	1	21.59[ab]	5	3.4	5	MS
256	2008-1-3	1	22.50[ab]	5	3.4	5	MS
	2008-36-1	1	24.53[ab]	5	3.4	5	MS
	2007-66-1	1	20.47[b]	5	3.4	5	MS
	2007-79-1	1	15.86[b]	3	2.2	3	MR
F-test			**				
CV(%)			40.92				

[1]The data were arcsine transformed before the analysis. Means in the same column followed by different small letters are significantly different at P≤0.05 by DMRT.

Table 11. Combined disease incidence of rice varieties inoculated with 8 *U. virens* isolates by conidia injection.

Variety	Disease incidence (%) [1]
Gangyou 827	55.06[a]
Jinxiangyou 830	54.79[a]
Fengyouxiangzhan	53.50[a]
Heyou 6	32.01[b]
Suaiyoulianhe 2	29.96[b]
Nongfengyou 256	23.85[c]
F-test	**
CV(%)	16.74

[1]The data were arcsine transformed before the analysis. Means in the same column followed by different letters are significantly different at P≤0.05 by DMRT.

Table 12. Combined disease incidence of 8 *U. virens* isolates inoculated to 6 rice varieties by conidia injection.

Isolates	Disease incidence (%) [1]
2008-33-1	58.09[a]
2007-6-1	51.63[b]
2007-11-1	42.84[c]
2008-1-3	41.71[c]
2007-66-1	41.48[c]
2007-79-1	34.60[d]
2008-36-1	33.74[d]
2008-2-2	28.17[e]
F-test	**
CV(%)	16.74

[1]The data were arcsine transformed before the analysis. Means in the same column followed by different letters are significantly different at P≤0.05 by DMRT.

U. virens isolates and moderately susceptible to 87.5% of them. The consistent reaction of this cultivar to *U. virens* observed in the fields with that observed in this experiment indicates that its resistance could be polygenic. The higher disease score observed in the experiment could result from excessive concentration of the inoculum applied to the rice plant and the unnatural inoculation process. With all these shortcomings of the artificial inoculation, it is therefore necessary to repeat the

Table 13. Reaction of rice varieties to *U. virens* isolates.

Variety	Percentage of *U. virens* isolate with the reaction					
	HR	R	MR	MS	S	HS
Gangyou 827	0	0	0	37.50	62.50	0.00
Jinxiangyou 830	0	0	0	25.00	75.00	0.00
Heyou 6	0	0	0	87.50	12.50	0.00
Suaiyoulianhe 2	0	0	0	75.00	25.00	0.00
Fengyouxiangzhan	0	0	0	25.00	62.50	12.50
Nongfengyou 256	0	0	12.5	87.50	0.00	0.00

HR=highly resistant; R=resistant; MR=moderately resistant; S=susceptible; HS=highly susceptible.

screening under field condition before the varieties could be labeled for their reaction to *U. virens.*

Conflict of interests

The authors did not declare any conflict of interests.

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