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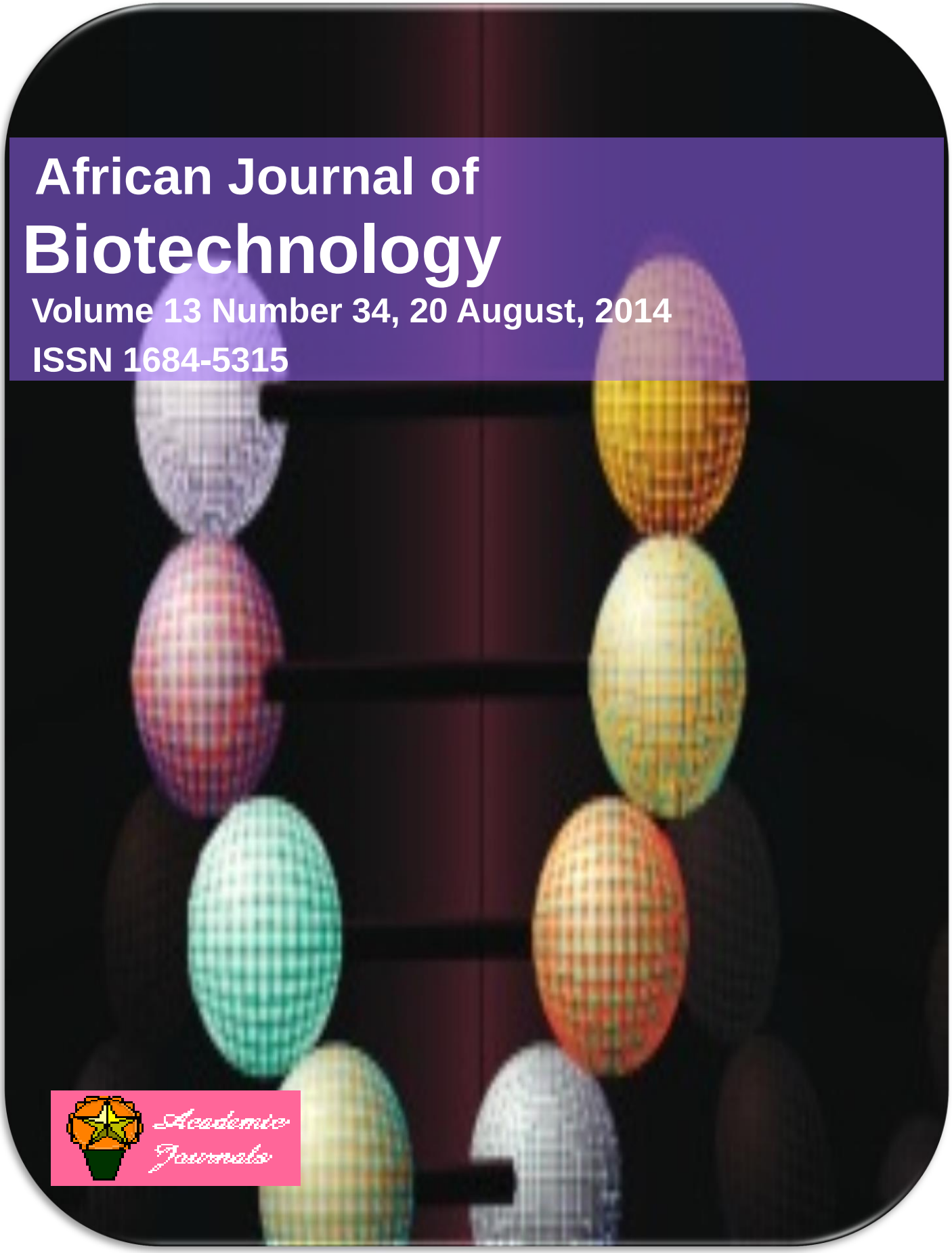

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Full Length Research Paper

Shoot regeneration, biochemical, molecular and phytochemical investigation of *Arum palaestinum* Boiss

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***Arum palaestinum* Boiss. populations are in danger of extinction in the wild. Thus, there is a need to establish a reliable strategy for multiplying this valuable medicinal plant. In the present study, seeds and tissue culture of *A. palaestinum* were subjected to biochemical, molecular and phytochemical analysis. Obtained results indicated that the best medium for shoots proliferation was Murashige and Skoog (MS) medium supplemented with 5 mg/L benzyl adenine (BA) and 0.1 mg/L naphthalene acetic acid (NAA). The regenerated shoots were rooted on half strength MS medium containing 1 mg/L NAA and 2 g/L charcoal. Tissue culture derived plantlets were successfully acclimatized under *ex vitro* conditions. Protein analysis referred that, the difference in protein profiles in the examined samples suggests that a real genetic change might have occurred. Obtained results of the inter simple sequence repeat (ISSR) revealed variation between the regenerated plants and mother plant while the phytochemical investigation revealed that, 10 phenolic compounds (seven flavones, one flavonol and two phenolic acids) were identified using HPLC analysis and five compounds were detected in the plant for the first time. Genetic characterization and chemical investigation of seeds and *in vitro* cultures reported herein, is the first report for *A. palaestinum*.**

Key words: Black calla lily, *in vitro* culture, inter simple sequence repeat (ISSR), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), isozyme, phenolic compounds.

INTRODUCTION

Arum palaestinum Boiss. (Black Calla Lily) is one of about 26 species of the *Arum* genus belonging to family Araceae (Boyce, 1993; Mayo et al., 1997; Al-Lozi et al., 2008; Makhadmeh et al., 2010) native to Europe,

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Northern Africa, Western Asia, with the highest species diversity in Mediterranean region (Dessouky et al., 2007a). Black lily is a typical “cryptic” species, since its appendix emits mainly ethyl acetate, producing a smell of rotten fruit. In many countries, the aerial parts of *A. palaestinum* are considered ornamental plant, animal fodder and edible after being soaked in salty water or dried. The plant is used in folk medicine to cure several chronic diseases such as stomach acidity, atherosclerosis, cancer, and diabetes (Al-Eisawi, 1982; Al-Lozi et al., 2008; Makhadmeh et al., 2010).

Previous work on the characteristics of secondary metabolites of the Araceae family indicated the presence of polyphenols, alkaloids, proanthocyanidins, flavones, flavone *C*-glycosides and flavonols (Williams et al., 1981; Kite et al., 1997). The phytochemical investigation of *A. palaestinum* resulted in the isolation of two *C*-glucoside flavones: isoorientin and vitexin. The effects of isoorientin on rat isolated aorta, ileum, trachea and uterus and on guinea pig uterus were studied by Afifi et al. (1999). A novel alkylated piperazine were also isolated and showed a significant cytotoxicity against cultured tumor cell lines (El- Desouky et al., 2007a). Polyhydroxy alkaloid compound in addition to; caffeic acid, isoorientin, luteolin, vicenin and 3,6,8-trimethoxy 5,7, 3', 4'-tetrahydroxy flavone were isolated by El- Desouky et al. (2007b). Recently, Aboul-Enein et al. (2012) assured potential antitumor effect of *A. palaestinum* extract. It is worth to mention that all published reports of phytochemical studies used leaves and flowers of *A. palaestinum* and provide evidences for strong antitumor activities of its extract.

The successful use of plant biotechnology techniques in production of secondary metabolites, mass propagation and conservation of rare species dates back to early eighty’s and is well discussed by Engelmann (2004). However, survey of published data indicated that there is only one published manuscript on *in vitro* culture of *A. palaestinum* via somatic embryogenesis (Shibli et al., 2012).

Genetic markers derived from electrophoretic analysis can be used to survey the level of genetic diversity within and among populations and also for taxonomic purpose (Hamrick and Godt, 1989). Isozyme analysis is a highly appropriate method for identifying genomic allele components as well as supplementing DNA analysis. Since the 1930s, electrophoresis in conjunction with the zymogram technique has been used as a tool for the study of heritable variation. Isozymes are widely used because of their relative efficiency and cost effectiveness, particularly in studies of intra and inter specific variation (Johnson et al., 2007; Siva and Krishnamurthy, 2005; Johnson et al., 2010; Smila et al., 2007).

Recently, several DNA markers have been successfully employed to assess the genomic stability/instability in regenerated plants. Among the markers, the inter-simple sequence repeat (ISSR) has been favored because of their sensitivity, simplicity, and cost effectiveness (Yang et al., 1996). The aim of this study was establishment of applicable tissue culture system coupled with monitoring of genetic stability of tissue culture derived clones (*in vitro* plants) for rapid mass propagation, conservation and future biotechnology based production of pharmaceutically bioactive ingredients of black calla lily. The second objective of this study was the genetic characterization and phytochemical investigations for both *in vitro* produced plants and *in vivo* plants, for a better understanding of genetic relationship and the discovery of new potent bioactive substances.

MATERIALS AND METHODS

Plant material

Seeds of *A. palaestinum* were collected from their growing habitats in Bergesh protected area, Irbid, Jordan, Latitude: 32°25'43.17 and Longitude: 35°46'47.01 in February 2012.

Tissue culture of *A. palaestinum*

Seeds of *A. palaestinum* were decoated under sterile conditions of air laminar flow cabinet. The decoated seeds were surface sterilized by immersion in 70% ethanol for 60 s, and then immersed in 20% sodium hypochlorite (NaOCl) solution for 20 min. Seeds were then rinsed three times with sterile distilled water and cultured on basal Murashige and Skoog medium (MS) (1962) containing 3% sucrose and 4.4 g/L of MS, salts without growth regulators and solidified with 2.8 g/L gelrite and kept in incubation room under dark condition for 48 h. The *in vitro* germinated seedlings (2- month-old) about 4-6 cm in height were used as a source of starting plant materials (Figure 1a). During germination, callus was proliferated directly from seeds in some samples as shown in Figure 1b then different explants (leaves, stems, root and corms) were excised from the *in vitro* seedlings (two months old) and cultured on six different regeneration media as illustrated in Table 1. All media contained 4.4 g/L MS basal salts, 30 g/L sucrose and solidified with 2.8 g/L gelrite. The proliferated shoots were multiplied on MS medium supplemented with 0.1 mg/L NAA and 5 mg/L BA (medium 6). Number and length of shoots, and roots were recorded. Shoots developed on regeneration media were rooted on half strength basal MS medium (2.2 g/L MS salts), containing 30 g/L sucrose and supplemented with 1 mg/L NAA and 2 g /L charcoal and solidified with 2.8 g/L gelrite and all culture were incubated in temperature controlled growth room at 27 ± 1°C for 16 h daily light system under light intensity (Ca 50 µmol m^{-2} s^{-1}) and subcultured monthly in fresh medium. Complete plantlets (shoots and roots) were transplanted to mixture of 1:3 vermiculite and soil in plastic pots and placed in greenhouse for acclimatization

Protein analysis

For SDS-PAGE protein patterns and Isozyme analysis, 300 mg of regenerated shoots from corms of *A. palaestinum* cultured on the six tested regeneration media and mother plant were extracted according to the method of Gottlieb (1981)

The separating gel of 10% acrylamide was prepared following the method of Laemmli (1970). The method of Weber and Osborne (1969) was used to determine the apparent (subunit) molecular weight of proteins dissolved or extracted in the presence of SDS

Figure 1. a) *In vitro* germinated seeds of *A. palaestinum*. **b)** Seeds derived callus during germination.

Table 1. Structure of regeneration media used.

Media code	BA (mg /L)	NAA (mg /L)	Thiamine (mg/L)	KH_2PO_4 (mg/L)	Glycine (mg/L)	Adenine sulphate (mg/L)
1	5	-	70	170	100	50
2	1	0.1	-	-	-	-
3	5	0.5	-	-	-	-
4	5	-	-	-	-	-
5	5	0.1	-	-	-	-
6	5	0.1	70	170	100	50

while isozyme gel was stained for α-Esterase enzyme according to the protocols described by Soltis et al. (1983).

Molecular analysis using ISSR

Fourteen ISSR primers were used for the control mother plant and tissue culture raised plantlets. PCR amplification was performed in 25 µl reaction mixture each containing 0.25 µl 0.5 U Taq DNA polymerase, 2.5 µl 0.2 mM dNTPs (dATPs, dCTPs, dGTPs and dTTPs), 5 µl (5X) colourless reaction buffer, 20.4 ng (3 µl) genomic DNA and 3 µl of 10 pmole primers, and 11.25 µl sterile distilled water. The thermocycler program for ISSR was 95°C for 3 min; 92°C for 2 min; 44 cycles of 43°C for 1 min; 72°C for 2 min; 72°C for 10 min and at 4°C for soaking; 100 bp DNA ladder (Biogene) was used. The banding profile of ISSR was scored using Labimage program. The polymorphism was estimated as follow:

Percent of polymorphism = (Number of polymorphic bands / Total Number of Bands) × 100.

Phytochemical investigation

The seeds (8 g) and the air dried *in vitro* shoots (47 mg), roots (140 mg) and callus (71 mg) of *A. palaestinum* were extracted with 70% methanol at room temperature for three times. The crude filtered extracts were concentrated under reduced pressure in a rotary evaporator to give a residue which dissolved in methanol. The isolation and identification of the compounds were carried out by using two dimension paper chromatography method with stander samples and confirmed by analyzing the extract on an Agilent HPLC 1200 series equipped with diode array detector (Agilent Technologies, Waldbronn, Germany). Chromatographic separations were performed using a waters column C18. The binary mobile phase consisted of (A) acetonitrile and (B) 0.1% acidified water with formic acid. The elution profile was: 0-1 min 100% B (isocratic), 1-30 min 100-70% B (linear gradient), 30-35 min 70-20% B (linear gradient). The flow rate was 0.3 ml/min and the injection volume was 5 µl. Chromatograms were recorded at 278 nm. This analysis enabled the characterization of phenolics on the basis of their retention time and UV spectra.

The retention time of the isolated compounds were compared with those of standard samples which were selected according to the compounds previously isolated from *A. palaestinum* and the Araceae family by the Phytochemistry and Plant Systematic Department, National Research Center.

Statistical analysis

All data were subjected to analysis of variance ANOVA to test the significance in the all experiments. The least significant difference (LSD) at $P< 0.05$ level was calculated according to the statistical

Table 2. Regeneration of shoots from corms of *A. palaestinum* cultured on six tested regeneration media.

Media type	Number of shoots	Length of shoots (cm)	Number of Roots	Length of root (cm)
1	$1.3^{a} \pm 0.47$	$3.7^{a} \pm 0.47$	$3^{a} \pm 0$	$1^{b} \pm 0$
2	$1^{a} \pm 0$	$1.3^{c} \pm 0.47$	No roots	-
3	$1.3^{a} \pm 0.47$	$5^{bd} \pm 3.6$	No roots	-
4	$1.3^{a} \pm 0.47$	$4.3^{ab} \pm 0.47$	$1^{b} \pm 0$	$0.5^{a} \pm 0$
5	$1^{a} \pm 0$	$2.3^{c} \pm 0.47$	$2^{c} \pm 0$	$0.5^{a} \pm 0$
6	$1.7^{b} \pm 0.47$	$6^{d} \pm 1.4$	No roots	-
F- value	0.850	16	4.756	6.818
Propabilty level (P<)	0.541	0.0001	0.017	0.008

Data (mean ± SD) sharing the same letter in the same column is not significantly different.

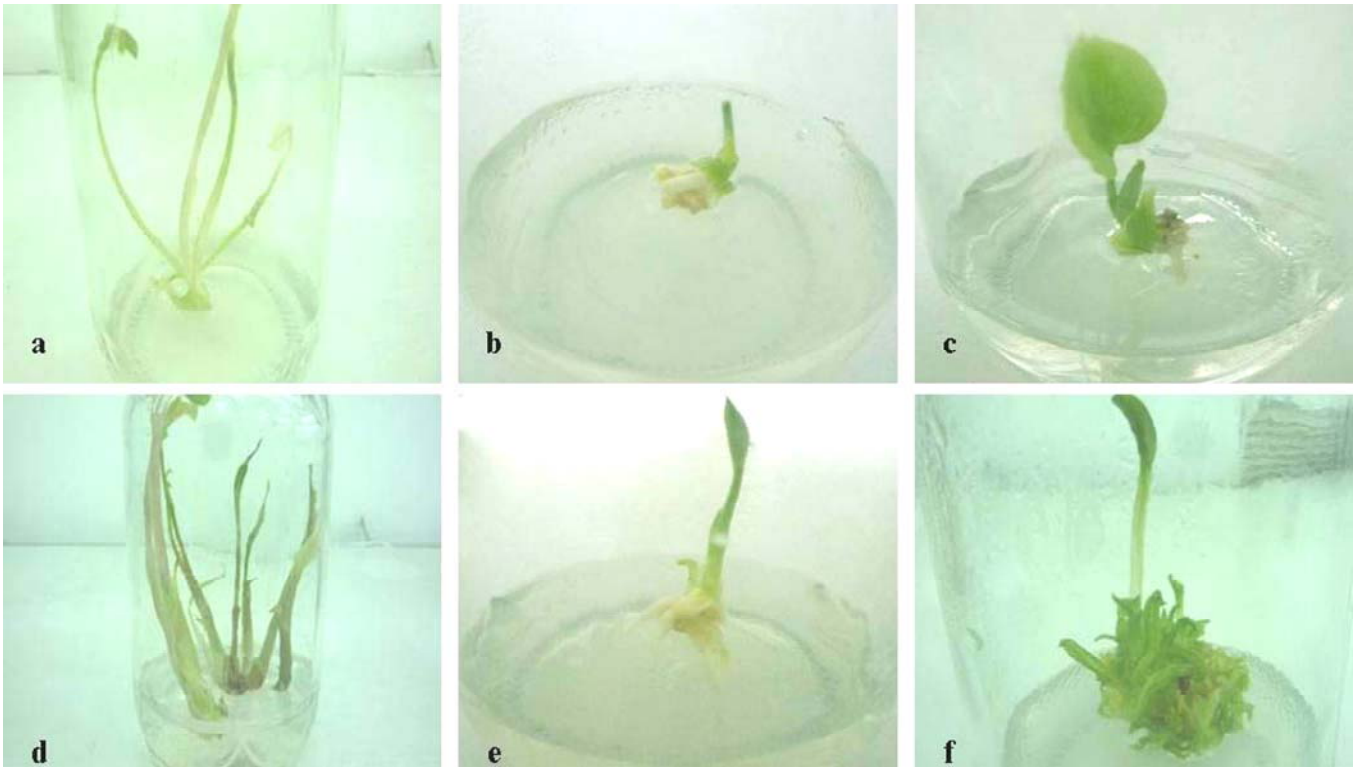

Figure 2. Regeneration of *A. palaestinum* from corms explants cultured on: **(a)** MS-medium with 5 mg/L BA +0.1 mg/L NAA (medium 6), **(b)** MS-medium with 1 mg/L BA + 0.1 mg/L NAA (medium 2), **(c)** MS-medium with 5 mg/L BA + 0.1 mg/L NAA (medium 5), **(d)** MS-medium with 5 mg/L BA + 0.1 mg/L NAA (medium 6), **(e)** Root proliferated on shoots on MS-medium contained 5 mg/L BA (medium 1) and **(f)** multiplication of the regenerated shoots on MS medium supplemented with 0.1 mg/L NAA and 5 mg/L BA.

analysis method described by Casanova et al. (2004).

RESULTS AND DISCUSSION

Tissue culture of *A. palaestinum*

The regeneration of new shoots from primary explants is a prerequisite for any regeneration protocol. In this study, the pre-existing buds started to develop earliest from only the corms and new shoots development was observed within eight weeks while all other plant material (explants) showed no growth response. Data obtained in Table 2 indicates that all the tested media for regeneration produced shoots and medium 6 (5 mg/L BA + 0.1 mg/L NAA) gave the highest shoots number (1.7) and length (6 cm) (Figure 2a and d) while medium 2 (MS + 1 mg/L BA + 0.1 mg/L NAA) gave the lowest shoots number (1) and length (1.3 cm) (Figure 2b and c). From Table 2, it could

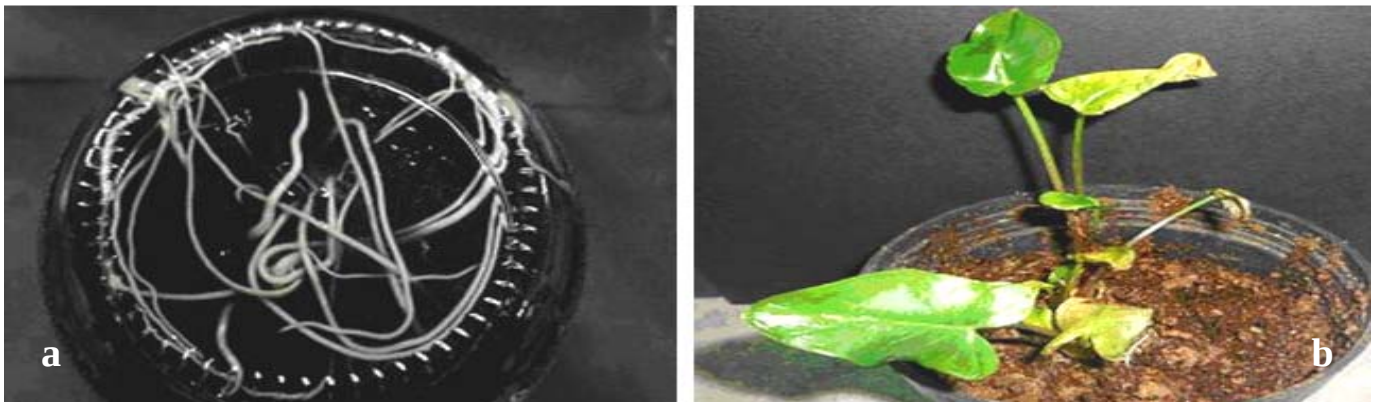

Figure 3. (a) Root formation. (b) Acclimatized complete plantlets of *A. palaestinum.*

also be observed that the highest number and length of roots (3 and 1 cm, respectively) was noticed with medium 1 (MS + 5 mg/L BA) (Figure 2e), whereas, medium 2, 3 and 6 did not show any response for rooting of shoots. The developed shoots were transferred to MS medium supplemented with 0.1 mg/L NAA + 5 mg/L BA and solidified with 2.8 g/L gelrite for multiplication of many long shoots (Figure 2f). The regenerated shoots were cultured on rooting medium that consisted of half strength MS medium + 2 g/L charcoal + 1 mg/L NAA which gave many long roots, its length was about 7 cm (Figure 3a). Rooted plantlets were acclimatized successfully with 95% survival rate (Figure 3b). The regenerated plantlets established in potting mixture were uniform and identical to donor plants with respect to growth characteristics and vegetative morphology.

Our results are in agreement with those of previous studies (Francis et al., 2007; Sivanesan and Jeong, 2007; Samantaray and Maiti, 2008) which showed that combination of cytokinins and auxins triggered the rate of shoot multiplication in various medicinal plants). The type of exogenous cytokinin used in the medium has a marked effect on the frequency of chestnut shoot proliferation (Carmen et al., 2001). In *Plumbago zeylanica,* BA was more effective for shoot bud proliferation than kinetin (Rout et al., 1999). In tomato, NAA showed the most positive effect on induction and elongation of lateral roots such an effect was also observed by Taylor et al*.* (1998). For rooting, activated charcoal may absorb toxic substances in the medium and improving root regeneration and development (Ziv, 1979; Takayama et al*.,* 1980). In this respect, Takayama et al*.* (1980) reported an inhibition of root formation of *Lilium* by BA and that inhibition was completely reversed by the addition of charcoal.

The hardening of *in vitro* raised plantlets is essential for better survival and successful establishment (Deb and Imchen, 2010). In this respect, Shibli et al. (2012) obtained plantlets from somatic embryos of *A. palaestinum* and reported that rooted plants were grown in greenhouse and acclimatized successfully with a 95% survival rate. In the present study, there is no feasible morphological difference among leaves of the *in vitro* raised plants, while some differences in root formation was observed with plantlets grown in media 2, 3 and 6.

Protein profiles

The protein profile system revealed the biochemical variation and evolutionary relationship among the plantlets grown on the six regeneration media and mother plant of *A. palaestinum* were demonstrated in Figure 4. The molecular weights of detected bands for all samples ranged from 7 to 79 KDa. Shoots grown on medium 1 only showed three bands at molecular weights 70, 61 and 46 KDa. There was one band detected at molecular weight 64 KDa in shoots grown on medium 2 and absent from the other samples. Also, band with molecular weight 20 KDa was absent in shoots grown on medium 2 and present in other samples examined. A polypeptide band of molecular weight 37 KDa was detected only in shoots grown in medium 3 and not present in all samples. Moreover, at molecular weight 34 KDa, band was absent in shoots grown on medium 5 and present in other samples examined. Similar protein profiles of mother plant and other plantlets grown in different media were observed at molecular weight 23 KDa. For donor plant, it could be observed that four bands were polymorphic bands at molecular weight of 79, 12, 16 and 13 KDa, respectively.

In the present study, the difference in protein profiles in the examined samples suggests that a real genetic change might have occurred due to the presence of growth regulators in the regeneration media used and these results are in agreement with those of Hendriks and Veries (1995) who detected a group of proteins (54 and 47 KDa) in embryogenic cultures of carrot. Similar finding was also observed by Beckmann et al*.* (1990) who reported that, SDS-PAGE analysis was used in the

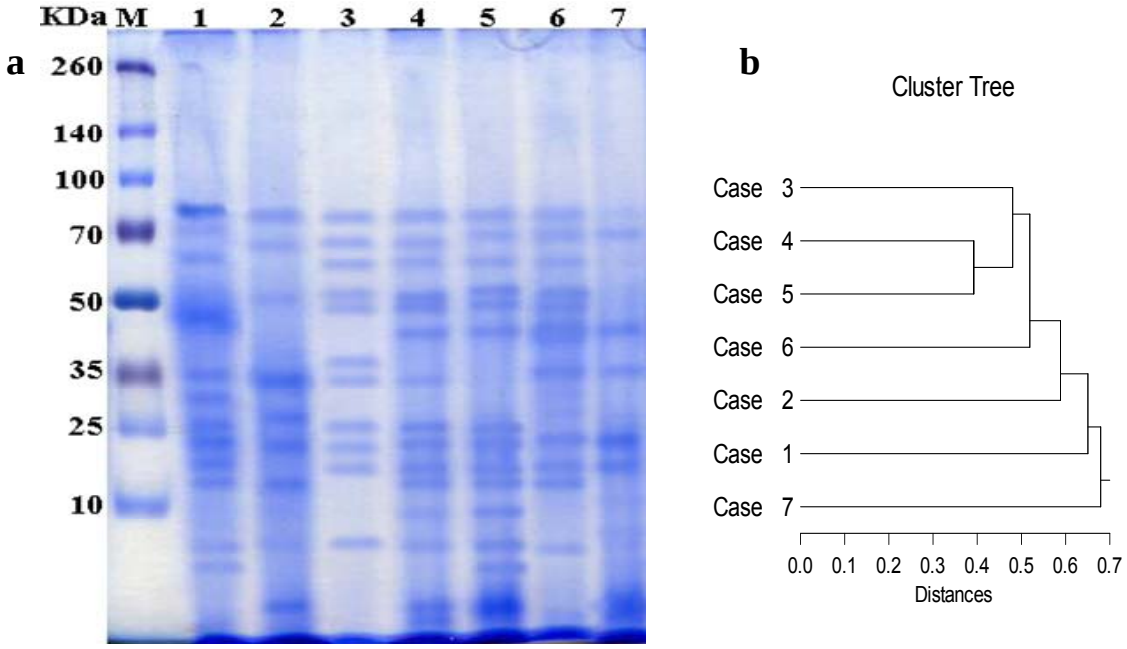

Figure 4. (a) SDS-PAGE of regenerated shoots of different *A. palaestinum in vitro* plants developed on the six tested regeneration media (1: 6) and control mother plant. Lane M refers to low molecular weight standard protein marker. **(b)** Dendrogram for regenerated plants and donor plant constructed from protein analysis data using un-weighted pair-group arithmetic average (UPGMA) and similarity matrices computed according to dice coefficients.

Table 3. Distribution of bands and relative mobilities (RF) of α-esterase of *A. palaestinum in vitro* plants and mother plant.

Primer	5'- Sequence -3'	Size range of the scorable bands (bp)	Total bands	No. of polymorphic bands	Polymorphism (%)
UBC-815	CTCTCTCTCTCTCTCTG	510-248	5	4	80
UBC-818	CACACACACACACACAG	552-317	3	0	0
UBC-824	TCTCTCTCTCTCTCTCG	827-304	7	7	100
UBC-825	ACACACACACACACACT	418-310	4	2	50
UBC-834	AGAGAGAGAGAGAGAGCTT	1324-234	6	2	33.33
UBC-840	GAGAGAGAGAGAGAGAYT	641-247	6	5	83.33
UBC-843	CTCTCTCTCTCTCTCTRA	1190-354	7	7	100
UBC-844	CTCTCTCTCTCTCTCTRC	748-204	8	4	50
UBC-845	CTCTCTCTCTCTCTCTRG	658-200	7	4	57.14
UBC-846	CACACACACACACACART	586-205	7	5	71.42
UBC-850	GTGTGTGTGTGTGTGTYC	756-258	5	4	80
UBC-857	ACACACACACACACACYG	393-191	4	2	50
UBC-864	ATGATGATGATGATGATG	916-315	4	3	75
UBC-873	GACAGACAGACAGACA	616-266	5	4	80
Total			78	53	
Average			5.6	4	65

identification of newly biosynthesized proteins. The obtained α-esterase isozyme banding patterns were typical for mother plant and plantlets raised from tissue culture and no polymorphism could be detected as shown in Table 3. The obtained data shows that band 1 (RF 0.125) was present in all *in vitro* plants and donor plant, while, band 2 (RF 0.173) was absent in all samples except regenerated plant grown in medium 3. However,

Table 4. ISSR amplification products of DNA extracted from *A. palaestinum in vitro* plants and mother plant.

Band number	RF Values	1	2	3	4	5	6	7
1	0.125	+	+	+	+	+	+	+
2	0.173	-	-	+	-	-	-	-
3	0.217	+	+	-	+	+	+	+

1 to 6, the tested regeneration media; 7 mother plant as a control; +, present; -, absent.

band 3 (RF 0.217) was absent from the same medium. In this connection, Saker and Rady (2003) reported that analysis of both esterase and peroxidase isozyme banding patterns does not give any polymorphism in tissue culture raised male and female papaya plants.

ISSR fingerprinting

In order to assess the genetic stability/instability of the regenerated plants, ISSR fingerprinting of the plantlets grown on the six regeneration media and donor mother plant of *A. palaestinum* was carried out. A total number of 78 scorable amplified DNA fragments ranging from 1324 to 191 bp were observed using the 14 ISSR primers, whereas 53 fragments were polymorphic. The 14 primers produced 65% polymorphism. The highest number of polymorphic bands (7) was obtained with primers UBC-824, UBC-843. The lowest number of polymorphic bands (2) was observed with primers UBC-825, UBC-234 and UBC-857 as shown in Table 4.

The highest percentage of polymorphism (100%) was observed with primers UBC-824 and UBC-843 while the lowest percentage of polymorphism (33.33%) was noticed with primer UBC- 834. No polymorphic bands were detected with primer UBC- 818. The obtained results showed that the regenerated plants showed apparent genetic variations when subjected to ISSR analysis, these results are in agreement with those of Hu et al. (2007) who noticed that ISSR primers could produce a high-frequency polymorphism in detection of somaclonal variation in *A. konjac*. Similar findings on genomic variation have been well documented in some other plants (Diwan and Cregan, 1997; Rahman and Rajora, 2001; Kawiak and Lojkowska, 2004). Recently, Biabani et al. (2013) employed 10 ISSR primers to assess genetic diversity among six populations of *Jatropha* from different Asian countries. 143 polymorphic bands were produced and polymorphism ranged between 46.2 and 60.8% between different genotypes.

Cluster analysis was done on the basis of similarity coefficients which ranged from 0-0.6 among the 6 tested regenerated plants and their donor mother plant as shown (Figure 5). The dendrogram constructed from UPGMA cluster analysis of the Dice similarity coefficients was calculated from ISSR data. The dendrogram based on genetic similarities separated the six samples of *A. palaestinum* into two main groups.

The regenerated plant 3 and donor plant 7 was grouped in the first cluster alone, and all other samples were grouped in the second cluster, which was separated into two sub-clusters, the first sub-cluster included *in vitro* plant 1 and the second included the other 3 samples (regenerated plants 4, 5 and 6). The three samples were classified into two sub-clusters, the first included regenerated plant 4 and the second included the other 2 samples sub-cluster.

Phytochemical investigation

Ten (10) compounds were detected and present in the seeds of *A. palaestinum* and its *in vitro* culture samples and identified as: apigenin, apigenin 6,8 di-*C*-glucoside, vitexin, isovitexin, orientin, isoorientin, luteolin 7-glucoside, quercetin, caffeic acid and isoferulic acid. The comparison between the HPLC analysis of seeds, shoots, roots and callus extracts with the identified compounds were summarized in Table 5.

Five compounds, (apigenin, apigenin 6, 8 di-*C*-glucoside, isoorientin, quercetin**,** and caffeic acid) were present in the shoot. Four compounds, (apigenin 6, 8 di-*C*-glucoside, orientin, isoorientin, and quercetin) were detected in the root, while two compounds, (apigenin 6, 8 di-*C*-glucoside and isoorientin) in the callus. Separated flavonoid peaks were initially identified by direct comparison of their retention time with those of standards. Standard solution was then added to the sample and peaks were identified by the increase in their intensity. This procedure was performed separately for each standard.

In general, a profound difference of the compounds was observed between the different analyzed samples. Some compounds are found in the donor plant and not detected in shoots, roots and callus raised from tissue culture. In the present study, five compounds, apigenin, apigenin 6,8 di-*C*-glucoside, isovitexin, quercetin and isoferulic acid were detected in the tested samples for the first time while the other compounds were isolated before by Afifi et al. (1999) and El-Desouky et al., (2007a).

Conclusions

In conclusion, survey of published data and open access patent data base indicated that there is no evidence for

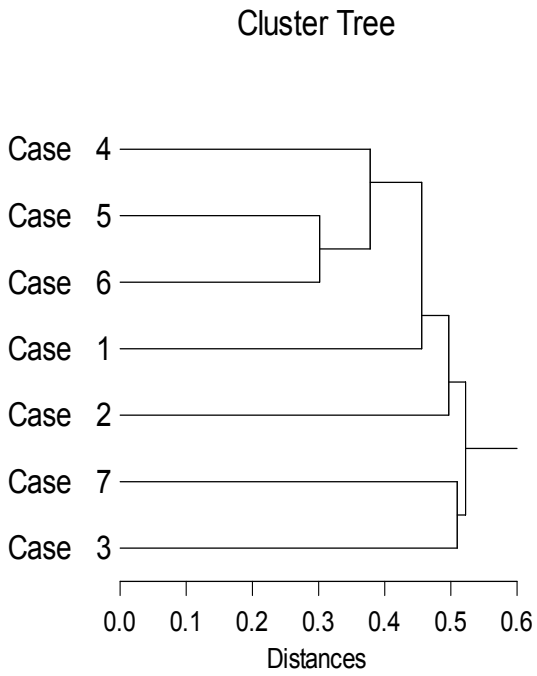

Figure 5. Dendrogram illustrating coefficient similarities among 6 regenerated plants (1:6) and their donor mother plant (7) of *A. palaestinum* based on ISSR data.

Table 5. HPLC analysis of seeds & *in vitro* cultures of *A. palaestinum.*

Compound	Retention time	Seeds	Regenerated Shoots	Roots of regenerated shoots	Callus	Previous work on mother plant
Apigenin	39	+	+	-	-	-
Apigenin 6,8 di-*C*-glucoside	23.26	+	+	+	+	+
Vitexin	26.7	+	-	-	-	+
Isovitexin	27.25	+	-	-	-	-
Orientin	37.1	+	-	+	-	-
Isoorientin	39.4	+	+	+	+	+
Luteolin 7-glucoside	32.9	+	-	-	-	-
Quercetin	3.4	+	+	+	-	-
Caffeic acid	22.3	+	+	-	-	+
Isoferulic acid	14.9	+	-	-	-	-

(+) Present; (-) absent.

preceding trials on protein analysis, DNA fingerprinting and phytochemical investigation of tissue cultures of *A. palaestinum* and only one manuscript on *in vitro* culture of *A. palaestinum* via somatic embryogenesis is published, so there is a huge shortage of information in this plant which we tried to cover in this study by the development of *in vitro* culture protocols and integrated investigations on genetic studies to better understand its genetic diversity, re-establishing and clonalization strategies.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Molecular identification of phosphate solubilizing bacterium (*Alcaligenes faecalis*) and its interaction effect with *Bradyrhizobium japonicum* on growth and yield of soybean (*Glycine max* L.)

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A phosphate solubilizing bacterium was isolated from the rhizosphere soil of upland rice and identified by 16S rRNA gene sequencing. The gene sequence showed 99% homology with *Alcaligenes faecalis*. Based on the gene sequence homology, it was identified as *A. faecalis.* Interaction effect of this bacterium on growth and yield of soybean was studied under glass house conditions by inoculating the bacterium either alone or in combination with *Bradyrhizobium japonicum* and *Bacillus megaterium* or both. The inoculated plants showed significantly taller plant height, more number of leaves, higher numbers of pods, plant dry weight and grain yield compared to un-inoculated ones (control). The triple inoculation was found superior compared to single as well as dual inoculations. Nitrogen and phosphorus content of the plant tissue was also higher in triple inoculation compared to others, which indicates their synergistic interaction in the rhizosphere of soybean.

Key words: *Alcaligenes faecalis*, *Glycine max,* phosphate solubilization, 16S rRNA gene sequencing.

INTRODUCTION

Next to nitrogen, phosphorus is the major plant nutrient required for early establishment and better growth of plant. Phosphorus also induces early maturity of the crop. Application of phosphate fertilizers significantly increased seed set, seed filling efficiency and kernel yield (Zehra, 2011). However, the phosphorus added to soil through phosphate fertilizers is fixed by soil minerals such as aluminum, iron and calcium, and form their respective phosphates eventually leading to phosphorus deficiency (Gyaneshwar et al., 2002). In such cases, a large fraction of soil microflora can dissolve insoluble phosphates in soil by secreting organic acids and make them available to plants as the pH of soil greatly influences phosphate solubilization.

Occurrences of phosphate solubilizing bacteria have been reported from different environmental niches

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(Castagno et al., 2011). Those isolated from alkaline soil showed tolerance to wide range of temperature and pH besides utilizing both organic and mineral phosphate to release absorbable phosphate ion to plants (Mohammad et al., 2009). Castagno et al. (2011) isolated different genera of phosphate solubilizing bacterium (PSB) through *Pantoea, Erwinia, Pseudomonas, Rhizobium* and *Enterobacter* from Salado river basin and characterized by 16S rRNA gene sequence analysis. *Aspergillus* and *Bacillus subtilis* have been found to be dominant species in the rhizosphere soil of beetle vine (Tallapragada and Seshachala, 2012). It is well known that 16S rRNA is a part of protein synthesizing machinery, which does not vary much from one organism to another. In molecular taxonomy, 16S rRNA gene sequencing technique is widely used for classifying bacteria isolated from different sources (Heilig et al., 2002; Woo et al., 2008; Patil et al., 2010; Naz et al., 2012).

Broader spectrum of phosphate solubilization and plant growth promotion resulted in the production of higher plant biomass (Panhwar et al., 2011). PSB applied with triple super phosphate increased plant height, number of tillers and mineral nutrient content in tissues of aerobic rice (Sarkar et al., 2012). Inoculation of PSB increased phosphorus uptake, growth and yield of upland rice (Panhwar et al., 2013). Soybean plants inoculated with *B. japonicum* together with pseudomonas strain (Phosphate solubilizer) resulted in 38% increased grain yield in pot culture experiments and 12% grain yield in field conditions (Aftab et al., 2010). Similarly, inoculation of *B. japonicum* increased phenolic compounds, organic acids, sterols and triterpenes in the aerial part of soybean (Carla et al., 2011; Luis et al., 2013). In this study, we isolated a phosphate solubilizing bacterium, from the root zone soil of upland rice, identified as *Alcaligenes faecalis* by 16S rRNA gene sequence analysis and explored its interaction effect with *B. japonicum* and *B. megaterium* on growth and yield of soybean.

MATERIALS AND METHODS

Isolation

Phosphate solubilizing bacteria were isolated from the root zone soil of upland rice by dilution plate method. Dilution (1:100) was made in sterile water and transferred 0.1ml on Pikovskays's medium dispensed in petri plates. These plates were incubated at 30°C for four days. The colonies forming clear zone around them were transferred on a fresh Pikovaskays's agar, purified and used for molecular identification.

Total genomic DNA isolation

Total genomic DNA was extracted by alkaline lysis method (Sambrook et al., 1989). The bacterial isolate was grown in Pikovakay's broth for 48 h at 30°C and 3 to 5 ml bacterial culture were pelleted with centrifugation at 12,000 rpm. The pellet was re-suspended in 650b µL of extraction buffer (10 mM Tris HCl pH 8.0, 20 mM EDTA and 250 mM NaCl) and incubated at 65°C for 30 min for lysis. To the extract, 100 µL of 5 M potassium acetate solution was added and placed on ice for 10 min for precipitation of protein and carbohydrates and clear supernatant was collected by centrifugation. DNA was precipitated by adding 0.6 volumes of chilled isopropanol and the DNA pellet was collected by centrifugation at 12,000 rpm. The pellet was washed twice with 70% ethanol, air dried and dissolved in 10 mM TE (10:1) buffer stored in aliquots at -20°C. The quality and quantity of the isolated DNA were checked with 0.8% agarose gel electrophoresis and spectrophotometrically.

Primer designing and PCR amplification

The primers were designed manually based on the already reported 16S rRNA sequences from the NCBI database (http://www.ncbi.nlm.nih.gov). A forward primer 5' GTTAGATCTTGGCTCAGGACGAACGC 3' and reverse primer 5' GATCCA GCCGCACCTTCCGATACG 3' were designed and used for the present study. The primers were custom synthesized by Sigma-Aldrich (Sigma, USA) and diluted accordingly for the polymerase chain reaction reactions. Annealing temperature for primer pair was standardized and PCR was performed in a 40 µL reaction volume containing 1X buffer with $MgCl_2$ (1.5 mM), dNTP's (200 µM), forward and reverse primers (0.5 µM each), *Taq* DNA polymerase (1 U Genei Bangalore) and template DNA (50 ng). Amplification was carried out with an initial denaturation at 96°C for 3 min followed by 35 amplification cycles consisting of 94°C for 1 min, 50°C for 30 s and 72°C for 1 min and a final extension step at 72°C for 10 min. Controls for PCR reactions were carried out with the same primers without providing template DNA. PCR products were separated on 1.0% agarose gel and documented using gel documentation system Hero Lab, Germany.

Cloning, plasmid isolation and sequencing

The PCR products were eluted from the gel using GenElute™ Gel Extraction Kit (Sigma, USA) and the eluted products were cloned into pTZ57R/T cloning vector using InsT/A clone PCR product cloning kit (MBI, Fermentas Life Sciences) after determining the appropriate vector: insert ratios. The ligation reaction was performed with 1.5 µL of 10X ligation buffer, 1 µL (50 ng) T/A cloning vector, 1 µL (5U) T4 DNA ligase in a 15 µL reaction volume at 16°C overnight. The ligated product was used to transform competent *Escherichia coli* (DH5α) cells using heat shock method (Sambrook et al., 1989) and plated on Luria Bertoni (LB) agar medium containing ampicillin (100 µg/ml) and X-gal, IPTG (50 µg/ml each). The recombinant colonies were initially screened by blue white selection, followed by colony PCR using M13 primers (Sambrook et al., 1989). Single positive colony was selected, inoculated in 3 ml LB broth containing ampicillin (100 µg/ml) and incubated overnight at 37°C. Cells were harvested by centrifuging at 12,000 rpm for 1 min and media was removed by aspiration, leaving the bacterial pellet as dry as possible. Plasmid was isolated using GenElute™ HP Plasmid MiniPrep Kit (Sigma, USA) following the manufacturer's protocol. The isolated plasmid was sequenced (SciGenom Labs Pvt. Ltd., India) using M13 forward and reverse primers.

Sequence analysis and homology search

Sequence results were analyzed with VecScreen online software from NCBI (nttp://www.ncbi.nlm.nih.gov) for removing the vector contamination. Forward and reverse primer sequences were checked against each other by generating the reverse complement of the "reverse" sequence using FastPCR Professional (Experimental test version 5. 0. 83) and aligning it with the "forward" sequence with the help of CLUSTAL W Multiple Sequence Alignment Program using the online software SDSC Biology Workbench (San Diego Supercomputer Center). The full length gene homology

Table 1. Influence of *A. fecalis, B. megaterium* and *B. japonicum* on growth, yield, nitrogen and phosphorus content of soybean.

Bacterial culture	Plant height (cm)	Number of pods	Number of leaves	Dry weight of plant		Dry weight (g) of seeds	Nitrogen content (mg/plant)		Phosphorus content (mg/plant)	
				Shoot	Root		Shoot	Root	Shoot	Root
Control	27.66^{c}	28.00^{b}	8.66^{d}	2.92^{c}	0.47^{c}	4.35^{d}	4.14^{c}	0.60	0.83^{b}	0.08^{b}
Alcaligenes fecalis	43.33^{b}	40.66^{a}	12.66^{bc}	5.85^{b}	1.06^{bc}	7.53^{bc}	8.45^{b}	0.84	1.50^{b}	0.12^{ab}
Bacillus megaterium	41.00^{b}	40.66^{a}	10.33^{cd}	5.72^{b}	1.28^{abc}	6.68^{c}	8.40^{b}	0.73	1.63^{b}	0.13^{ab}
Bradyrhizobium japonicum	46.33^{ab}	41.00^{a}	12.00^{bc}	6.44^{b}	1.29^{abc}	6.63^{c}	8.28^{b}	0.84	1.49^{b}	0.17^{ab}
A.faecalis + B.meagterium	40.66^{b}	39.00^{ab}	12.00^{bc}	6.14^{b}	2.12^{a}	8.21^{bc}	9.49^{b}	1.36	1.83^{b}	0.26^{ab}
A.faecalis + B. japonicum	44.33^{b}	40.66^{a}	13.66^{b}	6.75^{b}	1.79^{ab}	9.83^{ab}	10.07^{b}	0.88	1.76^{b}	0.18^{ab}
A.faecalis+ B. meagterium+ B. japonicum	53.00^{a}	45.33^{a}	21.66^{a}	10.87^{a}	2.15^{a}	10.64^{a}	16.99^{a}	1.67	3.24^{a}	0.34^{a}
LSD	6.80	11.26	2.70	1.77	0.89	2.20	1.83	NS	0.96	0.20

Means with same superscript along the column do not differ significantly at p=0.05 level by DMRT. NS, Non significant.

search was performed with Centre for Biotechnology Information (NCBI) (nttp: //www.ncbi.nlm. nih.gov/BLAST/) (Altschul et al., 1990).

Interaction effect of P solubilizers with *B. japanicum* on growth and yield of soybean

A. faecalis and *B. megaterium* along with *B. japanicum* were used in the green house experiment to explore their interaction effect on growth and yield of soybean. *A. faecalis* and *B. megaterium* were grown in Pikovakay's broth and the *B. japanicum* was on the yeast extract mannitol medium on a rotary shaker at 30°C for four days. Culture having appropriate population (~10^{7}-10^{8}cells/ml) was used for inoculation. The red sandy loam soil was mixed with a recommended quantity of farm yard manure (FYM) and filled in polyculture bags of 10"×16"size (4kgs/bag) and watered one day prior to sowing. The bacterial cultures (10 ml each) were inoculated as per treatment allocation given in Table 1. The vegetable soybean seeds were sown and allowed for germination. After germination, two plants per bag were maintained in each treatment. Observations for growth (plant height, number of leaves and number of pods) were recorded on 90th day, then the crop was harvested, dried in hot air oven at 60°C for five days to obtain constant weight and observation for plant biomass was recorded. The seeds were separated from pod and dry weight was recorded. Total nitrogen content of the plant was estimated by Micro Kjheldhal method and phosphorus content was estimated by vanadomolybdate yellow colour method (Jackson, 1973). The data obtained were statistically analyzed by analysis of variance (ANOVA) using MSTAT-C soft ware and the means were separated by Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

Isolation of agriculturally important microorganisms from different ecological niche is advantageous in efficient strain screening, which can be used for biofertilizers production. Formation of clear zone around the colony of bacteria is an indication of phosphate solubilization when grown on Pikovskay's agar (Mahantesh and Patil, 2011). PSB was isolated from the root zone of upland rice and the bacterium formed clear zone around the colony on Pikovskay's agar indicating phosphate solubilization. *A. faecalis* isolated from Dehradun valley soil samples solubilized phosphates (Shruti and Pathak, 2012). Further, the bacterium was identified by 16SrRNA gene sequence analysis. The genes encoding 16S rRNA in prokaryotes and 18S rRNA in eukaryotes are most widely used in molecular phylogenetics as these genes are universally distributed, functionally constant, sufficiently conserved and have adequate length (Madigan et al., 2009). Thus, the 16s rRNA gene sequence has emerged as a preferred genetic technique for the identification of poorly described strain (Farrelly, 1995; Goto et al., 2000; Clarridge, 2004). In this study, the primers designed yielded approximately 1.5 kb product which was separated on 1% agarose gel and cloned into T/A cloning vector (pTZ57R/T). The recombinant bacterial colonies obtained after transformation were confirmed through colony PCR, as well as, with isolated plasmid (Figure 1). The sequence analysis (BLASTn) showed 99% homology with earlier reported *A. faecalis*. Hence, the bacterium was confirmed as *A. faecalis*. Jimenez et al. (2011) reported similar BLAST analysis for characterizing free nitrogen fixing bacteria of the genus *Azotobacter* from soil samples.

Use of PSB as biofertilizers has currently increased phosphorus uptake in plants and

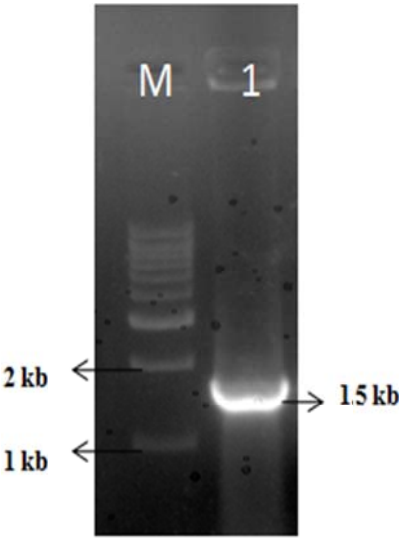

Figure 1. Amplification of 16S rDNA of *Alcaligenes faecalis* isolated from the rhizosphere soil of upland rice (M: marker, lane 1: 16S rDNA).

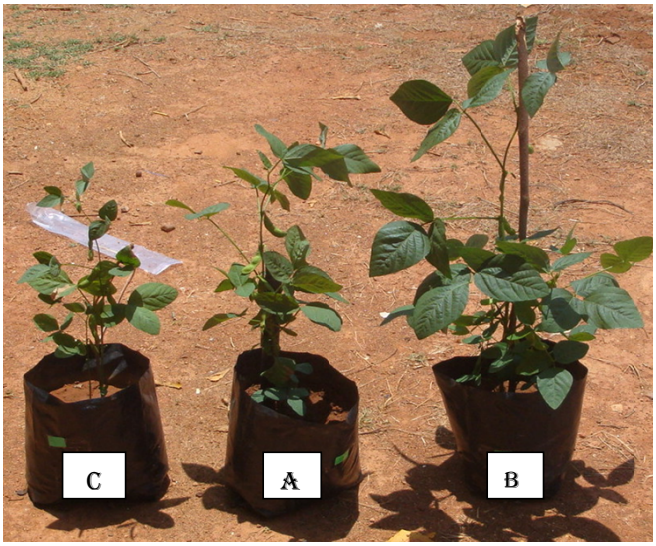

Figure 2. A. Treated with *A. faecalis*; **B.** treated with *A. faecalis* and *B. japonicum;* **C.** control.

improved yields in several crop such as *Lens culinaris Medic,* rice, sugarcane, *Lotus tenui* (Monika et al., 2009; Sarkar et al., 2012; Sundara et al., 2002; Castagno et al., 2011). Inoculation of PSB to aerobic rice had increased phosphorus uptake by plants and resulted in higher plant height and yield (Panhwar et al., 2011). In our study, inoculated treatments showed significantly increased growth in terms of plant height, number of leaves, number of pods, plant dry weight and grain yield compared to uninoculated plants. Inoculation of *A. faecalis* alone significantly increased the plant height compared to *B. megaterium*. Co-inoculation of *A. faecalis* +*B. megaterium* + *B. japonicum* significantly increased growth and yield of soybean (Table 1 and Figure 2) compared to single as well as dual inoculations. This indicates that the new isolate of *A. faecalis* is an efficient PSB compared to *B. megaterium*. Phosphate solubilizing bacteria alone or in combination with nitrogen fixing bacteria could promote growth and yield of plants (Castagno et al., 2011). Sarkar et al. (2012) reported significantly taller plant height and more number of tillers per plant due to PSB inoculation along with triple super phosphate to rice plants.

Nitrogen and phosphorus content in the tissue of soybean plant was found significantly higher in inoculated plants (Table 1) compared to un-inoculated ones. The highest was obtained from triple inoculation. However, nitrogen content of root did not vary significantly over the control. Similarly, highest shoot and root phosphorus content was observed in the triple inoculation treatment (Table 1) whereas; others were on par with the control plants. This indicated that the supplement of nitrogen by *B. japonicum* through biological nitrogen fixation and the availability of soluble phosphorus in rhizosphere of the plant is due to PSB inoculation (Qureshi et al., 2012) which resulted in increased growth and yield of soybean plant. Besides, bacteria involving better scavenging of soluble phosphorus would enhance plant growth through biological nitrogen fixation (Alia et al., 2013). This study suggests that the *A. faecalis* is an efficient PSB and interacts synergistically with *B. japonicum* to promote growth and yield of soybean crop.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Polyclonal antibodies of *Ganoderma boninense* isolated from Malaysian oil palm for detection of basal stem rot disease

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Basal stem rot (BSR) disease caused by the fungus, *Ganoderma boninense* has become a serious threat to the oil palm industry, especially in Southeast Asia (SEA). A highly selective and sensitive diagnostic tool for BSR is extremely required for early detection, and thus, development of immunological test using enzyme-linked immunosorbent assay-polyclonal antibody (ELISA-PAb) was evaluated. Results indicate that ELISA-PAb shows recognition of *Ganoderma* species associated with BSR except for *G. tornatum*. Cross-reactivity test with fungi commonly found in oil palm plantation revealed observation of some cross-reactions with some saprophytic fungi. ELISA-PAb shows better detection as compared to cultural-based method, *Ganoderma* selective medium (GSM) with an improvement of 18% at nursery trial. The present study also demonstrates sensitive detection on ELISA-PAb with an increment of 30% as compared to GSM test at field trial using oil palm roots and stems. Polyclonal antibodies raised against *G. boninense* with positive signals was achieved, however, not specific enough for detection of BSR disease caused by *Ganoderma*.

Key words: *Ganoderma boninense*, basal stem rot, polyclonal antibodies, enzyme-linked immunosorbent assay (ELISA).

INTRODUCTION

Palm oil is an important commodity to the world's largest palm oil producing countries, Malaysia and Indonesia. In Malaysia, the oil palm industry has significantly contributed to the rapid economic development of the

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Abbreviations: BSR, Basal stem rot; **ELISA,** enzyme-linked immunosorbent assay; **OD,** optical density; **MAb,** monoclonal antibody; **PAb,** polyclonal antibody; **EDTA,** ethylene diamine tetraacetic acid; **PBS,** Phosphate buffered saline; **PBST,** Phosphate buffered saline with tween 20; **HRP,** horseradish peroxidase; **IgG,** immunoglobulin; **ABTS,** 2,2'-azino-di-ethyl-benzothiazoline-6 sulfonic acid; **SDS-PAGE,** sodium dodecyl sulphate-polyacrylamide gel electrophoresis; **GSM,** *Ganoderma* selective medium.

country. Demand on the palm oil has lead to the increment of oil palm planted area in Malaysia which reached 5.08 million hectares, with an increase of 1.5% in 2012 against 5 million hectares recorded in 2011 (MPOB, 2013). Nevertheless, rapid growth of the oil palm industry has contributed to the fast movement and distribution of pests and diseases from other regions. Among others disease, basal stem rot (BSR) has pose a serious threat to oil palm plantation where infection can kill up to 80% stand palms in replanted areas (Ariffin et al., 2000; Turner and Gillbanks, 2003) or under planted areas with coconut palms (Idris et al., 2000; Turner, 1965).

In recent years, much attention has been given to BSR disease as it being the most destructive disease infecting oil palm in Southeast Asia (Turner, 1981). Earlier studies have made clear that, at least four different *Ganoderma* species is associated to BSR disease with *G. boninense* being the most pathogenic against oil palm (Idris, 1999). This disease has become a serious threat to oil palm industries in Malaysia which causes great losses of stand palm due to death (Ariffin et al., 2000). The disease can only be recognized at a very late stage with serious symptoms of foliar chlorosis and breakage at older fronds, presence of decayed tissues at palm base and production of fruiting bodies (Utomo and Niepold, 2000). The stem rotting caused restriction of water and nutrients uptake to the fronds; thus, promote the collapsing of palm trunk (Turner, 1981). In older palms, the disease was easily spread to neighbouring palms by root to root contact (Singh, 1991). BSR was also found in younger palms aged 10-15 years old; resulted to an unopened sheath leaves symptom (Turner, 1981). Once BSR was identified, younger palms normally died within 6-24 months, whereas, the matured palms survived lesser than two to three years (Idris, 1999, 2011). BSR disease was highly found in area replanted from coconuts and oil palms in inland area (Turner, 1965) and peat area (Azahar et al., 2011).

To date, there is effective controlling method or robust diagnostic tools for detecting the BSR disease at early stage. Generally, the detection of the disease at early stage is done in three conventional methods using drilling technique (Ariffin et al., 1993), chemo diagnostic test using ethylene diamine tetraacetic acid (EDTA) which was done to diagnose Thanjavur wilt disease caused by *Ganoderma lucidium* (Natarajan et al., 1986) and semi selective media for *Ganoderma* cultivation on agar plates (Ariffin et al., 1993). However, these methods were time consuming and gave low accuracy, hence, a rapid, economical and accurate method were urgently required to optimise fungicide use for prolonging the life span of the infected oil palm as the curative treatments currently are unavailable. A nucleic acid-based technique developed by Utomo and Niepold (2000), requisite on detection of specific DNA sequences in the genome and proper laboratory environment was required (McCartney et al., 2003). This method may produce false positive results if the sterilization and aseptic techniques are not practised correctly. Due to limitation of the sample preparation, specific antibodies offer more rapid diagnostic than nucleic acid-based techniques (Ward et al., 2004).

Immunological methods by manipulating antibodies have widely been used in detecting bacteria, viruses (López et al., 2003), fungi in roots, soil and plant materials (Cotado-Sampayo et al., 2008; Safarnejad et al., 2011; Walcott, 2003). Mostly antibodies produced by manipulating animals such as rabbits, mice and chicken, and most recently, recombinant antibodies produced by mammalian cell line was discovered (Frenzel et al., 2013). Antibodies are used by the immune system to identify and nullified foreign objects andn have been used to investigate presence of various fungi with different degrees of specificity (Thornton and Wills, 2013) as a diagnostic tool in various fields such as plant pathology, pharmaceutical and medicine (Alvarez, 2004).

The use of monoclonal and polyclonal antibodies in immunochemical techniques such as enzyme-linked immunosorbent assay (ELISA) offer greater simplicity and fast diagnostic than DNA probe analysis such as PCR (Bridge et al*.,* 2000; Darmono, 2000). Monoclonal antibodies are mostly more specific and sensitive than polyclonal antibodies in determining the target pathogen even in low concentration with a high degree of accuracy (Tsai et al., 1992). Successful works on monoclonal and polyclonal antibodies by ELISA has been reported previously. Diagnostic by monoclonal antibody (MAB) in mycology studies was carried out for the detection of *Puccinia striiformis* urediniospores that caused yellow rust disease in wheat plants (Skottrup et al., 2007), and for the detection on *Spiroplasma citri* and *S. kunkelii*, the plant pathogen for citrus stubborn disease and corn stunt disease (Jordan et al., 1989). Other successful detection using polyclonal antibody (PAB) was reported on *Ganoderma lucidum* from coconut palm (Rajendran et al., 2009), *Alternaria alternate* in tomato and potato plants (Smith, 1993) and also detection of *Aspergillus parasiticus* in contaminated corn, rice, wheat and peanut (Guo-Jane and Shou-Chin, 1999) and detection of Streptomyces species in soil samples (Sangdee et al., 2012). Polyclonal antibodies was commonly used for detection of human infection as in production of Tas transactivator for detection of foamy virus (Qiu et al., 2012), detection of *Escherichia coli* using Shiga Toxin 2 in human (He et al., 2013) and to study human collectin 11 (CL-11) levels somewhat related to human diseases and symptoms (Selman et al., 2011).

Presently, detection of *G. boninense* using immunological methods neither have not broadly been practiced nor utilised for screening of BSR disease. Development of polyclonal and monoclonal antibodies against *G. boninense* isolated from Indonesia were reported, which showed unevenness of detection (Utomo

and Niepold, 2000; Darmono, 2000). Study by Shamala et al. (2006) has successfully produced monoclonal antibody (MAb) against *G. boninense* using Malaysian oil palm isolate; however cross-reactivity highly occurred. Hence, in this study, our aim was to develop polyclonal antibodies against *G. boninense* using the vast virulent isolate to oil palm, which was discovered in highly infected oil palm plantation with BSR disease in Malaysia. In this paper, we describe the production and application of specific PAbs against *G. boninense* for BSR disease detection using modified ELISA method. The results from the experiments conducted in the nurseries and fields in Malaysian oil palm plantations describe their diagnostic potential.

MATERIALS AND METHODS

Preparation of *Ganoderma* antigen

Pure culture of *G. boninense* isolate PER71 was obtained from culture collection of GanoDROP unit, Malaysian Palm Oil Board, Bangi, Malaysia. Potato dextrose agar (PDA) was used for culture maintenance according to Wagner et al. (2003) in *G. lucidum* study. After 7-10 days of incubation, the actively growing mycelium was cut and transferred to sterile conical flasks containing 100 mL potato dextrose broth (PDB) and incubated at 28°C for 14 days. The mycelia cultures was harvested by vacuum filtration, subsequently rinsed with distilled water and blotted dry using sterile Whatman No.1 filter paper. Mycelium (0.5 g) was ground using a pre-cooled sterile mortar and pestle in the presence of liquid nitrogen. Then, suspended in 1.5 mL phosphate buffer saline (PBS: 8 gL^{-1} NaCl, 0.2 gL^{-1} KCl, 2.9 gL^{-1} Na_2HPO_4, 0.2 gL^{-1} KH_2PO_4, pH 7.4), vortexes thoroughly for a few seconds and centrifuged at 9000 rpm for 20 min at 4°C. Supernatant was separated and purified using ammonium sulphate (70%) precipitation. Precipitated protein was referred to as antigen and suspended in PBS buffer for further analysis.

SDS-PAGE analysis and protein profilling

Protein concentration was determined using Bradford assay (Bradford, 1976) based on bovine serum albumin (BSA) standard. Protein molecular mass was determined using 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Protein was run in equal concentration in the SDS-PAGE gel. Gel was stained with Coomassie Brilliant Blue G 250 and destained with destained-buffer (Blakesley and Boezi, 1977). Protein profiling of *G. boninense* was also conducted using Liquid Chromatography Mass Spectrophotometry (LC-MS) analysis to identify the amino acid profiling. Amino acid analysis was provided by Chemical Engineering Pilot Plant (CEPP), Universiti Teknologi Malaysia (UTM), Skudai, Johor, Malaysia.

Immunization and polyclonal antibodies (PABs) production

Ganoderma antigen was prepared in PBS and concentration was adjusted to 200 µg/mL for the injection. Three adult New Zealand white rabbits initially were given four intramuscular injections in 1:1 (v/v) Freuds complete adjuvant (FCA, Difco, USA). Further boosting immunization was done two weeks later with another injection of 200 µg/mL in 1:1 (v/v) Freuds incomplete adjuvant (FIA, Difco, USA). Rabbits received injections in each treatment from Day-0 until Week-18. Blood (20 mL) was taken from the rabbits two weeks after each injection, subsequently the titre of anti-serum was analysed for immunoreactivity towards *G. boninense* and detected by indirect ELISA. Blood samples were allowed to clot at 37°C for 1 h and stood overnight at 4°C to retract. Anti-serum was collected after a centrifugation at 1500 rpm for 20 min to remove the remaining red blood cells. Harvested anti-serum was stored at -20°C for further analysis.

Enzyme-linked immunosorbent assay (ELISA)

Fifty microliters of anti-serum (2 µg/mL) diluted in coating buffer, PBS (pH 7.4) was incubated overnight in the ELISA plate at 4°C. The plate was washed with 200 µL phosphate buffer saline with tween 20 (PBST) three times, blocked with 5% skim milk at 37°C for 2 h, and washed again with PBST three times. About 50 µL of anti-serum at different dilutions (1:10, 1:100, 1:1000 and 1:10,000) was incubated per well at 37°C for 1 h. After washing with PBST three times, 50 µL horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (IgG) (JacksonImmunoLab, New York) at 1:5000 dilution was added to each well and incubated at 37°C for another 1 h. Plate was washed another three times with PBST. Colour reaction was developed by adding the 50 µL/well azino benzothiazoline sulfonic (ABTS; 2, 2'-azino-di-[3-ethyl-benzothiazoline-6 sulfonic acid) and reaction was stopped by the addition of 50 µL/well of 2 M H_2SO_4. Hydrolysed substrate was read at 405 nm with microplate reader according to optical density (OD). Analyses on the data was done by plotted the standard curve from the series of concentration serial dilutions of serum (X-axis/log scale) against the absorbance (Y-axis/linear). All statistical analysis was done through analysis of variance (ANOVA) with the mean compared by the Least Significant Difference (LSD) at P-value ≤ 0.05 using Statistical Analysis System (SAS) software.

Cross-reactivity test with fungi associated in oil palm plantation

Specificity was determined by ELISA assay using the fungi commonly found in the oil palm plantation in Malaysia. Pure culture of fungi tested in this study was obtained from the culture collection of GanoDROP unit, Malaysia. Antigen preparation of each fungus was obtained according to the *Ganoderma* extraction as mentioned previously. Equal concentration of protein was prepared for ELISA-PAb test against *Ganoderma*. Fungi used for cross-reactivity test are *G. zonatum*, *G. miniatocinctum* and *G. tornatum*. Others fungi commonly found in oil palm plantations were also tested, these are *Penicillium sp,. Marasmius palmivorus*, *Thielaviopsis paradoxa*, *Trichoderma* spp., *Aspergillus niger, Trichoderma virens, Trichoderma harzianum, Curvularia* sp*., Helminthosporium* sp., *Pestalotiopsis* sp*., Schizophyllum* sp*., Fusarium* sp*., Botryodiplodia sp.* and *Melanconium* sp. All ELISA-PAb test on cross-reactivity was done in three replicates.

Nursery evaluation in seedlings artificially inoculated with *G. boninense*

In nursery test, oil palm (DxP) aged 3 months old, was challenged with *Ganoderma* via artificial inoculation with *G. boninense* using rubber wood block (RWB) sitting technique as described by Idris (1999). Blocks sized 6 x 6 x 12 cm, were prepared by incubating the *G. boninense* inoculum onto RWB for 3 months. A total of 30 palms were conducted in the test which consisted of two treatments: infected palms with *G. boninense* and uninfected palms (control). The experiments were laid out in completely randomized

design (CRD) with three replicates. Samples from leaves, stems and roots were collected and surface sterilization was performed prior to extraction of the protein. Preparation of the protein was done exactly according to antigen preparation. Protein concentration was then determined by using Bradford assay to define the minimum level of coating concentration of protein on wells with sufficient amount of antigen for immunization and ELISA protocol. Protein was stored in the -20°C for further analysis. This experiment was repeated in triplicates.

Field evaluation in oil palm infected with *G. boninense*

ELISA-PAb test was also carried out for evaluation of field samples. A total of 120 matured palm with healthy-looking and symptoms of *Ganoderma* incidence (presence of some *Ganoderma* symptoms such as basidiomycetes fruiting bodies, yellowish leaves, broken fronds on the petiole and skirting around the palm trunk, production of stunted shoots or unopened spear leaves) were spotted randomly and collected from three different oil palm plantations: Teluk Intan, Perak; Kluang, Johor; and Sepang, Selangor. Samples from leaves, stems and roots were collected and surface sterilization was done to minimize the contamination. Cultural-based technique using GSM (Ariffin and Idris, 1991) was done subjected to obtain pure culture of fungi from each sample. Tissue samples were ground, suspended in PBS buffer, filtered and precipitated prior to getting the protein. Protein concentrations were determined by Bradford assay and subsequently continued to ELISA-PAb test.

RESULTS AND DISCUSSION

Polyclonal antibodies

Crude protein of *G. boninense* was extracted with total concentration of 1.60-2.58 mg/mL. SDS-PAGE image of the crude protein revealed that *G. boninense* consists of protein ranging from 10-220 kDa. Native protein size in this study was relatively higher than a study reported by Darmono (2000) with 70 kDa of *Ganoderma*'s protein from Indonesian isolates. Wide range of protein sizes might be due to collation of extracellular, intracellular enzymes and others protein since *Ganoderma* can colonise oil palm hard-fibre with alterations in cellulose, hemicellulose and lignin contents (Abe et al., 2013). However, the enzymes mechanism of oil palm was not clearly explained.

A total of 16 amino acids were determined from crude protein of *G. boninense* by using LC-MS and amino acid analyser. Protein analysis showed that the proline (Pro) was the most abundant amino acid in *G. boninense* at 40.15 µmol/mL followed by glycine (Gly) at 30.0 µmol/mL, glutamic acid (Glu) at 28.5 µmol/mL and valine (Val) at 26.65 µmol/mL (Figure 1). However, ammonia and cyctein (Cys) were undetectable. A high amount of proline residue identified from crude protein of *G. boninense* may become a key answer to the aggressiveness and noxiousness of *G. boninense* to oil palm. As been described by Szabados and Savouré (2009), proline produced highly in plants during environmental stress such as drought, salinity and biotic stress and was important for its tolerance towards stress conditions. Presence of proline was considered as protection of subcellular structure and macromolecule against environment and natural enemies for recovery purposes. Hence, proline accumulation in most plants, demonstrated that, it has diverse role to confer osmotic tolerance and adverse effects as plant protection and development by scavenging reactive oxygen species (Kishor et al., 2005; Matysik et al., 2002; Rhodes et al., 1999). In mutualistic fungi, it was proposed that proline help plants to notice the stress at soonest by activating the plant biochemical reactions that lessen the stress impacts (Rodriguez et al., 2004). Meanwhile, study by Chen and Dickman (2005) on a fungal pathogen, *Colletotrichum trifolii*, reported that proline protects *C. trifolii* against stresses including UV light, hydrogen peroxide, salt, and heat. Interestingly, the restoration of pathogen requires only proline that protect pathogen from death.

Thus, this gave a suggestion that in *G. boninense*, proline might have a role in response adaptation and support the organism to withstand the plant's biological counterattack or other good fungal pathogen in order to initiate the host. Transgenic plants which are unable to produce proline, proved to have significantly lower stress tolerance (Kishor et al., 2005).

Crude protein of *G. boninense* was used as antigen to obtain specific antibodies from rabbits. ELISA test was applied to evaluate the optimal polyclonal antibody titre. Antibody titre is defined as the lowest dilution to bind significantly to the antigen and as a simplest method to assess whether an immune response has occurred in the immunised animals against *Ganoderma*'s specific antigen.

Result shows that low PAb concentration at dilution of 1:10,000 was sensitive enough for the detection (Figure 2). Result also suggests that, at weeks-8, the antibody was sufficiently being detected by the ELISA-PAb. In related study, higher titres of polyclonal were found with 1:15,000 of *Ganoderma* from Indonesian isolates (Utomo and Niepold, 2000) and 1:256,000 of banana streak virus from Nigerian isolate (Agindotan et al., 2003).

Three trials done for specificity test resulted to the detection of four species of *Ganoderma viz*. *G. boninense*, *G. miniatocinctum, G. zonatum* which generally were found associated with BSR disease in oil palm with 100% of identification except for *G. tornatum* (Table 1). All three *Ganoderma* excluding *G. tornatum*, were reported as pathogenic to oil palm after a Koch Postulate analysis (Idris, 1999).

It was suggested that, all pathogenic *Ganoderma* have high similarity of recognition site in the antigen-antibody interaction, both acted as a key (antigen) and lock (antibody) conformation. Meanwhile, the non-pathogenic, *G. tornatum* offers partially conserved fragment since the percentage of detection is lesser at 66.7% on ELISA-PAb test but none detection was obtained from GSM. The specificity test conducted in this study, suggested that the pathogenic and non-pathogenic *Ganoderma* cannot be

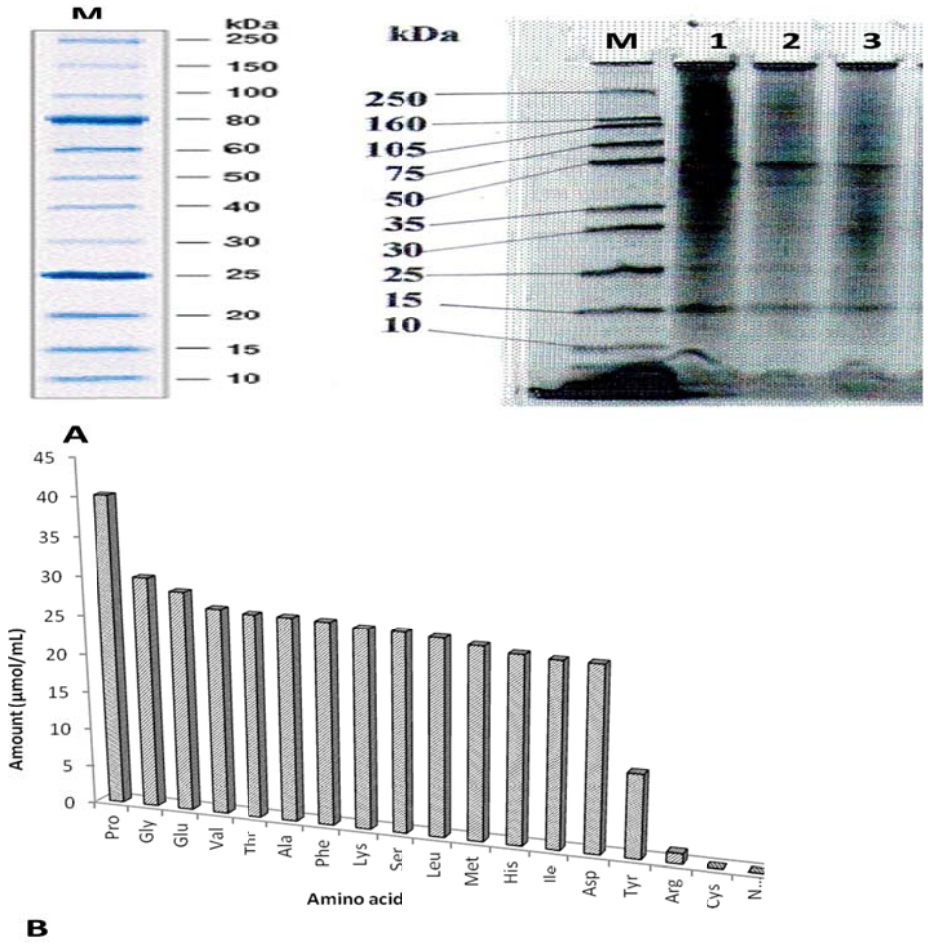

Figure 1. Characterization of *G. boninense* protein. **(A)** SDS-PAGE profile of *Ganoderma* antigen with the protein amount indicated in kilo Dalton (kDA). **(B)** Amino acid profiling using LC-MS analysis with the value indicated in µmol/mL.

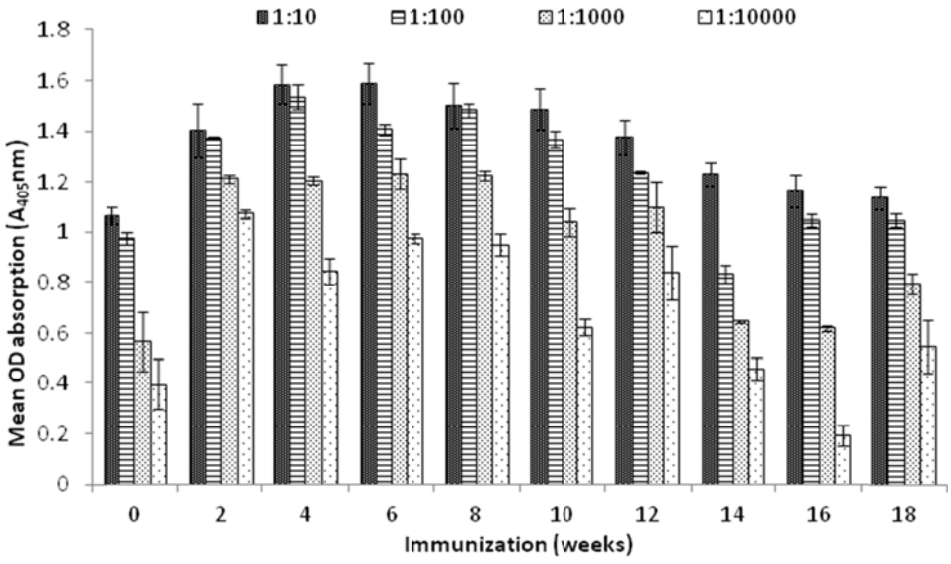

Figure 2. Determination of polyclonal antibodies titre. Substrate incubation was for 1 h at 37°C. All tests were done in triplicates.

Table 1. Cross-reactivity test of polyclonal antibodies using ELISA-PAb and GSM against various fungi isolated from oil palm plantations; N= 30.

Isolate	Pathogenicity test to oil palm	Mean of detection (%)	
		ELISA-PAb (%)	GSM (%)
G. boninense	Pathogenic (field disease)	100 ± 0[a]	100 ± 0[a]
G. zonatum	Pathogenic (field disease)	100 ± 0[a]	100 ± 0[a]
G. miniatocinctum	Pathogenic (field disease)	100 ± 0[a]	100 ± 0[a]
G. tornatum	Not pathogenic	66.7 ± 0.58[b]	100 ± 0[a]
Aspergillus niger	Not pathogenic	0 ± 0[c]	0 ± 0[b]
Penicillium spp.	Not pathogenic	100 ± 0[a]	0 ± 0[b]
Trichoderma virens	Not pathogenic	0 ± 0[c]	0 ± 0[b]
Trichoderma harzianum	Not pathogenic	0 ± 0[c]	0 ± 0[b]
Curvularia sp.	Pathogenic (leaf disease)	0 ± 0[c]	0 ± 0[b]
Helminthosporium sp.	Pathogenic (leaf disease)	0 ± 0[c]	0 ± 0[b]
Pestalotiopsis sp.	Pathogenic (leaf disease)	0 ± 0[c]	0 ± 0[b]
Schizophyllum sp.	Pathogenic (leaf disease)	0 ± 0[c]	0 ± 0[b]
Fusarium sp.	Not pathogenic	0 ± 0[c]	0 ± 0[b]
Marasmius palmivorus	Pathogenic (field disease)	100 ± 0[a]	0 ± 0[b]
Thielaviopsis paradoxa	Pathogenic (field disease)	100 ± 0[a]	0 ± 0[b]
Botryodiplodia sp.	Pathogenic (field disease)	0 ± 0[c]	0 ± 0[b]
Melanconium sp.	Pathogenic (field disease)	0 ± 0[c]	0 ± 0[b]

Means with different letters within a column are significantly different according to the t-test at $p<0.05$ using least significant difference (LSD). Note: PAb, polyclonal antibody; GSM, *Ganoderma* selective medium.

distinguished by using ELISA-PAb.

In this study, cross-reactivity test done using 17 various saprophyte fungi found in oil palm plantations revealed that *Penicillium* sp., *Marasmius palmivorus* and *Thielaviopsis paradoxa* were detected significantly using ELISA-PAb (Table 1). However, extensive cross-reactivity throughout *Ganoderma* and various fungi demonstrated the ability of false-positive values on unrelated fungus isolates. The occurrence of false-positive reaction is a serious drawback in the use of polyclonal antibodies (Griep, 1999; Utomo and Niepold, 2000).

In most cases of polyclonal antibodies as immune-assay especially for *Ganoderma* disease, cross-reactivity with saprophytic fungi is well-known since the fungi classified as complex organism comes with numerous antigen and may share with other unrelated or closely related fungi (Utomo and Niepold, 2000). However, the positive results on cross-reactivity to others fungi, might be because they were prominent fungi that commonly attack oil palm in a minor cases such as basal stem trunk caused by *Thielaviopsis paradoxa* and *Marasmius palmivorus,* causal of crown disease in oil palm (Turner, 1981). Meanwhile, *Penicillium* sp. known as ubiquitous fungi might be presence in the test due to the attribution of the antigen or cross-contamination since it was easily found in the nature environment.

The preparation of sufficiently polyclonal antibodies specifically to *Ganoderma* is very difficult as there is strong serological relationship with saprophytic fungi. Either for *Ganoderma* polyclonal or monoclonal anti-bodies, the illustration of the cross-reactivity with some fungus isolates have been reported by Shamala et al. (2006) and Utomo and Niepold (2000). By some reasons, the *Ganoderma* polyclonal antibodies failed to induce antibody response towards specific target protein which may be due to the poor antigenicity of an antigen produced and conservation of the peptide sequence in some species. It is particularly true for anti-peptide anti-bodies and in certain cases, high titre of antibodies generated against antigen may not recognize the peptide full-length either in Western or immunoassay (Biomatik, 2011).

Nursery evaluation

Samples taken from roots, stems and leaves were tested for *Ganoderma* infection using ELISA-PAb test and in-parallel with GSM method (Ariffin and Idris, 1991). *Ganoderma* PAbs produced in this study was found sensitive in distinguishing all field samples in roots and stem tissue. In the nursery trial, a total of 30 palms were tested and showed an average of 88.9% (ELISA-PAb) and 71.1% (GSM) of detection from roots samples in infected palms against healthy palm (0%) ($p<0.05$) (Figure 3). Similar results were observed from stem samples with an average of 82.2% using ELISA-PAb as

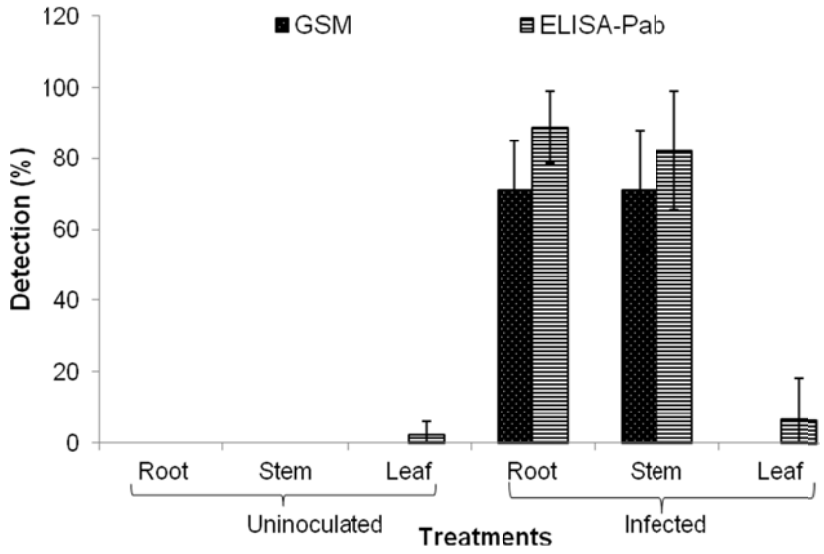

Figure 3. Nursery trial in detection of *Ganoderma* disease in oil palm seedlings using GSM and ELISA-PAb against uninoculated and artificially infected palms by *G. boninense*; N=90. GSM, *Ganoderma* Selective Medium.

compared with 71.1% using GSM ($p<0.05$) for artificially infected palms. For the remaining palms, no detection on the control (non-inoculated seedlings with *Ganoderma*) was observed. Results persisted negative or almost negative using leaves sample for both treated and non-treated palms.

Among the treatments, ELISA-PAb and GSM analysis only showed the highest percentage of detection on roots and stems but almost none using leaves samples. This may indicate that the slighter response of antibody in the leaves was observed compared to the roots and stems. As the antigen produced from the pure culture of *G. boninense* and not from systemic response of plant towards *Ganoderma*, hence, the response on leaves was lesser and not specific enough to be recognized by ELISA-PAb. The response of plants elicited by *Ganoderma* did not occur synchronously as the infection occurred based on root-to-root contact and subsequently, colonized the plant bole after the infection take place (Ariffin and Idris, 1991). During the infection, *Ganoderma* was either localized to the initial point of contact or completely enveloped the root at the point of contact through epidermis and exodermises (Flood et al., 2010). Rapid colonization of *Ganoderma* was observed through roots and into lower stem or bole by production of brown discoloration at infected area (Darmono, 1998, 2000; Flood et al., 2010).

To achieve accurate results, it was suggested that the sensitivity and accuracy may be obtained from chromatography methods such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC), as well as by molecular methods (Pirestani et al., 2011).

Field evaluation

For field evaluation, *Ganoderma* detection in apparently healthy-looking palms from three different plantations was done for an early diagnosis of diseased palms. Similar pattern of ELISA-PAb detection was also observed using samples taken from mature palms collected from three oil palm plantation at MPOB Research Station: Teluk Intan, Perak; Kluang, Johor and Sepang, Selangor (Table 2). A total of 120 palms were tested using ELISA-PAB concomitantly with GSM. Findings revealed that the positive signal was found in roots and stems collected from Teluk Intan, Perak for ELISA-PAb (100%) and GSM (70-90%) at $p<0.05$. Results on the samples accumulated from Kluang, Johor was also found similar to those of Teluk Intan, Perak as roots and stems were detected using ELISA-PAb (100%) against GSM (70-80%) at $p<0.05$.

Detection of ELISA-PAB and GSM were also found comparable for Sepang, Selangor; resulted in the detection of 100% (ELISA-PAb) and 80% (GSM) at $p<0.05$. Hence, this indicated that, ELISA-PAb was more sensitive and accurate as compared to GSM in detecting presence of *G. boninense* using both roots and stems sample. Conversely, ELISA-PAb and GSM failed to be detected in leaves. However, in order to increase the accuracy and consistency of *Ganoderma* detection, culture-based method, GSM is still needed to be applied in-parallel as a reconfirmation procedure. Effort is being focused on using high concentration of antiserum (1:100 or 1:1000) as to direct the target protein to be probed by antiserum itself, hence, remove most of the backgrounds. ELISA offers an easy, inexpensive and rapid assay as

Table 2. Field trial on detection of *Ganoderma* disease in matured oil palm using GSM and ELISA-PAb for healthy-looking palms in three different *Ganoderma* infected areas from different sample tissues; N=120.

Sample	Oil palm plantation	Mean of detection (%)	
		GSM	ELISA-PAb
Root	Teluk Intan, Perak	70 ± 4.83[a]	100 ±0[a]
Stem		90 ± 3.16[a]	100 ± 0[a]
Leaf		0 ± 0[b]	10 ± 3.16[b]
Root	Kluang, Johor	70 ± 4.83[a]	100 ± 0[a]
Stem		80 ± 4.22[a]	100 ± 0[a]
Leaf		0 ± 0[b]	0 ± 0[b]
Root	Sepang, Selangor	80 ± 4.22[a]	90 ± 3.16[a]
Stem		80 ± 4.22[a]	100 ± 0[a]
Leaf		0 ± 0[b]	0 ± 0[b]

Means with different letters within a column are significantly different according to the t-test at $p<0.05$ using Least Significant Difference (LSD). PAb, Polyclonal antibody; GSM, *Ganoderma* selective medium.

it requires small amount of sample tissues. Thus, ELISA polyclonal might be useful as pre-scan to handle many samples in time. Detection of *Ganoderma* disease in speciously infected oil palms is possible and strongly achieved with a combination of immunoassay, culture-based technique and molecular works.

Conclusion

This article provides an overview of polyclonal antibody approach and its application in detection of *Ganoderma* disease is one of decision-making tool for an early detection in nursery and field. The study is conducted as a preliminary research in developing polyclonal anti-bodies of *G. boninense*.

The findings from this study could be useful for future research work. Polyclonal antibodies of *G. boninense* can be produced, beforehand; more research needs to be carried out to achieve highly confidence of the generated polyclonal. Studies on biological and epidemiological aspects on the pathogen itself are essential in providing a better understanding of the natural occurrence of the disease. In future, provision of immunoassay-based kits would be helpful in the detection and development at nursery and field level and this would certainly mostly help the implementation of Integrated *Ganoderma* Management (IGM) against *G. boninense* disease in oil palm.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Molecular characterization of cultivated cowpea (*Vigna unguiculata* L. Walp) using simple sequence repeats markers

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Forty eight accessions of cultivated cowpea were assessed using 12 simple sequence repeat (SSR) markers. The unweighted pair group method with arithmetic mean (UPGMA) dendrogram constructed revealed three main clusters when truncated at 65% similarity coefficient. All accessions showed high genetic variation except four which are genetically similar at 100% similarity coefficient. A two dimensional principal coordinate analysis (PCA) also revealed high genetic relationships among the accessions used. The polymorphism information content (PIC) revealed that the number of alleles per locus ranged from 2 to 5 with a total of 37 alleles generated from the SSR primers. The PIC value ranged from 0.075 to 0.603 with a mean value of 0.344 from a total of 4.467. There was no significant correlation between the repeat number and the allele numbers (r=0.21) so also between repeat numbers and PIC (r=0.11). The PIC also revealed the comparative genetic diversity from three sub-regions in Africa; West Africa, North East and Central Africa and Southern Africa with thirteen accessions each. West African accessions were the most diverse with a PIC value of 4.4310, showing the greatest genetic diversity and most likely the center of origin of cultivated cowpea.

Key words: Cowpea, dendrogram, genetic diversity, polymorphism, simple sequence repeat (SSR) markers.

INTRODUCTION

A detailed study of the variation of a crop, both morphologically and genetically, in relation to the geographical distribution of such variation could help in speculating on the origin of such plant. Crops that have been cultivated for a long time revealed an area with intense variation or great diversity, since in that area there would have been time for large numbers of mutations and gene recombination to take place, as a result of interbreeding among different varieties (Padulosi et al., 2007; Padulosi et al., 2009; Feleke et al., 2006). It is generally observed that a very large numbers of varieties or high variation of the species is found towards the center of the distribution area of the crop, and this is accompanied by a corresponding thinning out of the variability towards the periphery

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(Kuruma et al., 2012; Pasquet, 2000; Feleke et al., 2006).

The arrival of cowpea in West Africa and the development of the cowpea / cereals farming system probably date back from 3 to 4 000 years. Wild cowpea *(subsp. dekindtiana*) could have been gathered as folder to feed cattle and later domesticated as early as 4000 BC in West Africa. During the process of domestication and selection of cowpea from its wild progenitor, characters lost and gained included seed dormancy together with a reduction of pod dehiscence on one hand and an increase in pod and seed size on the other (Adewale et al., 2011; Ogunkanmi et al., 2006; Ogunkanmi et al., 2007).

The selection of cowpea as a pulse as well as folder might have resulted in the establishment of the cultigroup *unguiculata* (Ibrahima et al., 2013; Pasquet, 2000; Kuruma et al., 2008). Selection for types with long peduncle for fibre as well as for folder or seed has resulted in the cultigroup *Textilis* (Ibrahima et al., 2013). Once the cultigroup *unguiculata* was established in West Africa, diversity developed and accumulated through mutation.

Through centuries of cultivation, short day cowpea cultivars became adapted to the cereal farming system, while day neutral cultivars later evolved from these short day cultivars and became adapted to the yam based farming system in the humid zones of West Africa (Manggoel and Uguru, 2011; Ogunkanmi et al., 2007). Through West Africa the cultigroup *unguiculata* was introduced to East Africa, was brought to Europe, there it was known to the Romans about 2300 BC, and to India about 2200 BC (Padulosi et al., 2009). The cowpea underwent further diversification in India and Southeast Asia, producing the cultigroup *Biflora* for its grain and for use as a cover crop, and the cultigroup *Sesquipedalis* with its long pods used as a vegetable (Manggoel and Uguru, 2011; Ogunkanmi et al., 2008) cowpea was probably brought to the Americas during the 17th century by the Spanish and Portuguese traders.

A simple and precise technique for measuring the overall genetic diversity of a crop is not yet available, and no single approach can be considered the best for measuring diversity (Amin et al., 2010; Charcosset and Moreau, 2004; Kuruma et al., 2008). The classification of cultivated crop plants and the determination of their interrelationships require morphological traits together with sophisticated analyses such as the molecular studies as many of the morphological characters commonly used are prone to environmental influences, thereby reducing the fine resolution require ascertaining phylogenetic relationships (Kuruma et al., 2008).

The number of morphological attributes that can be scored is generally limited due to environmental influence hence DNA markers have therefore been used extensively to study relationships within and between crop species as they provide a larger number of characters which are unaffected by environmental influence and consequently can provide unambiguous character state assignments (Aaron et al., 2010; Ibrahim et al., 2007).

Plant systematist have therefore cautioned that whenever possible, systematic/evolutionary relationships and genetic diversity levels should be assessed by more than one class of genetic markers such as morphological together with isozymes and/or DNA based markers (Pasquet, 2000). Molecular markers are therefore being used in many aspects of plant genetics and breeding (Andargie et al., 2011; Moalafi et al., 2010), taxonomy, variability of populations and mating systems. They are based on differences in DNA sequences between individual and they generally detect more polymorphisms than morphological and protein-based markers and constitute a new generation of genetic markers (Badiane et al., 2012; Prasanthi et al., 2012).

Among others for example, restriction fragment length polymorphism (RFLP) markers have been used to construct genetic linkage maps in cowpea (Fatokun et al., 1993b) and to study the taxonomic relationships in the genus *Vigna* (Fatokun et al., 1993a).

However the use of RFLP in germplasm studies is limited by several factors, for example they require relatively large amounts of DNA for the assay, they are time consuming and labour intensive. The microsatellites markers (SSR) on the other hand have many advantages over classical RFLP and RAPD since they require minute amounts of DNA and are relatively cheap and time saving. (Andargie et al., 2011; Aaron et al., 2010; Kuruma et al., 2012)

Microsatellites are stretches of DNA, consisting of tandemly repeating mono-, di-, tri-, tetra-, or penta- nucleotides units, that are arranged throughout the genomes of most eukaryotic species (Kuruma et al., 2012; Badiane et al., 2012; Kuruma et al., 2008). The uniqueness and value of microsatellites arises from their multi-allelic nature, co-dominant transmission, ease of detection by PCR, high information content, ease of genotyping and its relative abundance in genome. They are good for tracing pedigrees, because they represent single loci and avoid the problems associated with multiple banding patterns (multiplex) obtained with other marker system.

The objectives of this work however are to assess the level of diversity within cultivated cowpea and to determine to probable center of origin of cultivated cowpea in Africa using microsatellites markers.

MATERIALS AND METHODS

Plant materials and DNA extraction

Forty eight cultivated cowpea were selected for DNA analysis (Table 1). Two seeds from each accession were sown in pots containing good loamy soil and placed on the floor in a screen house at International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. After two weeks of planting newly opened fresh young leaves were picked from each accession for DNA extraction. 0.3 g fresh leaf sample was ground into fine powder and DNA extracted according to the procedure described by (Dellaporta et al., 1983). The DNA was diluted in 0.1 × TE (1mM Tris 0.1 mM EDTA, pH 8.0) to

Table 1. Origin and accession number of Cowpea lines used for fingerprinting.

Code number	Tvu no	Origin	Region	Cultivars group
1	8130	Ghana		Unguiculata
2	6939	Niger		Unguiculata
3	8001	Nigeria		Unguiculata
4	8510	B. Faso		Unguiculata
5	14532	Mali		Unguiculata
6	8082	CotedVoire		Unguiculata
7	1177	Uganda	W. Africa	Unguiculata
8	8049	Nigeria		Unguiculata
9	11412	Gambia		Unguiculata
10	14818	Senegal		Unguiculata
11	15206	Congo		Unguiculata
12	10843	Cameroon		Unguiculata
13	8650	Togo		Unguiculata
14	7146	Ethiopia		Unguiculata
15	11954	Sudan		Unguiculata
16	9700	Egypt		Unguiculata
17	13484	Kenya		Unguiculata
18	15267	Chad		Unguiculata
19	15247	Chad		Unguiculata
20	13826	CAR	NE and C Africa	Unguiculata
21	13850	CAR		Unguiculata
22	11980	Sudan		Unguiculata
23	9548	Egypt		Unguiculata
24	16029	Somalia		Unguiculata
25	13439	Kenya		Unguiculata
26	13830	CAR		Unguiculata
27	11773	Malawi		Unguiculata
28	11774	Malawi		Unguiculata
29	15388	Zimbabwe		Unguiculata
30	14895	Botswana		Unguiculata
31	15047	Zambia		Unguiculata
32	988	S Africa		Unguiculata
33	15443	Swaziland	Southern Africa	Unguiculata
34	15077	Zambia		Unguiculata
35	1995	S Africa		Unguiculata
36	16098	Zimbabwe		Unguiculata
37	15433	Swaziland		Unguiculata
38	15055	Botswana		Unguiculata
39	15429	Lesotho		Unguiculata
40	3658	China		Cylindrical
41	3657	China		Cylindrical
42	21	Philippine		Sesquipedalis
43	22	Philippine	Asia	Sesquipedalis
44	3655	China		Sesquipedalis
45	1498	India		Sesquipedalis
46	3653	China		Sesquipedalis
47	3656	N. Caledonia		Sesquipedalis
48	3652	Australia	Australia	Sesquipedalis

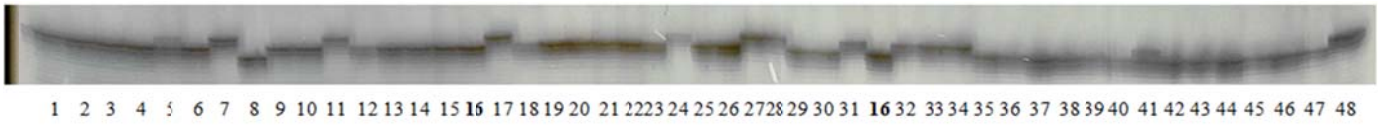

Figure 1. A gel photomicrograph showing the bands of SSR VM 74 with forty-eight cowpea lines. Note sample 16 was loaded twice at different places to serve as control.

10 ng/µL concentration.

Primer screening

A total of 120 SSR primers were screened and optimized for polymerphism and annealing temperature (Tm) using two accessions to ensure optimal primer performance.

Optimal PCR amplification across the two accessions was achieved with the range between 54 and 64°C annealing temperature. Thirteen primers that showed good and clear polymorphism with the PCR products were therefore used for this study.

PCR amplification

A 20 µL reaction volume containing 2.0 µL of 10x buffer, 4.0 µL of 10 ng/µL template DNA, 2.0 µL $MgCl_2$, 1.6 µL mixture of 10 mM dNTPs (dATP, dCTP, dGTP and dTTP), 9.2 µL of ultra pure water, 1.0 µL of SSR primers and 0.2 µL Taq (promega) was loaded in Perkin Elmer Mj cycler for DNA amplification. The PCR reaction was carried out with a profile of 18 cycles at 94°C for 1 min initial denaturing and extension at 72°C for 1 min. Annealing temperatures were progressively decreased by 0.5°C every cycle from 64 to 54°C. The reactions continued for 30 additional cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min and ended with a 10 min extension at 72°C after about 3 hours. 2.0 µL of PCR products was loaded in 3% agarose gel to check for polymorphism before running those that showed polymorphism on polyacrylamide gel electro-phoresis.

Polyacrylamide gel electrophoresis of PCR products and data analysis

PCR products were separated on a sequencing gel containing 70 ml freshly prepared 6% polyacrylamide solution, 350 µL ammonium persulphite (APS) and 35 µL TEMED. The gel was run at constant power of 50 W, 2500 V and 60 mA for 3 h. The gel was later fixed, stained, and developed using silver staining kit (Promega corp. Madison WI). Fragments that were clearly resolved on gels (Figure 1) were scored as 1 or 0 that is, present or absent respectively on all the forty eight accessions of cultivated cowpea. The bands that could not be confidently scored were regarded as missing data. Clearly resolved DNA bands were amplified by 12 SSR primers and used for the clustering analysis.

Data analysis

Pairwise distance (similarity) matrices were computed using sequential, hierarchical and nested (SAHN) clustering option of the NTSYS-pc version 2.02j software package (Rohlf, 1993). The program generated a dendrogram, which grouped the test lines on the basis of Nei genetic distance using unweighted pair group using mathematical average (UPGMA) cluster analysis.

The polymorphism information content (PIC) provides an estimate of the discriminatory power of locus or loci, by taking into account, not only the number of alleles that are expressed, but also the relative frequencies of those alleles. PIC values were calculated by the algorithm: PIC = $1-\Sigma^n Pi^2$ where *i* starts from 1 and Pi^2 = frequency of the ith alleles (Ott, 1999). PIC values range from 0 (monomorphic) to 1 (very highly discriminative, with many alleles each in equal and low frequency). The two dimension Principal Component Analysis (PCA) programmes of the Statistical Analysis System software package (SAS) version 8.2 was used. Only the distributions of the accessions along the first two principal components were considered in this paper.

RESULTS

A dendrogram (Figure 2) for the 48 cowpea lines was constructed by the UPGMA on the basis of the genetic similarity and Jaccard's Coefficient. The population clustering exhibits three main clusters when truncated at 65% similarity coefficient. The accession numbers, origin, codes, region and the cultivars name are given in Table 1. Codes 14, 16, 30 and 37-formed accessions from cluster three, two of which originated from Southern Africa (Botswana-30 and Swaziland-37) and the other two (14 and 16) from North East Africa (Ethiopia and Egypt), respectively.

The second cluster contained five accessions; two (9 and 12) were from Gambia and Cameroon, two (18 and 24) from Chad and Somalia and one (40) *Cylindrical* from China respectively. The first cluster is large and contained the remaining accessions including the seven *sesquipedalis* from Asia and the two *cylindrical*. The dendrogram distinguished all accessions except four (11 and 41) and (20 and 29) which are genetically similar.

A two dimension principal coordinate analysis did not detect significant sub group among the forty eight lines, they scattered randomly in the coordinates irrespective of the geographical location (Figure 3). However, West African accessions (Code 1-13) distributed more widely than others an evidence for West Africa great diversity of cultivated cowpea.

The PIC revealed that the number of alleles per locus ranged from 2 to 5 with a total of 37 from 12 primers (Table 2). The mean number of allele per locus was 2.92. Four primers (VM 39, VM 98, VM 27 and VM 78) had least number (2) of alleles each. The PIC value for the 12 SSR primers ranged from 0.075 to 0.603 with a mean value of 0.344 from a total of 4.467. There was no significant correlation between the repeat number and the allele numbers (r=0.21) so also between repeat numbers and PIC (r=0.11). Twelve primers that amplified clear polymorphic bands on the polyacrylamide gels were used

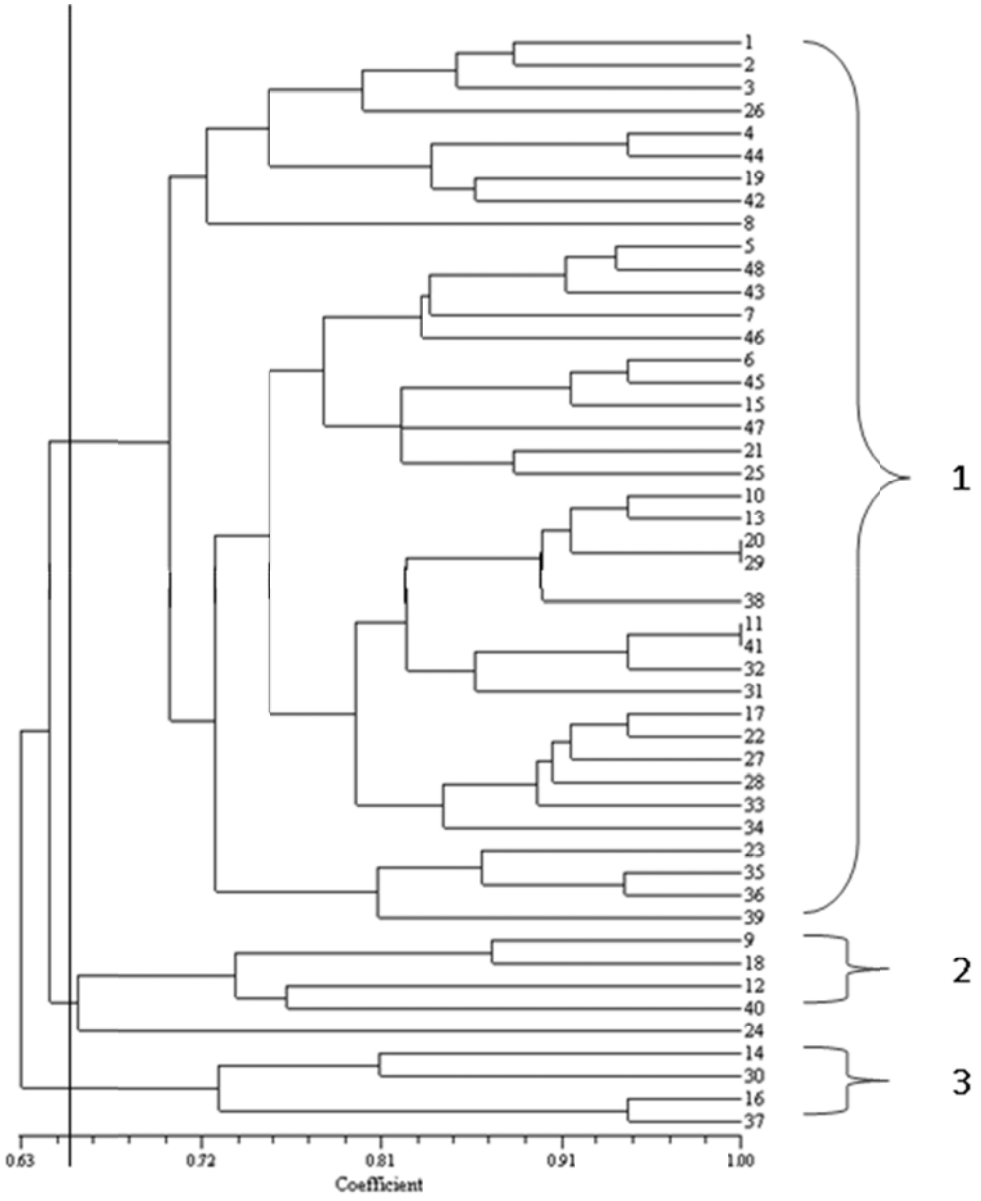

Figure 2. Dendrogram showing the distribution of Forty-eight cowpea accessions with twelve SSR primers.

to analyze the forty eight cowpea lines. These primers, their repeat type, repeat number, allele number and polymerphism information content were listed in Table 2. The information on their polymorphic ability is summarized with a bar chart (Figure 4).

Table 3, reveals the genetic diversity within three sub-region in Africa; West Africa, North East and central Africa and Southern Africa with 13 accessions each. The PIC from the three regions revealed PIC from West African accessions to be 4.4310, Southern Africa have 3.9539 while North East and Central Africa with 3.9872, this is another evidence of great diversity from West Africa region.

DISCUSSION

The use of genetic diversity - on farm through field experimentation or in sophisticated gene transfer procedures remains arguably the best route to secure our food and that of our children. The genetic diversity contained in different varieties provides farmers with options to develop, through selection and breeding new and more productive crops that are resistant to pests, diseases and stress. It refers to the variation at the level of individual genes (polymorphism), and provides a mechanism for populations to adapt to their ever-changing environment. The more variation, the better the chance that at least some

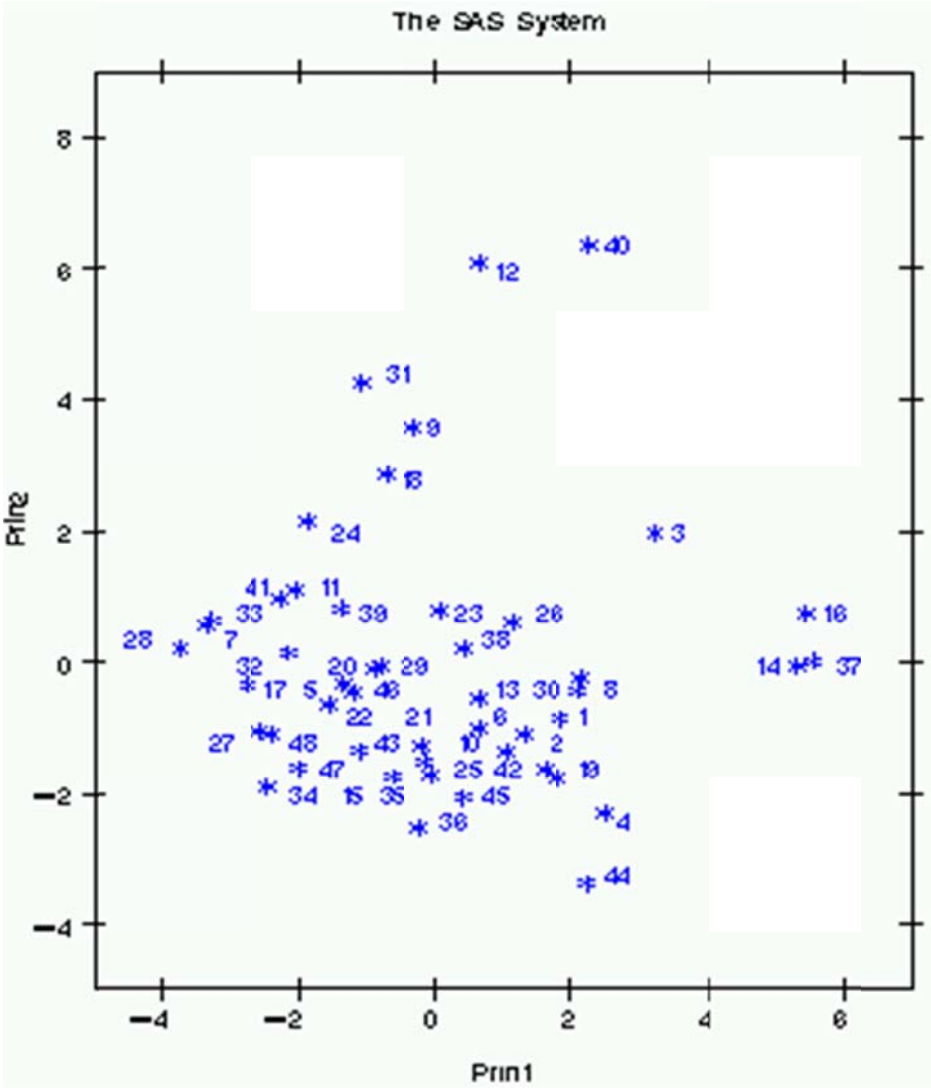

Figure 3. Principal component analysis for the 48 accessions of cowpea using twelve SSR primers.

Table 2. Number of alleles and polymorphism information content (PIC) of the cowpea microsatellite primers.

Primers	Repeat sequence	Repeat number	Number of alleles	PIC
Vm 98	AC /CT	9	2	0.323
Vm 9	AG/CT	21	4	0.519
Vm 37	AG/CT	13	4	0.557
Vm 27	AG/CT	14	2	0.172
Vm 39	AC/TG	13	2	0.376
Vm 22	AG/CT	12	3	0.226
Vm 36	AG/CT	13	4	0.603
Vm 71	AG/CT	12	3	0.480
Vm 74	AC/TG	8	3	0.386
Vm 78	AG/CT	5	2	0.075
Vm 35	AC/TG	11	5	0.502
Vm 34	AG/CT	14	3	0.248
Total			37	4.467

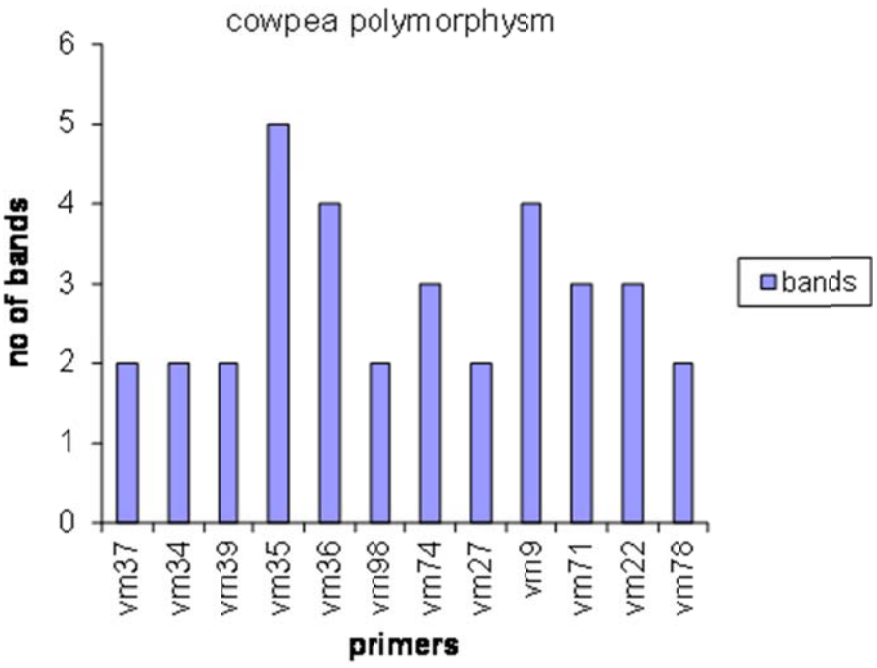

Figure 4. Bar chart showing the number of alleles detected by each primer.

Table 3. Polymorphism Information Content (PIC) of cultivated cowpea from three regions in Africa (North East and Central Africa, West Africa and Southern Africa).

Primer	Diversity Index		
	NE and CA (13)	WA (13)	SA (13)
Vm 37	0.2387	0.3228	0.3820
Vm 34	0.2387	0.3228	0.2387
Vm 39	0.4351	0.382	0.2387
Vm 35	0.4816	0.4714	0.5370
Vm 36	0.3609	0.7264	0.5127
Vm 98	0.3228	0.4297	0.2387
Vm 74	0.4360	0.2387	0.435
Vm 27	0.2387	0.1417	0.1417
Vm 9	0.5229	0.3820	0.5960
Vm 71	0.4731	0.4350	0.3609
Vm 22	0.0000	0.3398	0.2725
Vm 78	0.2387	0.2387	0.0000
Total	3.9872	4.4310	3.9539
Mean	0.3323	0.3693	0.3295

of the individuals will have an allelic variant that is suited for the new environment, and will produce progeny with the variant that will in turn reproduce and continue the population into subsequent generations.

Pasquet (2000), Ng (1995) and Aaron et al. (2010) reported that the area of maximum diversity of cultivated cowpea is found in West Africa in an area encompassing the savannah region of Nigeria, southern Niger, part of Burkina Faso, Northern Benin, Togo and the Northwestern part of Cameroon. In this study, the West African accessions distributed more widely than accessions from other regions, indicating more diverse accessions from that region than others, and may likely be the center of diversity.

Supporting this is the PIC values from the three regions. It revealed the genetic diversity within three sub- regions in Africa; West Africa, North East and central Africa and

Southern Africa with 13 accessions each. The PIC from the three regions varies with accession from West Africa having the highest PIC value of 4.4310, North East and central Africa having PIC of 3.9872 and Southern Africa with PIC of 3.9539. This suggests that genetic variation among lines from WA based on micro-satellite analysis is higher when compared with that observed among accessions from other regions. According to Padulosi et al. (2007), Padulosi et al. (2009) and Ogunkanmi et al. (2007) an area with intense variation may probably be the one where the crop must have been cultivated for a long time as a result of interbreeding and introgression among different varieties. This result is in agreement with the work of (Ogunkanmi et al., 2006) where he postulated West Africa as the center of origin of cowpea based on morphological data.

The 12 microsatellite markers used in this study detected 37 alleles among the 48 cowpea accessions with marker VM 27 detected the smallest number of alleles. In (Li et al., 2001) VM 27 was also reported to detect the lowest number of alleles among 90 cultivated cowpea lines and one wild cross compatible relative. The number of alleles detected in yard long bean ranges from 2 to 7 (Ogunkanmi et al., 2006) tomato 1 to 5 (Broun and Tanksley, 1996), Maize 2 to 11 (Senior and Heun, 1993), Barley 3 to 37 (Becker and Heun, 1995), and wild cowpea 4 to 13 (Ogunkanmi et al., 2008) as against 2 to 5 in this study. This suggests that genetic diversity in vegetable cowpea lines based on microsatellite analysis is higher and have higher genetic base when compared with that observed among cultivated cowpea lines used in this study. It is interesting to note that VM 39 which detected the highest number of alleles in the work of (Ogunkanmi et al., 2006), now showed the least number of alleles as in VM 27 above. The ability to use the same SSR primers in different plant species depends on the extent to which primer sites flanking SSRs are conserved between related taxa and the stability of the SSR over evolutionary time. The high discriminating power of SSRs is also an important factor in the analysis of variation in the gene pool of crops. Wayne et al. (1996) and Fatokun et al. (1999), in their study with rice established that 28% of the allelic variability was lost during the process of cultivar development from landraces. This is evident from the understanding of domestication process involved in the evolution of crop plants. Allelic variance are lost or reduced as plants are domesticated and hence narrow genetic base.

However, the high level of similarity among two pairs of accessions as detected by microsatellite markers (Figure 2) may be due to seed mix up during the process of labeling or handling in the gene bank. It could also be that the similar accessions came from same plant stand and subsequently found their way to the gene bank hence they are given different identification numbers.

To this end, microsatellites markers have been proved to be highly informative and provide an efficient and accurate means of detecting genetic variation in cowpea.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Use of spent compost in the cultivation of *Agaricus blazei*

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Two compost formulations, based on Braquiaria straw (*Brachiaria sp.)*, a conventional one and a spent one, were tested in the cultivation of ABL 99/30 and ABL 04/49 strains of *Agaricus blazei*. The experimental design was in a completely randomized factorial scheme with four treatments (two strains of *A. blazei* x two types of compost) and 30 repetitions. Each experimental unit consisted of a box with 10 to 10.5 kg of moist fresh compost. According to the results obtained, the loss of organic matter of the composts was affected by the *A. blazei* strain and the type of compost used. The traditional compost lost a higher organic matter content compared to the spent compost, and the ABL 99/30 strain caused a higher loss of organic matter in the composts compared to the ABL 04/49 strain. Yield, biological efficiency, mass and number of basidiomata produced were similar between the conventional and the spent compost, as well as the chemical analysis of the produced basidiomata. However, the *A. blazei* strains showed some differences among each other, the basidiomata of strain ABL 04/49 obtained a higher percentage of crude protein in their composition, compared to the ABL 99/30, in both composts. Thus, the utilization of spent compost in the cultivation of *A. blazei* did not impair the basidiomata yield nor their nutritional value, demonstrating it to be a good option to be used as an ingredient in the compost formulation for the *A. blazei* cultivation.

INTRODUCTION

The use of spent compost (substrate resulting in the end of the production cycle) for the production of new mushroom cultivation cycles is a promising alternative, which aims at replacing the soil and organic substrates

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commonly used in mushroom production, and whose advantages are the reduction of the cost production and the environmental impact caused by these materials extraction from the environment (Pardo-Giménez and Pardo-González, 2009; Pardo-Giménez et al., 2010).

Mushrooms have a great commercial importance due to their nutritional and medicinal properties. Mushrooms are considered food of a high nutritional value as they have low lipid content, a considerable amount of phosphorus and present a high level of proteins and dietary fibers (Furlani and Godoy, 2007).

Shibata and Demiate (2003), while carrying out the nutritional analysis of two strains of *A. blazei*, obtained the following basidiomata chemical composition means: 37.4% protein, 8.82% fiber, 7.49% ash, 0.99% lipid and 45.30% carbohydrate.

The composting process and the appropriate chemical composition of the substrates and supplements used in the compost are fundamental to reach a desirable yield in the mushroom cultivation. The use of agroindustrial residues for the formulation of composts is intended to minimize the cost of mushrooms production (Silva et al., 2009).

In Brazil, the cultivation of *A. blazei* occurs in a very similar way to the cultivation of *A. bisporus*. Athough the species present certain similarities, it is necessary to develop specific technologies for the cultivation of *A. blazei* in order to increase its yield, considering the low yield reached in this mushroom cultivation when compared to the one obtained by the cultivation of *A. bisporus* (Kopytowski Filho, 2006; Dias, 2010).

According to Kopytowski Filho (2006), the cultivation of *A. blazei* can be divided as follows: composting phase I, which corresponds to the period of composting process in yard; composting phase II, which is the process including the compost pasteurization and conditioning, and the composting phase III, which corresponds to the stages of inoculation and colonization of the compost; the covering and harvesting are carried out afterwards. The composting process is generally critical to obtaining good quality compost; however the high yield reached depends significantly on phase II (Sánchez, 2004).

In Brazil, materials such as cereal straws (wheat, rice, and barley), grasses (brachiaria, coast-cross and tifton), animal bedding (horses and poultry), nitrogen sources (organic and/or mineral), limestone and plaster are used as substrates for compost formulation in *Agaricus* cultivation (Minhoni et al., 2005; Kopytowski Filho, 2006).

Usually, the material supply for composts formulation varies according to its availability depending on the season of the year (Andrade et al., 2008). Several materials have been used in the compost preparation for the cultivation of *A. blazei*; the uses vary according to their availability in the different country regions and season of the year.

However, little is known about the reutilization of these materials for a new cycle of *A. blazei* production, yield and nutritional composition of basidiomata produced. Thus, this study aims at assessing the yield, biological efficiency, number of mushrooms, mass of mushrooms and the bromatological analysis of mushrooms produced, using two strains of *A. blazei* and two formulations of composts based on brachiaria straw (*Brachiaria* sp.): conventional compost and a spent one.

MATERIALS AND METHODS

The experiment was carried out in the facilities of the Mushrooms Module, Plant Production Departament, FCA/UNESP, Botucatu-SP, with two types of composts (traditional and spent) (Table 1) and two strains of *A. blazei* ABL 99/30 and ABL 04/49.

Seeds production

The strains ABL 99/30 and ABL 04/49 of *A. blazei* used were both kept in the Mushrooms Module Matrix Bank, Plant Production Department, FCA/UNESP, Botucatu-SP. Initially, 0.5 cm diameter disks were transferred from the primary matrix, under aseptical conditions, to other Petri dishes with compost - agar (CA). After inoculation, the Petri dishes were transferred to an incubator where they were kept for 10 days in darkness at 28 ± 1°C for colonization. The Petri dishes colonized were split in eight equal parts; each part of this segment was inoculated in flasks containing sorghum grains (400 g), plaster, and calcium carbonate. The sorghum grains were initially boiled in water for 40 minu. After draining the excess of water, 20 g kg^{-1} calcium carbonate and 160 g kg^{-1} of plaster were added relative to the moist weight of grains cooked. The lower part of the flasks cap were fitted with filter paper in order to allow aeration and prevent contaminations after autoclaving. The flasks were incubated in an incubator, in darkness at 28 ± 1°C for 12 days. The inoculum was produced by packing the substrate prepared in high density polyethylene (HDPE) bags, using about 1200 g of sorghum grains per plastic. The plastic bags contained Tyvek® filters in the upper parts, thus, allowing the gas exchanges.

The substrates prepared were autoclaved at 121°C for 3 h. Then, the bags were kept at rest for 24 h in order to reduce the temperature to about 25°C. Then, the inoculation of each plastic bag was undertaken at temperature of 28 ± 1°C for 15 days. By the end of the incubation period, the substrates were colonized by the fungus, and then called spawn, and ready to be inoculated in the compost.

Composting

Composting phase I was carried out on concrete floor, with open sides and natural ventilation. Before forming the furrows, brachiaria straw was moistened and overturned every two days for a total period of 10 days. The furrows were formed by a layer of straw (20 cm high), followed by a layer of sugarcane bagasse (20 cm high) until they reached 1.8 × 1.8 m, height and width respectively. Limestone, urea and soy bran were added to both furrows according to each treatment. Table 2 presents the amount of each ingredient added in the formation of the furrows for the 2 types of composts.

The composts were overturned, and water was added manually with a hose in order to keep the moisture between 70 to 75%. Altogether, six overturns were carried out, totaling 14 days in

Table 1. Content of moisture, mass and percent of carbon and nitrogen, and the C/N relation of the ingredients used in the traditional and spent composts.

Ingredient	Moisture (%)	Carbon (%)	Carbon (Kg)	Nitrogen (%)	Nitrogen (Kg)	C/N
Traditional compost						
Sugarcane bagasse	64.90	50.00	70.20	0.52	0.73	96.15
Brachiaria	18.36	48.10	62.83	1.26	1.65	38.17
Soy Bran	12.83	50.00	3.92	7.80	0.61	6.41
Urea	0	27.00	0.41	45.00	0.68	0.60
Spent compost						
Sugarcane bagasse	64.90	50.00	52.65	0.52	0.55	96.15
Brachiaria	18.36	48.10	47.12	1.26	1.23	38.17
Soy Bran	12.3	50.00	2.62	7.80	0.41	6.41
Urea	0	27.00	0.27	45.00	0.45	0.60
Spent Compost	71.6	9.35	5.31	0.50	0.28	18.70

C/N = Carbon/nitrogen relation. Traditional Compost = substrate used in the mushrooms production, consisting of sugarcane bagasse, brachiaria straw, soy bran and urea. Spent compost = substrate used in the mushrooms production, consisting of sugarcane bagasse, brachiaria straws, soy bran and urea added of spent compost (substrate obtained by the end of the cultivation cycle).

Table 2. Traditional and spend composts formulation.

Ingredient (kg)	Compost			
	Traditional		Spent	
	Moist weight	Dry weight	Moist weight	Dry weight
Sugarcane bagasse	400.00	140.40	300.00	105.30
Brachiaria straw	160.00	130.62	120.00	97.97
Soy bran	9.00	7.85	6.00	5.23
Urea	1.50	1.50	1.00	1.00
Plaster	-	8.00	-	8.00
Limestone	-	9.00	-	9.00
Spent compost	-	-	200.00	56.80
Total Mass of Compost	570.50	297.37	627.00	283.30
Total Mass of Carbon	-	137.36	-	107.97
Total Mass of Nitrogen	-	3.67	-	2.92
Initial C/N relation	-	37.42	-	37.00

phase I. In phase II, the composts were transferred into perforated plastic boxes, which measured 56.5 × 46.5 × 28.5 cm (length, width and height respectively). The boxes were randomly placed inside a climate-controlled chamber (Dalsem mushrooms) for the pasteurization (8 h at 62 ± 2°C) and conditioning (8 days at 48± 2°C). In the end of phase I and II (Table 3), three samples of each compost were collected and dehydrated at 65°C for 48 h to analyze carbon, nitrogen, organic matter and pH. The results are presented in Table 4.

Inoculation of composts

The inoculation of composts was carried out manually by adding 1.5 g of *A. blazei* seed per kg^{-1} of moist compost. The composts were split and transferred (10 to 10.5 kg of moist compost) to other polyethylene boxes internally covered with polyethylene transparent plastic containing orifices in the lower part. The boxes were randomly placed in an incubator (Dalsem Mushrooms) and kept for 16 days at 28 ± 1°C.

The soil used in the covering layer was classified as Dystrophic Red Nitosol (Carvalho, 1983) from the Fazenda Lageado (FCA / UNESP). The soil pH was corrected to 7.0 by adding calcium carbonate, 20 days before the compost covering. Altogether, 840 L of soil were used, and 30% (360 liters) of charcoal (1 to 2 cm thick) was added. The soil pasteurization was carried out at 62°C for 8 h, in an incubator (Dalsem Mushrooms). About 15 kg of soil were added to each box to act as cover layer. The soil was previoulsly moistened with the assistance of a hose to keep the moisture at about 70%. After the addition of the cover layer, the compost was covered with transparent plastic, and incubated for six days at 22 ± 1°C. After the cover layer had been colonized, the plastic was

Table 3. Composting process phases.

Days	Activity	Procedure	Phase
-10	Pre-moistening	Straw moistening	Pre-composting
-7		Overturn and moistening of straw	
-5		Overtun of straw	
-3		Overtun of straw and addition of bagasse	
0	Setting of furrows	Additon of 1st half of soy bran, urea and limestone.	Composting Phase I
2	1st Turn over	Additon of 2nd half of soy bran, urea and limestone.	
4	2nd Turn over	Additon of 1st half of plaster	
7	3rd Turn over	Additon of 2nd half of plaster	
9	4th Turn over	Eventual correction of moisture	
11	5th Turn over	Eventual correction of moisture	
14	6th Turn over	Eventual correction of moisture	
15	Pasteurization	Temperature of 62°C ± 2 for 8 h	Composting Phase II
16	Conditioning	Temperature of 48°C ± 2 for 8 days	
27		Compost with 25°C ready to be inoculated	

Table 4. Content of moisture, nitrogen, organic matter and carbon, C/N relation and pH of traditional and spent composts, in the end of composting phase I and II.

Compost	Traditional	Spent
End of phase I		
Moisture (%)	77.54	73.72
N (%)	0.33	0.32
C (%)	10.19	10.03
O.M (%)	18.34	18.04
C/N	32/1	32/1
pH	7.43	7.53
End of phase II		
Moisture (%)	72.60	71.07
N (%)	0.44	0.40
C (%)	11.38	9.85
O.M (%)	20.49	17.53
C/N	26/1	25/1
pH	7.65	7.67

N, Nitrogen; O.M, organic matter; C, carbon; C/N, carbon/nitrogen relation.

removed. During the production of basidiomata, water was added in the cover layer with the assistance of a hose to keep the moisture at about 75%.

By the end of the mushrooms harvesting period, the composts loss of organic matter was calculated by using 6 boxes of each tratment, from which the cover layers were removed, and the composts moisture and mass content was later determined in the end of the mushrooms production.

Variables analyzed

Number and fresh mass of mushrooms

The number and fresh mass of mushrooms were daily determined during harvest. A semi-analytical scale was used to determine the mushrooms fresh mass.

Yield and biological efficiency

Yield was expressed as the fresh mass of mushrooms / fresh mass of compost × 100, and the biological efficiency as the fresh mass of mushrooms / dry mass of compost × 100. The mushrooms fresh mass was determined in the end of harvest and the compost fresh mass was determined in the end of composting phase II.

Organic matter loss

The loss of organic matter was expressed as the compost dry mass in the end of composting phase II - compost dry mass in the end of the production / compost dry mass in the end of composting phase II × 100. The organic matter loss of the composts is presented in Table 5.

Nutritional analysis of *A. blazei* strains

Nutritional analyses of mushrooms were carried out at the Faculdade de Medicina Veterinária e Zootecnia - FMVZ/ UNESP, Laboratory of Bromatology, Botucatu-SP. Two samples of dehydrated mushrooms of each treatment were collected during the production and the contents of crude protein, ether extract, ash and crude fiber were determined according to Silva and Queiroz (2002). The conversion factor 4.38 is used to determine protein in mushrooms (Furlani and Godoy, 2007).

RESULTS AND DISCUSSION

The F values of variance of the organic matter loss of traditional and spent composts according to the *A. blazei* strain used are presented in Table 5. The type of compost and the strain of *A. blazei* used influenced the percentage of organic matter loss of composts. Table 6

Table 5. F values obtained in the analysis of variance of organic matter loss of traditional and spent composts according to the *A. blazei* strain used.

Parameter	Organic matter loss
Compost	12.99**
Strain	24.84**
Compost x strain	0.38^{ns}
Variation coefficient %	16.64

**Significance level < 1%; *significance level < 5%; ns: no significant difference.

Table 6. Organic matter loss of traditional and spent composts according to the *A. blazei* strain used.

Strain	Compost	
	Traditional	Spent
ABL 99/30	42.0^{Aa}	32.8^{Ab}
ABL 04/49	29.8^{Ba}	23.3^{Bb}

*Means followed by the same capital letters inside a column and small letters inside a row do not differ significantly (Tukey, 5%). Mean obtained from 6 repetitions.

Table 7. F values obtained in the analysis of variance for the fresh mass of basidiomata (MB), number of basidiomata (NB), yield (Y) and biological efficiency (BE) of ABL 99/30 and ABL 04/49 strains of *Agaricus blazei* , cultivated in two types of composts, traditional and spent.

Parameter	MB	NB	Y	BE
Compost	0.125^{ns}	0.095^{ns}	0.125^{ns}	1.163^{ns}
Strain	96.99**	57.57 0**	96.972**	96.965**
Compost x Strain	2.55^{ns}	1.797^{ns}	2.517^{ns}	3.433^{ns}
Variation coefficient %	20.28	23.69	20.27	20.37

**Level of significance < 1%; * level of significance < 5%; ns, no significant difference.

presents the organic matter loss of each compost according to the *A. blazei* srain used for the mushrooms production. Generally speaking, the ABL 99/30 strain caused a higher organic matter loss of composts (37.40%) than the ABL 04/49 strain (26.55%), while in the avarage, the traditional compost lost a higher organic matter content (35.90%) compared to the spent compost (28.05%).

In Table 7, the effect of the *A. blazei* strains over the variables, fresh mass of basidiomata, number of basidiomata, yield and biological efficiency of mushrooms produced was verified. The type of compost and the interaction compost x strain did not cause effects over the variables analyzed.

The values obtained of mass and number of basidiomata, yield and biological efficiency of ABL 99/30 and ABL 04/49 strains of *A. blazei*, grown in both traditional and spent composts, are present in Table 8. Differences were verified between the variables analyzed as for the *A. blazei* strain used, being that the ABL 99/30 strain was superior to ABL 04/49, regardless of the kind of compost grown, in all variables analyzed. The results show that ABL 99/30 strain was superior in 35.16, 32.55, 35.07 and 35.16% as for mushrooms mass, number of mushrooms, yield and biological efficiency, respectively, in relation to the ABL 04/49 strain when they were cultivated in the traditional compost.

When *A. blazei* strains were grown in the spent compost, the ABL 99/30 strain was superior to ABL 04/49 for the same variables in 26.35, 23.66, 26.40 and 26.33%,

Table 8. Total mean values for number and fresh mass of basidiomata, yield and biological efficiency of 04/49 and 99/30 strains of *Agaricus blazei*, obtained in function of the kind of compost used.

Strain	Compost	
	Traditional	Spent
Mass (g)		
ABL 99/30	1303.23^{Aa}	1253.43^{Aa}
ABL 04/49	845.03^{Ba}	923.17^{Ba}
Number of Basidiomata		
ABL 99/30	68.20^{Aa}	65.63^{Aa}
ABL 04/49	46.00^{Ba}	50.1^{Ba}
Yield (%)		
ABL 99/30	13.03^{Aa}	12.54^{Aa}
ABL 04/49	8.46^{Ba}	9.23^{Ba}
Biological efficiency (%)		
ABL 99/30	47.56^{Aa}	43.37^{Aa}
ABL 04/49	30.84^{Ba}	31.95^{Ba}

Means followed by the same capital letters inside a column and small letters inside a row do not differ significantly (Tukey, 5).

respectively. Mamiro et al. (2007) studied the use of the spent compost, mixtures of spent compost with non-composted substrate and the supplementation period of the compost in the cultivation of *A. bisporus* and obtained the lowest indexes of productivity (4.9 kg/m^2) and biological efficiency (25.7%), by using spent compost without compost supplementation and the highest indexes of productivity (27.2 kg/m^2) and biological efficiency (144.3%) were reached when a 50/50 mixture of spent compost with non-composted substrate was used and the Target® (commercial nutrient for mushrooms) supplement was added at the moment of laying the cover of the compost.

Giménez and González (2009) used a mixture of spent substrate of *P. ostreatus* with spent substrate of *A. bisporus* for new cultivation cycles of *P. ostreatus* and obtained the best behaviour for the production parameters when they used combinations of 9:1 and 8:2 (p/p) (spent substrate of *P. ostreatus* and spent substrate of *A. bisporus*, respectively).

The values for frutification precociousness, frutification index, yield and biological efficiency obtained were next to the ones reached by the control treatment carried out by the authors by using an approppriate commercial substrate. Mamiro and Royse (2008) evaluated the effect of mixtures of spent compost and non-composted substrate in different ratios for the cultivation of *A. bisporus* on yield, biological efficiency and mass of the mushrooms and obtained higher results when they used 50/50 and 75/25 mixtures of non-composted substrate and spend substrate, respectively. They reached values of 10.9 kg/m² for yield and 61.5% for biological efficiency when they used the materials in a 50/50 ratio. When the ingredients were mixed in a 75/25 ratio, the results were 67.3% of biological efficiency and 11.9 kg/m² of productivity.

The kind of compost used for the cultivation of mushroms did not influence the mass and number of mushrooms and did not affect their yield and biological efficiency. A similar fact occurred with Zied et al. (2009) who worked with different composts formulations for the cultivation of *A. blazei* and did not found significant differences in the variables studied (mass of the mushrooms, number of mushrooms, yield and biological efficiency) in relation to the kind of compost used for the cultivation of the mushrooms.

The F values obtained in the analysis of variance for the dry matter, crude protein, ether extract, ash and crude fiber of ABL 99/30 and ABL 04/49 strains of *A. blazei*, cultivated in both types of compost are presented in Table 9. The results show that the type of compost used for the mushrooms cultivation affected the contents of dry matter and ether extract of mushrooms produced. The content of ether extract and of mushrooms crude protein were influenced by the strain of *A. blazei* used, and the effect of the interaction compost x strain was also verified on the composition of ether extract of mushrooms produced.

Table 10 presents the results obtained in the bromatological analysis of the mushrooms produced, these results show the ABL 99/30 and ABL 04/49 strains were similar regarding the content of dry matter of mushrooms. However, the mushrooms cultivated in the spent compost presented a higher content of dry traditional compost.

In relation to the crude protein of mushrooms, it was verified that the mushrooms of ABL 04/49 strain were superior when compared to the mushrooms of 99/30 strain, regardless of where the composts were cultivated. The first ones presented mean values of 24.84% of crude protein, while the second ones presented 22.91% as mean. The type of compost used didn´t alters the content of crude protein of mushrooms produced.

Basidiomata of ABL 99/30 strain presented lower content of ether extract when cultivated in the traditional compost (0.68%), and higher content in the spent compost (1.21%). On the contrary, the basidiomata of ABL 04/49 strain presented higher content of ether extract (1.17%) when cultivated in the traditional compost, and the smallest content (0.96%) was obtained when they were cultivated in the spent compost.

There were no significant differences in the percentage of ash and crude fiber of mushrooms produced, no effect over these variables were verified regarding the strain of *A. blazei* adopted or the type of compost used for the cultivation of mushrooms. Andrade et al. (2008) using

Table 9. F values obtained in the analysis of variance of dry matter, crude protein, ether extract, ash and crude fiber of ABL 99/30 and ABL 04/49 strains of *A. blazei*, cultivated in two types of composts, a traditional and a spent one.

Variance cause	Dry matter	Crude protein	Ether extract	Ash	Crude fiber
Compost	60.098**	5.173^{ns}	82.843**	1.112^{ns}	0.144^{ns}
Strain	6.089^{ns}	59.677**	47.078**	1.954^{ns}	0.107^{ns}
Compost x Strain	6.753^{ns}	0.535^{ns}	423.706**	1.626^{ns}	3.400^{ns}
Variation coefficient %	0.18	1.48	2.52	4.53	6.29

**Significance level < 1%; *significance level < 5%; ns: no significant difference.

Table 10. Content of crude protein, ether extract, ash and crude fiber obtained in the bromatological analyzes of the basidiomata produced according to the strains of *Agaricus blazei* and the type of compost used.

Strain	Compost	
	Traditional	spent
Dry matter (%)		
ABL 99/30	91.6^{Ab}	92.79^{Aa}
ABL 04/49	92.18^{Ab}	92.77^{Aa}
Crude protein (%)		
ABL 99/30	23.1^{Ba}	22.72^{Ba}
ABL 04/49	25.21^{Aa}	24.46^{Aa}
Ether extract (%)		
ABL 99/30	0.68^{Bb}	1.21^{Aa}
ABL 04/49	1.17^{Aa}	0.96^{Bb}
Ash (%)		
ABL 99/30	5.98^{Aa}	6.46^{Aa}
ABL 04/49	6.53^{Aa}	6.48^{Aa}
Crude fiber (%)		
ABL 99/30	8.26^{Aa}	8.82^{Aa}
ABL 04/49	9.09^{Aa}	8.24^{Aa}

*Means followed by the same capital letters inside a column and small letters inside a row do not differ significantly (Tukey, 5%)

three formulations of composts for the production of four strains of *A. bisporus*, verified that the strain and the type of compost used influenced the production of mushrooms and also caused variations in the contents of crude protein, ash and crude fiber of mushrooms produced.

Conclusion

The use of the spent compost in the *A. blazei* cultivation can be considered a viable alternative since its use did not alter variables such as mass and number of mushrooms, yield and biological efficiency of mushrooms, and also did not compromise the nutritional composition of the mushrooms produced. Furthermore, according to the results obtained, the use of spent compost in new cultivation cycles of *A. blazei* is an alternative for the reduction of the production costs and the accumulation of these materials in the environment.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Field efficacy of inorganic carrier based formulations of *Serratia entomophila* AB2 in *Sesamum indicum* var. Kanak

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Serratia entomophila **is a well-known bacterium of agricultural importance for its nutrient (P and Zn) solubilization, plant growth promoter (IAA) production, antifungal activity and larvaecidal activity against coleopteran and lepidopteron pest. In the present study, an attempt was taken to reduce the use of chemical pesticide and fertilizer by using two inorganic carriers (talcum powder and vermiculite) based formulation of *S. entomophila* in sesame. From the experimental results it was evident that the vermiculite based formulation of *S. entomophila* AB2 proved a better shelf life than that of talcum based formulation at 180th day. Mean-while, both of the inorganic carrier based formulations of *S. entomophila* AB2 showed better results than the unformulated *S. entomophila* AB2 product and 100% NPK (60:60:50). Experimental data ensured that vermiculite based formulation work more efficiently in controlling lepidopteron pest attack by 53% than that of talcum powder based formulation 31%. Productivity of sesame was also increased more with vermiculite based formulation (249%) in comparison to talcum powder based formulation (138%). It may be inferred that cumulative effect of high rate of seed germination, nutrient solubilization and reduced rate of pest attack resulted into 4.8 time enhanced yield in sesame in case of vermiculite formulated product. On the basis of the result of this experiment, it can be recommended that vermiculite (80 g/100 g of product) based formulation of *S. entomophila* AB2 applied at 3.6 qt hec^{-1} in sesame could be an effective measure of lepidopteron pest control as well as biofertilizer for qualitative and quantitative increase of sesame.**

Key words: *Serratia entomophila* AB2, formulation, talcum powder, vermiculite pest control, productivity, integrated crop management (ICM).

INTRODUCTION

Serratia entomophila is well known for its agricultural importance (Babalola, 2010). *S. entomophila* is mainly popular as natural biocontrolling agent of New Zealand grass grub (Coleoptera), *Costelytra zealandica* (Grimont et al., 1988). A Mexican strain of *S. entomophila* Mor.4.1 was reported to control white grub (Coleoptera), *Phyllophaga blanchardi* (Nunez-Valdez et al., 2008). *Serratia* sp. EML-SE1 was isolated in Korea from dead larva of diamondback moth (Lepidoptera), *Plutella xylostella* (Jeong et al., 2010).

Along with larvaecidal activity, *S. entomophila* was also explored for its multidimensional properties. *S. entomophila* was found to have significant contribution for increasing soil organic matter (SOM) and maintenance of soil ecology in pastures (Villalobos et al., 1997). Preconditioned cultures of *S. entomophila* were observed to survive better over untreated control in saline stress due to increased Glycine betaine and choline content (Sheen et al., 2013). *S. entomophila* M6 was demonstrated to neutralize heavy metals (Ji et al., 2012). Recently, genome sequencing projects have revealed great potential of *S. entomophila* as secondary metabolite producer (Bode, 2011).

The use of bacterial inoculants for agriculture is limited for a couple of reasons but the most notable among them is the poor efficacy of the product under field conditions (Prior, 1989). Acceptibility of the product largely depends on formulations of biopesticide or biofertilizer with increased selflife of microbial inoculant and efficient release for ensuring its subsequent availability to the target species. Scientists used different base compounds as suitable carrier to inoculate for formulation such as, talcum for fluorescent pseudomnads (Nandakumar et al., 2001); talcum and peat for *Pseudomonas chlororaphis* and *Bacillus subtilis* (Nakkeeran et al., 2004). An extended self life (8-10 months) with vermiculite based formulation was observed with *P. fluorescens* (Vidhyasekaran and Muthamilan, 1995) and *Azospirillum brasilense* (Saleh et al., 2001). The formulations of fluorescent *Pseudomonas* strain R62 and R81 were used to increase significantly plant growth and productivity in field condition (Sarma et al., 2009a). Broadcasting of talcum based formulation of *P. flurescens* strains (Pf1 and FP7) on paddy field significantly reduced sheath blight, and thereby, increasing yields (Nandakumar et al., 2001). Incorporation of commercial chitosan based formulation LS254 and LS255, comprising of *P. macerans* and *Bacillus subtilis* into soil at the ratio of 1:40 (Formulation:Soil) increased plant biomass and yield (Vasudevan et al., 2002).

The bacterial strain *S. entomophila* AB2, used in this study, was originally isolated from epizootic *Heliothis armigera* larvae (Chattopadhyay et al., 2011). The strain was characterized for nutrient (P and Zn) solubilization (Chattopadhyay et al., 2012a), plant growth promoter (IAA) production (Chattopadhyay and Sen, 2012b) along with antifungal (Chattopadhyay and Sen, 2013) and larvaecidal activity against lepidopteron pest. Studies on systemic infestation of this strain (Chattopadhyay and Sen, 2013) through plant parts encouraged its soil application. Therefore, the isolate *S. entomophila* AB2, as a single biological agent for integrated nutrient management (INM) and integrated pest disease management (IPDM) may have the potential to be a lucrative alternative to inorganic amendments (fertilizer, pesticides and fungicides) in integrated crop management (ICM) which need to be verified in field conditions. This communication makes an attempt to understand the feasibility of formulations involving a single indigenous strain, *S. entomophila* AB2 having multidimentional agricultural attributes for reducing the use of chemical pesticide and fertilizer in ICM practices. For this study, sesame was used as test crop. Two different inorganic carrier (talcum powder and vermiculite) based formulations were tested in field condition along with unformulated culture and NPK (60:60:50). Effectiveness of formulations was checked through a set of parameters: bacterial release in rhizosphere, self-life, pest control and productivity.

MATERIALS AND METHODS

Bacterial culture

The bacterial strain *S. entomophila* AB2, used in this study was isolated from epizootic *H. armigera* larvae (Chattopadhyay et al., 2011). The 16S rRNA gene sequence was registered to Gene Bank (Accession no. GU370899). The strain was characterized for nutrient (P and Zn) solubilization (Chattopadhyay et al., 2012a), plant growth promoter (IAA) production (Chattopadhyay and Sen, 2012b) along with antifungal (Chattopadhyay and Sen, 2013) and larvaecidal activity against lepidopteron pest.

Fermentation condition

Bacterial culture was maintained at -20°C as glycerol stock (50%). The working strain was grown in 100 ml flask containing broth medium (4 g sucrose, 1 g yeast extract, 0.2 g urea and 0.2 g NPK; pH 7.1) as seed culture. Fermentation was carried at 28°C for 72 h in a glass fermenter (MCU-200, B. Braun Biotech International, India) at 240 rpm using the same medium. Cells were harvested after entering into stationary growth phase (Visnovsky et al., 2008).

Product formulation

For product formulation two different inorganic carrier were used: talcum powder (TP; magnesium silicate, $Mg_3Si_4O_{10}(OH)_2$) and vermiculite (Ver; Phyllosilicate, $(MgFe,Al)_3(Al,Si)_4O_{10}(OH)_2 \cdot 4H_2O$). Sodium salt of carboxymethyl cellulose (CMC) was added in the formulations as an adhesive agent. After 3rd repeat sterilization, 80 g of carrier material was mixed with 18 ml of fermented broth (1.5×10^{10} cfu ml^{-1}), glycerol (1 ml 50% v/v) and CMC solution (1 ml 0.1 mg ml^{-1}) aseptically to generate 100 g of product (Vidhyasekaran and Muthamilan, 1995). The formulation was dried aseptically under the shade to reduce the moisture content to approximately

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Table 1. Product formulation and field trial experiments.

Test sample	Formulation	Field treatment
TS1	Control	Untreated
TS2	100% NPK (60:60:50)	100% NPK provided
TS3	90 ml fermented broth (*S. entomophila* AB2, 1.5×10^{10} cfu ml^{-1}) + 5 kg sterilized powdered soil	5 kg broadcasted in one plot area (4.0 m × 3.5 m)
TS4	18 ml fermented broth (*S. entomophila* AB2, 1.5×10^{10} cfu ml^{-1}) + 1 ml glycerol (50%, v/v) + 1 ml CMC (0.1 mg ml^{-1}) + 80 g talcum powder	500 g broadcasted in one plot area (4.0 m × 3.5 m)
TS5	18 ml fermented broth (*S. entomophila* AB2, 1.5×10^{10} cfu ml^{-1}) + 1 ml glycerol (50%, v/v) + 1 ml CMC (0.1 mg ml^{-1}) + 80 g vermiculite	500 g broadcasted in one plot area (4.0 m × 3.5 m)

18% and packed in sterilized polythene bags. The formulation contained 3.5×10^8 cfu g^{-1} of experimental bacterial load when packed. Formulation details are given in Table 1.

Field trials

Field trial experiments were conducted in three consecutive Ravi seasons. Experimental plots were kept idle for 6 months prior to seed sowing, to avoid the effects of any pesticide, chemical or biological, or any other application for soil treatment. The field soil was brought to a fine tilt by ploughing and 3.5 × 4.0 m plots were laid out. Randomized complete block design (RCBD) model was followed for the experiments.

Untreated (TS1) experimental plots were maintained as control whereas; other plots treated with 60:60:50 NPK (TS2). Unformulated experimental strain (TS3, 90 ml 1.5×10^{10} cfu ml^{-1}) cultures mixed with 5 Kg of powdered soil to broadcast over one plot area (4.0 × 3.5 m). For talcum powder based (TS4) and vermiculite based (TS5) formulations, 500 g of formulated product mixed with 4.5 kg of powdered soil to broadcast over one plot area. Treatment details were listed in Table 1. All experimental plots were irrigated, as per requirement to maintain the moisture level at 15%. In each case, broadcasting was done 1 h before sunset (Ghidiu and Zehender, 1993). After preparation of the field, surface sterilized seeds of sesame (*Sesamum indicum* var. Kanak) were sowed. Row to row distance was maintained at 30 cm whereas plant to plant distance was maintained 20 cm.

Inoculant availability assessment

After treatment, 1 g of soil of each treatment from day 10 and of intervals were suspended in 9.9 ml extraction buffer in tubes, containing 0.1% (w/v) tetra-sodium pyrophosphate and Tween 80 as an aid for proper cell dispersal. The tube containing sample was vortexed for 30 sec and placed inclined in an orbital shaker for 1 h at 10 rpm. The serially diluted sample was plated onto caprylate thallous agar (CTA) medium (O'Callaghan et al., 2002) supplemented with antibiotic Ampicillin (A) and Gentamicin (G) to measure the viable *S. entomophila* AB2 population.

Product self life assessment

For enumeration of viable inoculants from packed formulations same procedure was followed, at intervals from day 10. The serially diluted sample was plated onto caprylate thallous agar (CTA) medium (O'Callaghan et al., 2002) supplemented with antibiotic Ampicillin (A) and Gentamicin (G) to measure the viable AB2 population.

Pest control assessment

Experiments were carried out in open fields and therefore infested by different pest naturally. Only larvae of lepidopteron pests, particularly *H. armigera*, *Spodoptera litura* and *P. xylostella* were enumerated. Pest scouting was done in every alternative day after starting of fruit set and was continued up to harvesting. Total number of larvae was considered. Pest scouting was done in three consecutive Ravi season along with the field trial experiments.

Productivity assessment

For productivity assessment rate of seed germination (SG), growth parameters (average measurement of branch number (BN); shoot length (SL); shoot weight (SW) per plant) and yield parameters (average pod number/plant (PN); seed number/pod (SN); seed yield/plot (SY) were measured. The plants were air dried for a period of 7 days for measuring dry weight.

Statistical analysis

Standard deviation for each treatment was determined. The experimental data were statistically analyzed using ANOVA. Duncan's multiple range test (DMRT) was used to determine group mean value when ANOVA found significance at $P < 0.05$. Pesticidal activity was evaluated, through pest scouting and mortality rate evaluation, on the basis of severity of infestations (Amer et al., 1999).

RESLTS AND DISCUSSION

Effect of formulations on inoculant availability at rhizosphere

There was a significant difference in the viable count of inoculant from soil samples of TS3 with TS4 and TS5 (Figure 1). As found at day 10, soil treated with TS3 showed maximum count of viable inoculant (2.5×10^6 cfu

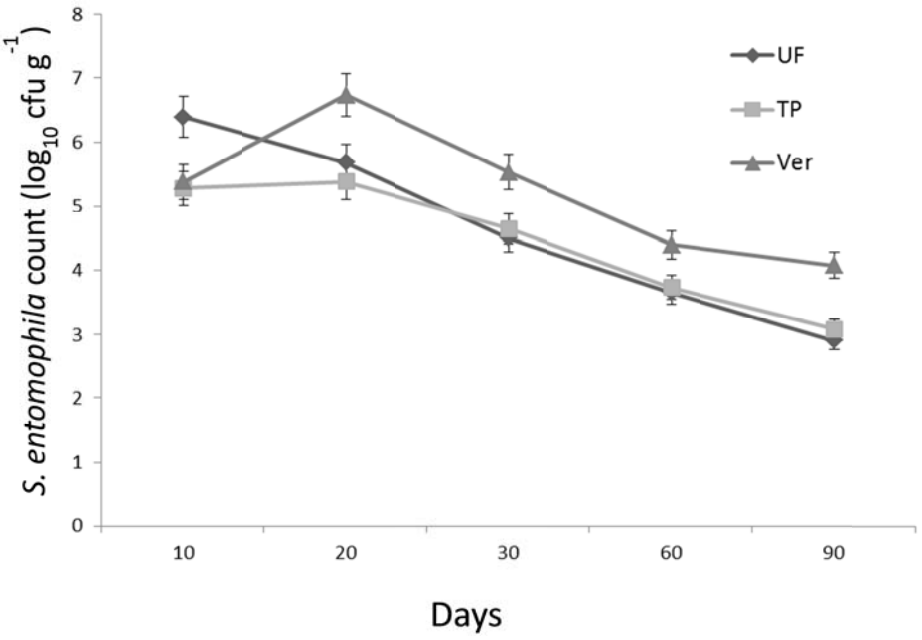

Figure 1. Efficacy of the different formulations of working isolate (UF, unformulated; TP, talcum powder based formulation; Ver, vermiculite based formulation) to release the bacterial isolates into the rhizosphere.

g^{-1}), in comparison to formulated samples TS4 (1.9×10^5 cfu g^{-1}) or TS5 (2.4×10^5 cfu g^{-1}) whereas, at day 20 the inoculant count from soil of TS3 was low (4.8×10^5 cfu g^{-1}), but higher while treated with TS4 (2.4×10^5 cfu g^{-1}) or TS5 (5.5×10^6 cfu g^{-1}). Thus, the results indicated slow release of inoculant from formulations and the vermiculite based formulation (TS5) was found to release microbial inoculant more efficiently. However, a gradual decrease of inoculant count from soil was observed thereafter.

Standardization of formulation is a challenging part and often a success limiting step in development of biocontrol products (Paau, 1998). It becomes further critical, if the active microorganism is non-spore former (O'Callaghan and Gererd, 2005). Successful release of *S. entomophila* was recorded at various soil moisture level while worked with clay based prill (O'Callaghan et al., 2002) or granule (O'Callaghan and Gererd, 2005). In the present study, *S. entomophila* AB2 population in rizosphere declined slowly with formulated samples in comparison to unformulated one. Vermiculite based formulation indicated more sustenance of inoculants.

Effect of formulations on inoculant self life

To determine the shelf life of the formulations, viable inoculant count of stored TS4 and TS5 products were estimated at monthly interval up to six months (Figure 2). Both the formulations had an initial bacterial loading of 3.5×10^8 cfu g^{-1}. On 30^{th} day, it decreased to 3.1×10^8 cfu g^{-1}. But, at 90 days of storage, inoculant load dropped by 5-fold and 10-fold in vermiculite and talcum based formulations respectively. The declining trend was observed thereafter up to the study period (6 month). It was evident that vermiculite based formulation of *S. entomophila* AB2 showed a better self life (3.6×10^6 cfu g^{-1}) than that of talcum based formulation (2.4×10^4 cfu g^{-1}) at 180^{th} day.

While soil was inoculated with unformulated *S. entomophila* 626, it was found that the rate of population decline increased with soil temperature though populations remained above the minimum level of detection after three months and soil moisture had little effect on survival (O'Callaghan et al., 2002). A biopesticide, containing a high density culture of the *S. entomophila* (Invade™), has been developed for control of grass grub in New Zealand (Jackson et al., 1992). But the liquid Invade™ product required to be maintained under refrigeration to avoid cell death during storage (Jackson et al., 1992). To overcome this problem Johnson et al. (2001) developed a system for stabilizing the bacterium in a biopolymer matrix, which can then be incorporated into clay-based granules. Later on, *S. entomophila* has been incurporated into prill formulations to improve distribution and application to pasture (O'Callaghan et al., 2002). Measurement of release of *S. entomophila* from prills in soils subjected to various watering regimes demonstrated that free soil water is important for distributing bacterial

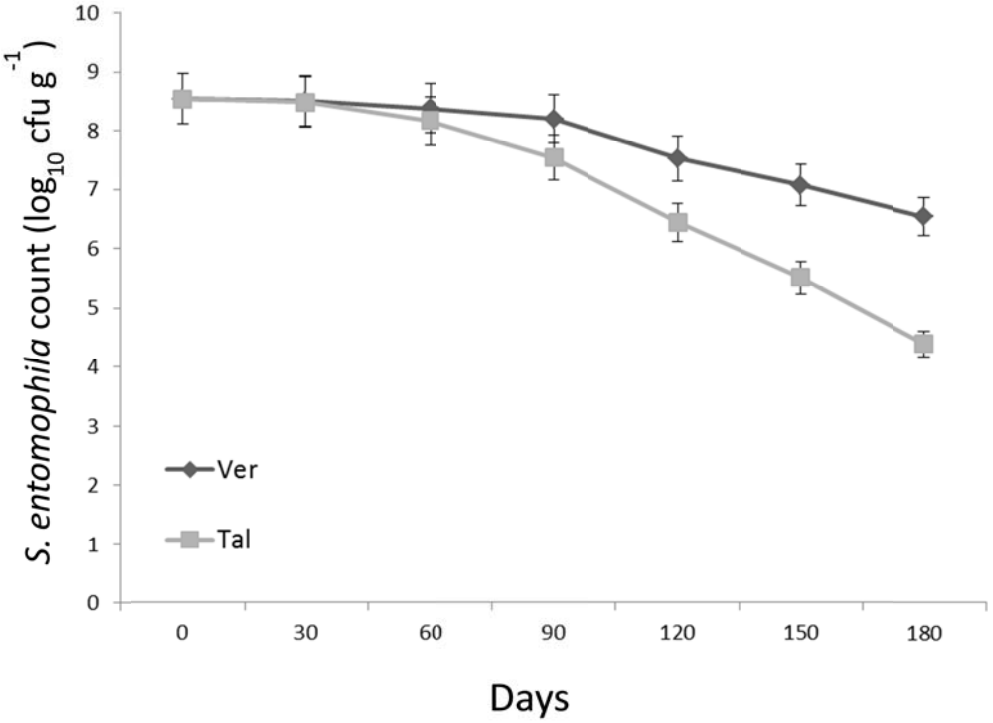

Figure 2. Self life of the two different formulations of working isolate (TP, talcum powder based formulation; Ver, vermiculite based formulation).

inoculum throughout soil profile. Another granular formulation of *S. entomophila* (Bioshield™) was developed by Townsend et al. (2004). In the present study vermiculite based formulation of the working strain *S. entomophila* AB2 ensured its significant viability up to experimental period of 6 months.

Effect on pest control

Highest pest attack was evident in 100% NPK (60:60:50) (TS2) which was found 115% more in comparison to control (TS1). A significant decrease in pest scouting (119%) was observed in plots treated with unformulated strain (TS3) in comparison to control (TS1). Experimental data ensured that vermiculite based formulation work more efficiently in minimizing lepidopteron pest (*H. armigera* 45%, *S. litura* 50%, *P. xylostella* 53%) than that of talcum powder based formulation (*H. armigera* 30%, *S. litura* 44%, *P. xylostella* 31%) (Figure 3).

It was reported that broadcasting of talcum based formulation of *P. flurescens* strains (Pf1 and FP7) on paddy field significantly reduced sheath blight, thereby, increasing yield (Nandakumar et al., 2001). Similarly, the present study clearly demonstrates, that even the plot treated with *S. entomophila* AB2 alone (TS3) can provide an effective measure for controlling lepidopteron pest infection.

Effect on productivity

The rate of seed germination in different soil treatments was observed (Figure 4). It was found that the rate was much low in TS1 (73.8%), TS2 (81.8%) and TS3 (83.8%) than formulations TS4 and TS5 showing almost 100% germination (97.4%). The profound effect of plant growth was recorded in terms of BN, SL and SW upon treatment with *S. entomophila* AB2 formulation (Figure 4). Particularly, the vermiculite based formulation (TS5) showed maximum effect and the increment was recorded in SL (155.54%), SW (218.87%) over the control (TS1). From the experimental data, it was also evident that productivity was more with vermiculite based formulation (SW 78%, SY 138%) than that of talcum powder based formulation (SW 119%, SY 249%) except SG in comparison with control. Cumulative effect of high rate of seed germination, reduced rate of pest attack resulted to 4.8 time enhancement of yield in sesame (Figure 4).

Effect of microbial consortium for seed germination is well studied (Pandey and Maheshwari, 2007; Babalola et al., 2007; Chen and Nelson, 2008; Naik and Sreenivasa, 2009). Formulations of *Pseudomonas* through application of the working isolate *S. entomophila* showed significant increase of seed germination in *Vigna mungo* (Sarma et al., 2009a). Similar trend was achieved AB2 in seed germination experiment. From earlier reports formulations of fluorescent *Pseudomonas* strain R62 and R81 were

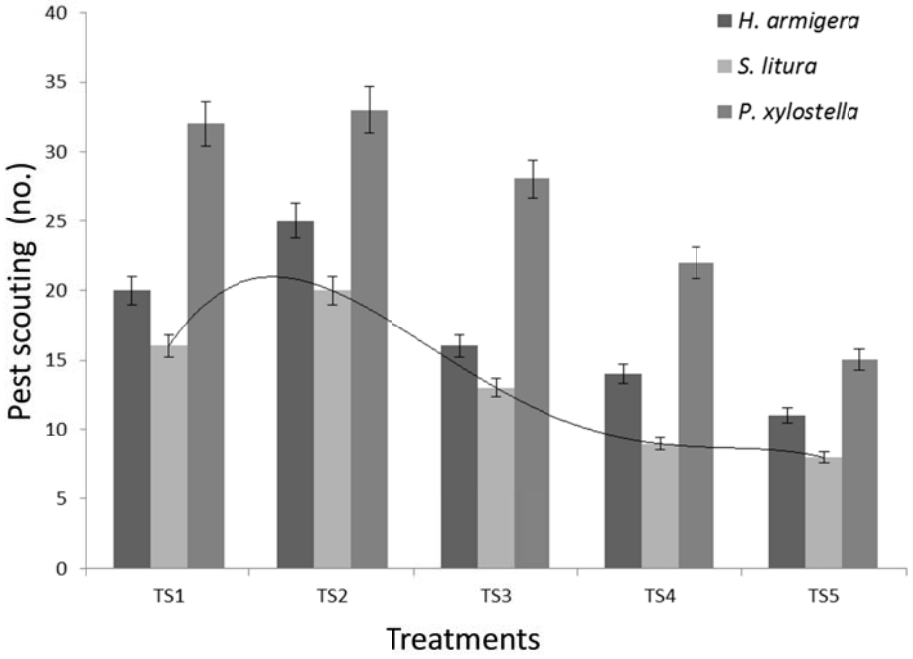

Figure 3. Effect of field treatment with control (TS1), 60:60:50 NPK (TS2), unformulated strain (TS3), talcum powder based formulation (TS4**)** and vermiculite based formulation (TS5) on providing protection against lepidopteron pest**s**.

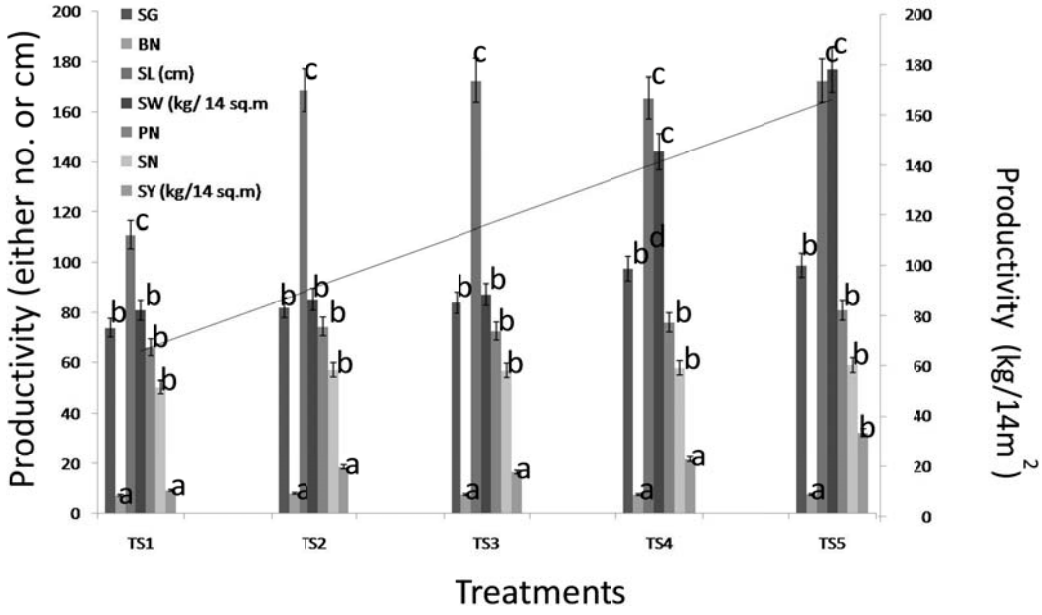

Figure 4. Effect of field treatment with control (TS1), 60:60:50 NPK (TS2), unformulated strain (TS3), talcum powder based formulation (TS4) and vermiculite based formulation (TS5) on productivity in terms of seed germination (SG), branch number (BN), shoot length (SL), shoot weight (SW), pod number (PN), seed number (SN) and seed yield (SY).

known to increase plant growth and producti-vity signi-ficantly in field condition (Sarma et al., 2009b). Since, the isolate *S. entomophila* AB2 was found to solu-bilize macro- and micro-nutrients (P and Zn)(Chattopadhyay et

al., 2011) it could be assumed that the nutrient availability was reflected in productivity.

Conclusion

The strain *S. entomophila* AB2, as a single biological agent for INM and IPDM seems to be a lucrative alternative to chemical fertilizer, pesticides and fungicides in ICM. The present study describes field trial of *S. entomophila* AB2 through inorganic carrier formulations, as soil inoculant. In addition to maintain its self life, the vermiculite based formulation can enhance field efficacy by improving establishment of microbial inoculant in soil microenvironment. Cumulative effect of high rate of seed germination, reduced rate of pest attack resulted into 4.8 time enhancement of yield in sesame. On the basis of the result of this study it can be recommended that vermiculite (80 g/100 g of product) based formulation of *S. entomophila* AB2 could be used at the rate of 3.6 qt hec^{-1} for quality and yield improvement of sesame. The information presented here may otherwise be useful for rice, pulse and cotton crops, where lepidopteron pest like *S. litura* (cutworm), *H. armigera* (bollworm) and *P. xylostella* (diamond back moth) outbreaks are common.

Conflict of Interests

The author(s) have declared no conflict of interests.

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African Journal of Biotechnology

Full Length Research Paper

Crude oil degrading potential of *Pennisetum glaucum* (L.) R. Br

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Pollution by crude oil and its products is one of the most prevalent environmental problems that cause greenhouse effects and global warming. The crude oil-degrading potentials of *Pennisetum glaucum* was investigated using 0.2, 0.9, 5.0 and 6.0% v/w concentrations of crude oil, which were employed to pollute soil planted with the seeds of the plant. These treatments were repeated in soil without seeds and the control had no crude oil pollution. Total petroleum hydrocarbons (TPH) were determined for all soil samples using gas-liquid chromatography. Microbial count was carried out on soil rhizosphere using standard methods. The results show that percentage TPH degraded in soil planted with *P. glaucum* was 100, 99.53, 99.44 and 99.47 for 0.2, 0.9, 5.0 and 6.0% v/w concentrations, respectively. *P. glaucum* alone degraded 0.56, -0.29, 0.39 and 0.31% for the same treatments. The total viable count of microorganisms from the polluted, vegetated soil samples was significantly ($P < 0.05$) higher than that of the unvegetated ones. *P. glaucum* might have enhanced the biodegradation of crude oil by stimulating the proliferation of microorganisms in the soil and hence may be used for phytoremediation of crude oil polluted soils.

Key words: *Pennisetum glaucum*, crude oil, total petroleum hydrocarbons (TPH), microorganisms.

INTRODUCTION

The environmental impact of crude oil spillage has become a global problem since it produces greenhouse gases such as carbon dioxide, methane, oxides of nitrogen and sulphur, particulate matter and other substances which contribute to global warming. Carbon dioxide concentrations have increased by 40% since pre-industrial times, primarily from fossil fuel emissions and secondarily from net land use change emissions. The ocean has absorbed about 30% of the emitted anthropogenic CO_2, causing ocean acidification (IPCC, 2013).

Oil spills have caused destruction to plants, animals, arable and uncultivated lands as well as the entire ecosystem. Total petroleum hydrocarbons (TPHs) are some of the most common groups of persistent organic contaminants found in crude oil (Huang et al., 2005).

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People who live in oil producing areas are exposed to polluted food and water. Crude oil polluted soil may remain unsuitable for plant growth for years. Natural restoration of polluted land takes time and as such several methods such as bioremediation and phytoremediation have been evolved to increase the rate of hydrocarbon degradation in polluted sites.

Bioremediation is the use of microorganisms to degrade or transform toxic contaminants into non toxic substances, while phytoremediation is the use of higher terrestrial plants for the same degradation or transformation. These methods are economically viable, environmentally friendly, non-invasive and deliver intact, biologically active soil (Wenzel, 2009).

The phytodegradation of organic compounds can take place inside the plant or within the rhizosphere of the plant. Many different compounds can be removed from the environ-ment by this method, including solvents in ground water, petroleum and aromatic compounds in soils and volatile compounds in the air (Newman and Reynolds, 2004). Removal of petroleum hydrocarbons from soil in phytoremediation is often attributed to the microorganisms living in the rhizosphere under the influence of plant roots (Luepromchai et al*.,* 2007). The stimulation of microbial activity brought about by the interaction between microorganisms and root exudates is known as rhizosphere effect. Root exudates mediate interaction between plants and microbes. Plants with extensive rooting system explore large volumes of soil, support larger bacterial population in the rhizosphere and produce exudates which can directly affect the activity of the rhizobacterial population.

Millet (*P. glaucum* (L.) R. Br. (Clayton and Renvoize, 1982) belongs to the Poaceae family and is native to tropical and warm temperate regions of the world. It is an annual grass with an extensive fibrous root system. Among the four grasses selected to rehabilitate the degraded ecosystem of an oil shale mined land of Maoming Petro - chemical company, China, *P. glaucum* × *P. purpureum* had the lowest survival rate of 62%, while *Vetiveria zizanoides* had the highest survival rate of up to 99% (Xia, 2004).

Wuana et al. (2013) reported that in a cadmium/lead contaminated soil, growth rates of *P. glaucum* were sigmoid, with growth rates appearing to decelerate with dose of cadmium and lead. They also added that soil to millet transfer factors showed that cadmium was more phytoavailable to millet than lead. The fibrous root structure of grasses is known to possess an extensive widely branched root system that provides a larger surface area for colonization by microorganisms than the tap root system (Diab, 2008).

Microorganisms have been reported to play major roles in bioremediation of crude oil contaminated soils (Rahman et al*.*, 2002; Isikhuemhen et al., 2003; Chikere et al., 2009; Fariba et al., 2010; Nwadinigwe and Onyeidu, 2012). Plant roots secret compounds that modulate underground microbial diversity (Baderi and Vivanco, 2009). The continued presence of plant roots and their exudates may be required for the degradation of hydrocarbons in crude oil polluted soil. Phytoremediation is important to oil producing nations where oil spillage is rampant and devastates the environment. Not much work has been carried out on hydrocarbon degrading potentials of *P. glaucum*. The objectives of this study therefore were, to investigate the role of *P. glaucum* in the degradation of crude oil in polluted soil, to determine the quantity of total petroleum hydrocarbon (TPH) degraded and to determine the microbial count of microorganisms in the soil rhizosphere of *P. glaucum* polluted with crude oil. The knowledge gained from this work may help affected nations and environmentalists in combating the menace of crude oil pollution, reduction of greenhouse emissions and in restoring the fertility of crude oil polluted land.

MATERIALS AND METHODS

Perforated black polythene bags (volume, 39.745 L) were filled, each with 16 kg of top soil collected at a depth of 10 cm, from the Botanic Garden, University of Nigeria, Nsukka. The set up was divided into parts A and B. Part A had no seed while part B had a seed planted in each bag. To simulate spillage, eight soil bags were polluted with 30 ml (0.2% v/w) of crude oil, 42 days after planting. The same was repeated with 150 ml (0.9% v/w), 750 ml (5.0% v/w) and 1000 ml (6.0% v/w) of crude oil separately, instead of 30 ml. Both parts A and B were polluted in the same manner. The control had no crude oil. The crude oil was obtained from Shell Petroleum Development Company, Oporoma, Bayelsa State, Nigeria. The experiment was completely randomized and carried out in three replicates. The bags were kept under the sun and were watered by rain fall since the experiment was carried out during the rainy season.

Determination of total petroleum hydrocarbons (TPH)

The unused crude oil was analyzed by gas-liquid chromatography (GLC) to determine the total petroleum hydrocarbon (TPH) composition. All the soil samples, vegetated and unvegetated, were collected 60 days after pollution (DAP) and also subjected to GLC to determine the TPH. The method used was the modified method of Shirdam et al. (2009). The soil samples were air dried at 25°C (room temperature) for 72 h. Two grams of the sample were weighed; 20 ml of hexane were added to weighed sample, stirred and left for 30 min. Approximately 1 cm of glass wool was passed into the column. Two grams of activated silica gel were heated in the oven at 130°C for 9 h and passed into the column to settle on the glass wool. Activated sodium sulphate (0.5 g) was added, 10 ml of dichloromethane (DCM) were poured into the column and the tap was opened to allow the DCM to run through. The sample was poured and immediately 10 ml of hexane were poured and allowed to run. The eluate was collected in a clean sampling bottle and labeled. In order to run in an Agilent GLC, the eluate was concentrated to 1 ml, poured into a GLC vial bottle and placed into the GLC to run. The GLC was equipped with a flame ionization detector (FID). For the unused crude oil, 2 ml were poured into a

Table 1. Total petroleum hydrocarbon distribution (ppm) in soil, unvegetated and vegetated with *Pennisetum glaucum,* polluted with different concentrations of crude oil.

Straight chain group	Conc. of hydrocarbon in unused crude oil	Polluted, vegetated soil samples (% v/w)				Polluted, unvegetated soil samples (% v/w)			
		0.2	0.9	5.0	6.0	0.2	0.9	5.0	6.0
C_8	341.79	-	-	-	-	-	-	-	-
C_9	1070.02	-	-	-	-	-	-	-	-
C_{10}	1392.12	-	-	-	-	-	-	-	-
C_{11}	1949.83	-	-	-	-	-	-	-	-
C_{12}	2124.50	-	-	-	-	-	12.03	-	-
C_{13}	1991.34	-	-	13.15	-	-	-	-	-
C_{14}	2247.70	-	-	25.68	-	-	36.70	-	-
C_{15}	2542.92	-	-	33.70	-	-	19.08	14.03	13.36
C_{16}	2607.72	-	-	32.92	-	-	21.44	49.82	15.48
C_{17}	2832.61	-	-	31.51	27.40	-	17.48	158.70	40.77
C_{18}	3804.47	-	-	27.49	19.84	-	-	92.13	52.71
C_{19}	4245.75	-	-	32.86	26.80	-	-	60.50	99.99
C_{20}	3549.56	-	-	35.54	-	-	-	23.75	80.21
C_{21}	5985.27	-	-	-	-	-	-	-	-
C_{22}	5549.01	-	125.76	-	30.82	31.72	-	33.33	94.36
C_{23}	4221.22	-	61.58	-	28.29	25.68	-	30.67	28.07
C_{24}	4833.37	-	30.42	48.61	45.74	107.88	-	25.90	77.43
C_{25}	5523.29	-	61.70	63.11	123.27	172.44	-	78.56	-
C_{26}	1675.04	-	-	-	16.30	-	-	-	-
C_{27}	1064.24	-	-	-	-	-	-	-	-
C_{28}	228.85	-	-	-	-	-	-	-	-
C_{29}	58.68	-	-	-	-	-	-	-	-
C_{30}	40.15	-	-	-	-	-	-	-	-
C_{31}	110.76	-	-	-	-	-	-	-	-
C_{32}	93.35	-	-	-	-	-	-	-	-
Total TPH	60083.56	0.00	279.46	344.57	318.46	337.72	106.73	567.38	502.38

-, Means absence of hydrocarbons

separating funnel. Twenty-five milliliter of hexane were added to the sample for the extraction and the eluate was collected in a sampling bottle. The oil was poured back to the funnel and 25 ml hexane was added. The process was repeated and the eluate passed through 50 g of Na_2S0_4 to remove water and concentrated to 1 ml. One micro liter of the concentrate was injected into the GLC and the retention time was compared with those of the standard total petroleum hydrocarbon concentrations. The injector temperature was 280°C while that of FID detector was 340°C. The column used for analysis was DB-5 with 30 m length and 0.25 mm internal diameter. The initial column temperature was kept at 50°C for 5 min, increased to 250°C with 10°C min^{-1} slope and kept at 250°C for 40 min.

Determination of percentage TPH degraded

The total TPH obtained under each column (Table 1) is the sum of the remaining hydrocarbons after degradation, under the column. The total TPH under the unused crude oil is the standard and is regarded as 100%. The TPH degraded for each treatment is obtained by subtracting the

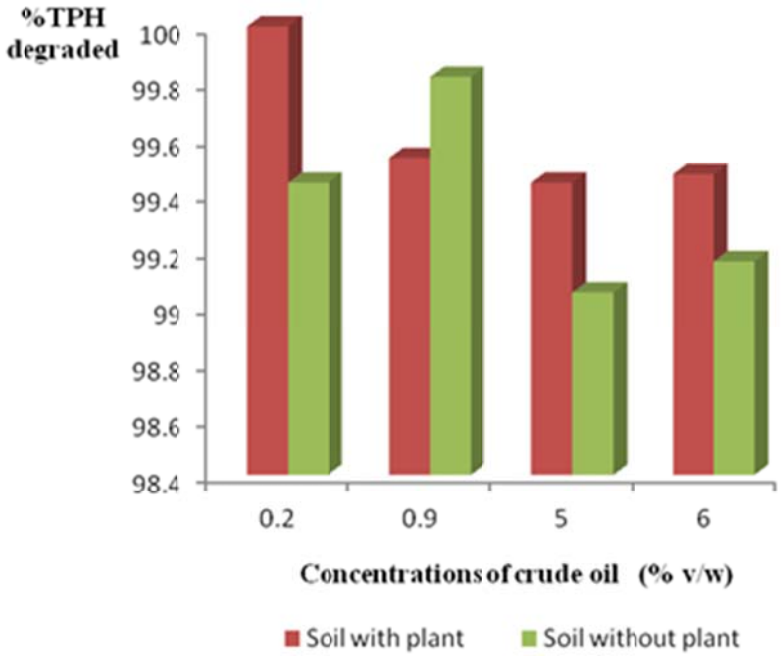

Figure 1. Percentage Total Petroleum Hydrocarbon degraded in the soil with and without *Pennisetum glaucum,* polluted with different concentrations of crude oil (% v/w).

remaining TPH under the treatment, from the standard. Percentage TPH degraded under each column is obtained by dividing the total TPH degraded under each treatment by the total TPH in the standard and multiplying by 100. Since vegetated soil contained both *P. glaucum* and microorganisms while unvegetated soil contained only microorganisms, it means that any degradation in vegetated soil was carried out by both the plant and microorganisms and any degradation in unvegetated soil is carried out by microorganisms. Therefore, the percentage TPH degraded by the plant alone is obtained by subtracting the percentage TPH degraded in unvegetated soil from the percentage TPH degraded in vegetated soil (This subtraction resulted in -0.29% found in the result, for 0.9% crude oil treatment).

Microbial count of rhizosphere

The total viable count (TVC) of microorganisms in the rhizosphere was carried out 60 days after pollution, in the Department of Pharmaceutical Microbiology, University of Nigeria, Nsukka, according to Henrik (1994). Sterilization was carried out by autoclaving at 121°C for 15 min. Each soil sample was serially diluted from 10^{-1} to 10^{-5}. For total viable count, 28 g of nutrient agar medium, which consisted of meat extract (1.0 g/L), yeast extract (2.0 g/L), peptone (5.0 g/L), sodium chloride (5.0 g/L) and agar (15.0 g/L), was dissolved in 1 L of distilled water. The inoculation was carried out by the pour plate method. One ml of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} of the sample was pipetted into sterile Petri-dishes, separately. After allowing the autoclaved media to cool to 45°C, 10 ml of molten agar medium was added to the plate containing the inoculums and was homogenized to ensure complete dispersal of the sample. The plates were allowed to set before incubating them in an oven at 37°C for 24 h. The results of the TVC were subjected to Analysis of Variance (ANOVA) and means were compared using Duncan's multiple range tests at $P < 0.05$ (Edafiogho, 2006).

RESULTS

Total petroleum hydrocarbons

The original unused crude oil sample which is the standard, showed the presence of high concentrations of straight chain hydrocarbons of C^8 - C^{32} (Table 1). Some other hydrocarbons like C^1 - C^7 and C^{33} - C^{40} were neither detected in the standard nor in any of the polluted soil samples, by the GLC. No straight chain groups, C^8 - C^{11}, C^{21} and C^{27} - C^{32} were detected in all the polluted soil samples (vegetated and unvegetated), even though they were found in the standard. No hydrocarbon was detected in the control and in the vegetated soil polluted with 0.2% v/w crude oil. Small quantities of hydrocarbons were detected in the vegetated and unvegetated soil when compared with the quantities detected in the standard. Percentage TPH degraded in vegetated soil, polluted with 0.2, 0.9, 5.0 and 6.0% v/w crude oil treatments were 100% (that is, all the hydrocarbons were degraded), 99.53, 99.44 and 99.47%, respectively, while the percentage TPH degraded in unvegetated soil polluted with the same quantities of crude oil were 99.44, 99.82, 99.05 and 99.16%, respectively (Figure 1). Therefore, *P. glaucum* alone degraded 0.56, -0.29, 0.39, and 0.31% for the same treatments, respectively (Percentage TPH degraded by the plant alone is obtained by subtracting the percentage TPH degraded in unvegetated soil from the % TPH degraded in vegetated soil).

Table 2. Microbial count (cfu /g) of soil polluted with different concentrations (%v/w) of crude oil, with or without *Pennisetum glaucum.*

Concentration of crude oil (% v/w)	Total viable count (cfu/g)
Vegetated, polluted soil samples	
Control (vegetated soil, without pollution)	$6.97 \times 10^6 \pm 11547^a$
0.2	$9.13 \times 10^7 \pm 88191^b$
0.9	$1.87 \times 10^7 \pm 81297^c$
5.0	$6.10 \times 10^7 \pm 57735^d$
6.0	$1.26 \times 10^7 \pm 81936^e$
Unvegetated, polluted soil samples	
Control (unvegetated soil, without pollution)	$5.60 \times 10^6 \pm 31797^f$
0.2	$8.82 \times 10^6 \pm 42702^g$
0.9	$3.71 \times 10^6 \pm 63333^h$
5.0	$1.44 \times 10^6 \pm 29059^i$
6.0	$8.54 \times 10^6 \pm 81742^g$

Values represent means ± standard error. Mean values with different letters in the column are significantly different at $p<0.05$.

Microbial count

The results of the total viable count (TVC) of microorganisms showed that the vegetated, polluted soil samples recorded a significantly ($P < 0.05$) higher TVC when compared with unvegetated, polluted soil samples (Table 2). For vegetated, polluted soil, the highest (significant at $P < 0.05$) TVC was recorded for 0.2% v/w crude oil treatment, followed by that of 5.0% v/w treatment, while the lowest was that of the control. For the unvegetated, polluted soil, the highest TVC was recorded for 0.2 and 6.0% v/w, followed by that of the control, while the lowest was that of 5.0% v/w treatment (significant at $P < 0.05$).

DISCUSSION

Some straight chain groups of hydrocarbons like C^1 - C^7 were volatile and so could not be detected by GLC both for the unused crude oil sample and the polluted soils. No hydrocarbons were detected in the vegetated soil polluted with 0.2% v/w crude oil, probably because all the hydrocarbons were phytodegraded by a combination of *P. glaucum* and microorganisms. The 0.2% crude oil spill was perhaps too small for the numerous microorganisms that had enough exudates from the plant. Hence, all the hydrocarbons (100%) were completely degraded. Since many hydrocarbons were not detected (examples, C^8 - C^{11}, C^{21} and C^{27} - C^{32}) or detected in smaller quantities in all the polluted soil samples, when compared with the unused crude oil sample, it showed that phytodegradation took place in the work. Hence, in the present experiment, the percentage TPH degraded in all the polluted soil samples, with or without *P. glaucum* ranged from 99.05 to 100%. Generally, more degradation of hydrocarbons took place in the presence of *P. glaucum* than in its absence, except for 5.0% polluted, unvegetated soil, where there was more phytodegradation for C^{15}, C^{20}, C^{24} as well as for C^{22}, C^{23}, C^{24}, C^{25} (for 0.9% v/w pollution) and for C^{26} (for 6.0% pollution). The reason for this unexpected behavior is not clear, although it may have to do with the concentration of the crude oil spilled and the type and quantity of microorganisms involved in the phytodegradation. In any case, degradation in the absence of the plant was high (99.05 to 99.82%). Therefore, microorganisms in the soil must have been responsible for this degradation of crude oil in the absence of the plant, to the extent that in unvegetated soil polluted with 0.9% v/w crude oil, there was so much degradation by the microorganisms that it appeared that the plant played no significant role in the phytodegradation, hence, it gave - 0.29% for the phytodegradation by the plant alone. Perhaps 0.9% oil spill is the right quantity which the microorganisms can degrade efficiently without the supply of exudates from the plant.

These findings are similar to the work of Diab (2008) who reported that 30% reduction of total petroleum hydrocarbons (TPH) was observed in the soil rhizosphere of *Vicia faba,* as compared to 16.8 and 13.7% reduction in *Zea mays* and *Triticum aestivum*, respectively. Dominiguez-Rosado and Pichtel (2004) found that the used motor oil (1.5% w/w) they employed to contaminate soil seeded with mixed clover was completely degraded after 150 days. They further reported that 67% of the oil

was removed with a mixture of sunflower/mustard, but with the addition of NPK fertilizers, the oil was completely degraded. In addition, the grass/maize treatment resulted in a 38% oil degradation, which increased to 67%, with fertilizer application. In the present work, the percentage hydrocarbons degraded by the plant alone was quite small compared with the percentage hydrocarbons degraded by a combination of the plant and its associated microorganisms. Therefore, a combination of microbial degradation (bioremediation) and phytodegradation may perhaps make phytoremediation more efficient. This agrees with the report of Wenzel (2009) who confirmed that the efficiency of phytoremediation relies on the establishment of vital plants with sufficient shoot and root biomass growth, active root proliferation and / or root activities that can support a flourishing microbial consortium assisting phytoremediation in the rhizosphere.

In the present work, the TVC of microorganisms of the vegetated, polluted soil was higher than that of the unvegetated polluted soil. The observed increase in microbial activity in vegetated soils may be attributable to root exudates and oxygen input from roots of the plant as it was observed by Escalante-Espinosa et al. (2005). This is in agreement with the work of Odokuma and Inor (2002), who reported that bioaugumentation using bacteria (*Bacillus* and *Azotobacter*) improved the growth of *Phaseolus* sp. in crude oil-polluted soil. Chikere et al. (2009) also reported that bacteria contributed during bioremediation of crude oil - polluted soils. In the present work, 0.2% polluted, vegetated soil gave the highest (100%) TPH degradation and had the highest microbial load, while the 5% polluted, unvegetated soil gave the lowest (99.05%) TPH degradation and had the lowest microbial load. Therefore, it may be assumed that the higher the microbial load, the higher the hydrocarbon degradation. Comparatively less degradation took place for 5 and 6% oil spills, perhaps because the microorganisms had to tackle with higher quantities of crude oil spills, in the absence of the plant and its exudates, in the case of unvegetated soil.

Johnson et al. (2005) and Mueller and Shann (2006) reported that microbial communities in planted soils are greater and more active, than in unplanted soils. Fariba et al. (2010) indicated that fungal strains played the main role in the degradation of petroleum polluted soils but the roots of plants enhanced the process. Plants can enhance the biodegradation of hydrocarbons by stimulating the rhizosphere microbes into greater activity (Nie et al., 2009) through the supply of oxygen (Escalante-Espinosa et al., 2005), root exudates, enzymes that are capable of transforming organic pollutants and by altering the biotic, physical and chemical conditions of the soil (Nie et al., 2009). Hence, in the present work, both the plant and microorganisms are involved directly and indirectly in the degradation of petroleum hydrocarbons into less toxic products that are less persistent in the environment than the parent compounds. Therefore, in phytoremediation, the emission of CO_2, methane, oxides of nitrogen and sulphur, aerosols, as well as particulate matter, etc., which are released into the environment in an oil spill, are mitigated, thereby helping to reduce greenhouse effect and global warming. The roots of plants loosen the soil and transport nutrients and water to the rhizosphere, thus additionally enhancing the microbial activity. In conclusion, therefore, *P. glaucum* contributed to the phytodegradation of the crude oil polluted soil. Although the actual percentage degradation of hydrocarbons contributed by the plants alone, was quite small compared with the contributions made by a combination of the plant and soil microorganisms, yet the plant phytostimulated the activities of microorganisms in their bioremediative work by means of the rhizosphere activities.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Growth response of region specific *Rhizobium* strains isolated from *Arachis hypogea* and *Vigna radiata* to different environmental variables

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Six different strains isolated from *Vigna radiata* (IARI-1 UU-2, UU-4, UU-7, UU-10 and UU-13) and seven strains from *Arachis hypogea* (IARI-16, UU-17, UU-18, UU-19, UU-20, UU-21 and UU-22) were selected to assess their capability to tolerate environmental variables like pH, temperature, salinity (NaCl), iron (Fe), phosphate (K_2HPO_4), and nitrate ($NaNO_3$). The bacteria under investigation expressed noticeable ability to grow under these stress factors examined. The rhizobial isolates were compared to two reference rhizobial strains of specific host collected from IARI, India. *Rhizobium* UU-4 from *Vigna radiata* was found to be most tolerant species to high and low temperature, high salinity, relatively higher phosphate and higher nitrate concentrations but was sensitive to lower pH. UU-2 from the same host tolerated maximum to lower temperature, alkaline pH, phosphate deficiency and to higher concentration of iron but was very sensitive to a little raise in salinity. UU-10 was tolerant to lower pH, low nitrate, relatively higher phosphate, non-saline conditions and low iron concentration in the media. UU-13 grew well only in the presence of higher phosphate and low iron and was sensitive to lower temperature, acidic pH, phosphate deficiency and to the presence of nitrate in the media. UU-1 was most sensitive to higher temperature, alkaline pH, non-saline condition, higher concentration of phosphorous and nitrate, and was tolerant to higher level of salinity. UU-22 from *A. hypogea* was most tolerant to lower as well as higher temperatures, but sensitive to salinity and higher nitrate concentration. UU-21 was very sensitive to low temperature, salinity, presence of iron and also grew only when the nitrate concentration in the media was more than 50 µg/ml, but was tolerant to alkaline pH. UU-16 grew well in salinity free conditions, tolerant to low iron and also grew well in higher nitrate (up to 200 µg/ml) and phosphate deficiency, but was very sensitive when the concentrations of iron became higher. UU-17 preferred lower pH, lower temperature, moderate salinity, higher iron, phosphate deficiency and also grew comparatively well in presence of nitrate. UU-20 though was sensitive to lower pH and phosphate deficiency, was tolerant to higher salinity and also grew well in presence of nitrate. Based on the above results, *Rhizobium* strains UU-22 from *A. hypogea* and UU-13 from *V. radiata* may be effective for nodulation as well as yield of two leguminous crops.

Key words: Rhizobia, region specific, environmental stress, *Arachis hypogea*, *Vigna radiata.*

INTRODUCTION

Intensive agriculture, which is largely based on the use of nitrogen chemical fertilizer, is the opposite of sustainable agriculture based on repositioning of nitrogen used by plant growth through supply of organic residue and succession of legume crop (Popelka et al., 2004; Acharya et al., 1953). Besides legumes are important in such agriculture practices being a chief source of protein and also produced beneficial effects for soil fertility and conservation due to biological nitrogen fixation. Inoculation of efficient strains of rhizobia is important when a legume is introduced in a region. The efficiency of the legume-rhizobia symbiosis is affected by various environmental factors (Thies et al., 1995; Palmer and Young 2000; Yuhashi et al., 2000). *Rhizobium* is a number of genetically diverse and phylogenetically heterogenous groups of bacteria. Recently, it has been reported that rhizobial cultures are also used as growth promoters for non-leguminous plants (Hossain and Mårtensson, 2008). It has been well reported that *Rhizobium* inoculants are highly sensitive to slightest change in environmental conditions, especially in respect of soil reaction due to variation in pH, moisture conditions and variation in temperature (Michiels et al., 1994; Evans et al., 1993). Thus, *Rhizobium* strains from outside a particular agro-climatic zone often fail to achieve the desired result (Azad, 2004). Therefore, it becomes necessary to isolate and screen the native *Rhizobium* strains and testing the efficacy of *Rhizobium* biofertilizer with regards to their infective capability, production of effective nodules in the host and their contribution to growth and yield attributes of the inoculated crops (Saikia et al., 2006).

Environmental stresses play an important role in level of legume production. Among stress factors, salinity, pH, temperature, iron, nitrate and phosphate are most important in regulating the natural distribution of plant, is a very serious problem in many agricultural areas. Different stress limits legume growth, especially when the crop relies on symbiotically fixed N (Velagaleti et al., 1990). The isolation and characterization of rhizobial strains tolerant to stress condition may allow the prediction of their eventual behavior as a community in soils and in this way may lead to a better interaction with the plant for its later introduction into unfavorable soils. With the purpose of isolation of *Rhizobium* strains from different agro-climatic condition, in the present work, different *Rhizobium* strains were characterized and their growths under different environmental stress like at low and high pH, temperature, salinity (NaCl), iron (Fe), phosphate (K_2HPO_4) and nitrate ($NaNO_3$) conditions were studied.

MATERIALS AND METHODS

Isolation of *Rhizobium* strains

Thirty days old selected legume plants were uprooted, washed in distilled water and the well-formed, healthy and pinkish nodules on the tap roots were carefully cut out. The nodules were immersed in 95% (v/v) ethanol for 10 s, sterilized for 5 min in 0.1% acidified mercuric chloride ($HgCl_2$, 1g L^{-1}; conc. HCl, 5 ml L^{-1}) and washed six times with sterile distilled water to get rid of the chemical (Chen and Lee, 2001). Each nodule was crushed using a sterile glass rod in an aliquot of sterile distilled water. Serial dilutions of the suspension were made and an aliquot of appropriate dilution was plated on yeast-extract mannitol agar medium (YEMA) and incubated at 28±2°C for four to seven days (Bogino et al., 2008). Distinct colonies were picked up and transferred to agar slants for further purification. Confirmation of the *Rhizobia* was ascertained by streaking on YEMA medium supplemented with Congored (0.025%, w/v), bromothymol blue test, and EPS production (Hameed et al., 2004; Sethi and Adhikary, 2009). The *Rhizobia* stand out as white and translucent colonies (Subbarao, 1977). One week old rhizobial colonies kept on YEM agar media (1.5% agar) were used for preparation as inoculants. For this purpose, loop of the respective colonies were inoculated in sterile YEM medium in liquid broth. Strains were routinely maintained on YEMA slants at 4°C (Castro et al., 1997). In addition, two strains of *Rhizobium* of the respective hosts isolated and maintained at the Microbiology laboratory of IARI (Indian Agricultural Research Institute), New Delhi were used as negative control. Growth of all the 26 native and 2 IARI strains of *Rhizobium* was estimated at 12 h intervals up to stationary growth phase and growth was measured as the absorbance of the culture suspension at 600 nm.

Selection of strains

Totally, 13 strains of *Rhizobium* were isolated from *V. radiata* and *A. hypogea* cultivated southern region of Odisha state, India and maintained in culture in YEM media. In addition, two strains of *Rhizobium* of the respective hosts isolated and maintained at the Microbiology laboratory of IARI (Indian Agricultural Research Institute), New Delhi, India were used as negative control. Based on the higher growth rate, six *Rhizobium* isolates from *A. hypogea* and five isolates from *V. radiata* were chosen and their growth pattern under various environmental variables was examined in culture. Strain number UU stands for Utkal University and IARI-Indian Agricultural Research Institute.

Growth response of *Rhizobium* species from *V. radiata* and *A. hypogea* under various environmental variables

Based on the higher growth rate, seven *Rhizobium* isolates from *A. hypogea* and six isolates from *V. radiata* were chosen and their growth pattern under various environmental variables was examined in triplicate in culture. These were: pH of the medium ranging from 5-10, at different temperatures (4, 25, 28, 30, 35 and 45°C), salinity ranging from 0 to 1 M and in presence and absence of various concentration of nitrate ($NaNO_3$), phosphate (K_2HPO_4) and iron (Fe- citrate). Growth response of the selected *Rhizobium* isolates at various pH levels (from 5 to 10), temperature gradients (4-45°C), salinity (0 to 1 M), nitrate (0 to 1 mg/ml), phosphate

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(0 to 20 mg/ml) and iron (0 to 300 µg/ml) was examined. The different concentrations of the treatments were prepared in YEMA medium and the organisms were grown by inoculating uniform amount of culture suspension into the experimental tubes. Corning hard glass test tubes of the size (18 x 200 mm) plugged with non-absorbent cotton wool was used and totally 10 ml of suspension including the inoculums culture were incubated in an incubator for up to 72 h. Triplicates were set for each set of experiments and mean of 3 closely concordant determinations were calculated and presented in text.

RESULTS

Growth pattern of six *Rhizobium* strains from *Vigna radiata* subjected to various temperature for example, 4, 25, 28, 30, 35 and 45°C, pH (5, 6, 7, 7.8, 9 and 10), NaCl (ranging from nil to 1 M), sodium nitrate (nil to 1 mg/ml), phosphate (nil to 20 mg/ml) and iron as citrate (nil to 300 µg/ml) was examined. Unless otherwise stated, the cultures were maintained at 28°C and pH 7.8 throughout the growth period (Figure 1A to F). Maximum growth of all the strains was obtained at 30°C with little change in temperature range of 25 to 35°C. Growth of IARI-1, UU-2 and UU-7 were considerably affected at 45°C in comparison to other strains, however, UU-4, UU-10 and UU-13 showed almost similar growth at the temperature range of 25 to 30°C with little less at lower temperature (Figure 1). Similarly, all these isolates grew well at pH 7.8 and increase or decrease of pH of the culture to acidic or alkaline range showed detrimental effect on the growth of these isolates; although at 4 and 45°C, and pH 5 and 10, respectively of the culture did not support good growth of the *Rhizobium* isolates. UU-2, UU-7 and UU-13 were quite tolerant to pH from 7 to 9 (Figure 1). All these isolates grew well in presence of 0.025 M NaCl (control). Upon increase of the NaCl concentration up to 0.1 M in the media except for IARI-1 and UU-10, the growth of all other strains decreased in presence of higher concentration of NaCl. Growth of all the isolates though were affected in absence of NaCl, none of them could tolerate up to 1 M NaCl, and in many, for example, UU-10 and UU-13, even growth was drastically reduced in presence of 0.5 M NaCl (Figure 1). Growth of all the isolates except IARI-1 and UU-4 was progressively enhanced in the presence of up to 0.05 mg/ml of $NaNO_3$ in the medium except in the case of UU-10; where, more than 0.02 mg/ml of nitrate did not support higher growth.

To the contrary in UU-2 and UU-7, highest growth was obtained even in presence of 0.05 mg/ml of nitrate. Further increase in the growth of all these strains was decreased and growth was static in UU-7 and UU-10 in the presence of 1 mg/ml of nitrate (Figure 1). When phosphate was not supplemented in media, growth of almost all the strains was decreased up to 45%. Similarly, with increase of the phosphate concentration in the media, growth was affected than that of control culture and the adverse effect was proportionate with increase of phosphate up to 10 mg/ml. With further increase, growth of all the isolates were severely affected (Figure 1).

Since Odisha soil is rich in iron, tolerance of the *Rhizobium* isolates from *V. radiata* to increase in iron concentration is of immense importance. The results show that UU-2, UU-4 and UU-7 tolerated and grew higher in presence of up to 10 µg/ml of iron citrate. With further increase in iron concentration, growth of all strains was adversely affected. The adverse effect of iron was comparatively less pronounced in UU-10, UU-2, UU-4 and UU-7 up to 200 µg of iron/ml. However, with further increase of iron up to 300 µg/ml, the growth of all the strains were decreased up to 80 to 90% (Figure 1).

Quite different from the growth response of *Rhizobium* from *V. radiata*, the rhizobia from *A. hypogea* showed less tolerance to change in the environmental stresses as above, but grew well in presence of higher concentrations of the phosphate in the medium. Though optimum growth of these rhizobia from *A. hypogea* was seen at 28°C with little higher temperature to 30°C, growth of UU-17, UU-18, UU-19, UU-21 and UU-22 were adversely affected by 6 to 14% and less. With further increase to 35°C, UU-16 was more sensitive and decreased the growth by 40% and all the other six strains growth decreased from 21 to 33%. Almost similar decrease in growth from 12-37% was seen at 25°C. With further decrease of temperature to 4°C or increase up to 45°C, the growth of all these strains decreased from about 56 to 68% (Figure 2). Similarly, with the increase in pH of the culture from 7.8 to 8, growth of all the IARI-16, UU-18, UU-20 and UU-22 decreased from 4 to 6%, and the decrease was more pronounced with further increase of pH up to 10 and also with decrease of the pH in the order of 7, 6 and 5 proportionately; at pH 5, growth of all these strains was inhibited by 47 to 81% and at pH 10, decrease of the growth was in the range of 30 to 53%. The results show that all the rhizobia from *A. hypogea* were more tolerant to alkaline pH than acidic conditions (Figure 2).

All the seven rhizobial strains from *A. hypogea* were sensitive to slight change in the NaCl concentration of the medium. In the absence of NaCl, growth was inhibited by 28 to 47%. With increase of the NaCl concentration in the medium, growth of all the strains was progressively decreased with proportionate increase in concentration of the salt, and at 1 M, growth of all the organisms was inhibited by 64 to 81% (Figure 2) showing that unlike rhizobia from *V. radiata,* rhizobia from *A. hypogea* were unable to tolerate in the saline conditions of the soils. The organisms grew well in media in the absence of nitrate. Growth of IARI-16 and UU-22 remained unchanged in presence of up to 0.02 mg/ml of nitrate, however, in all other strains, growth was decreased by 6 to 8% in presence of the low concentration of nitrate (Figure 2C). With the increase of NaCl in a medium from 0.5 up to 1 mg/ml, growth of all these strains decreased proportionately to the increase of the nitrate concentration and at 1 mg/ml in the media; growth of these strains was decreased from 66 to 82%. All the *Rhizobium* strains from *A. hypogea* were slightly sensitive to increase in

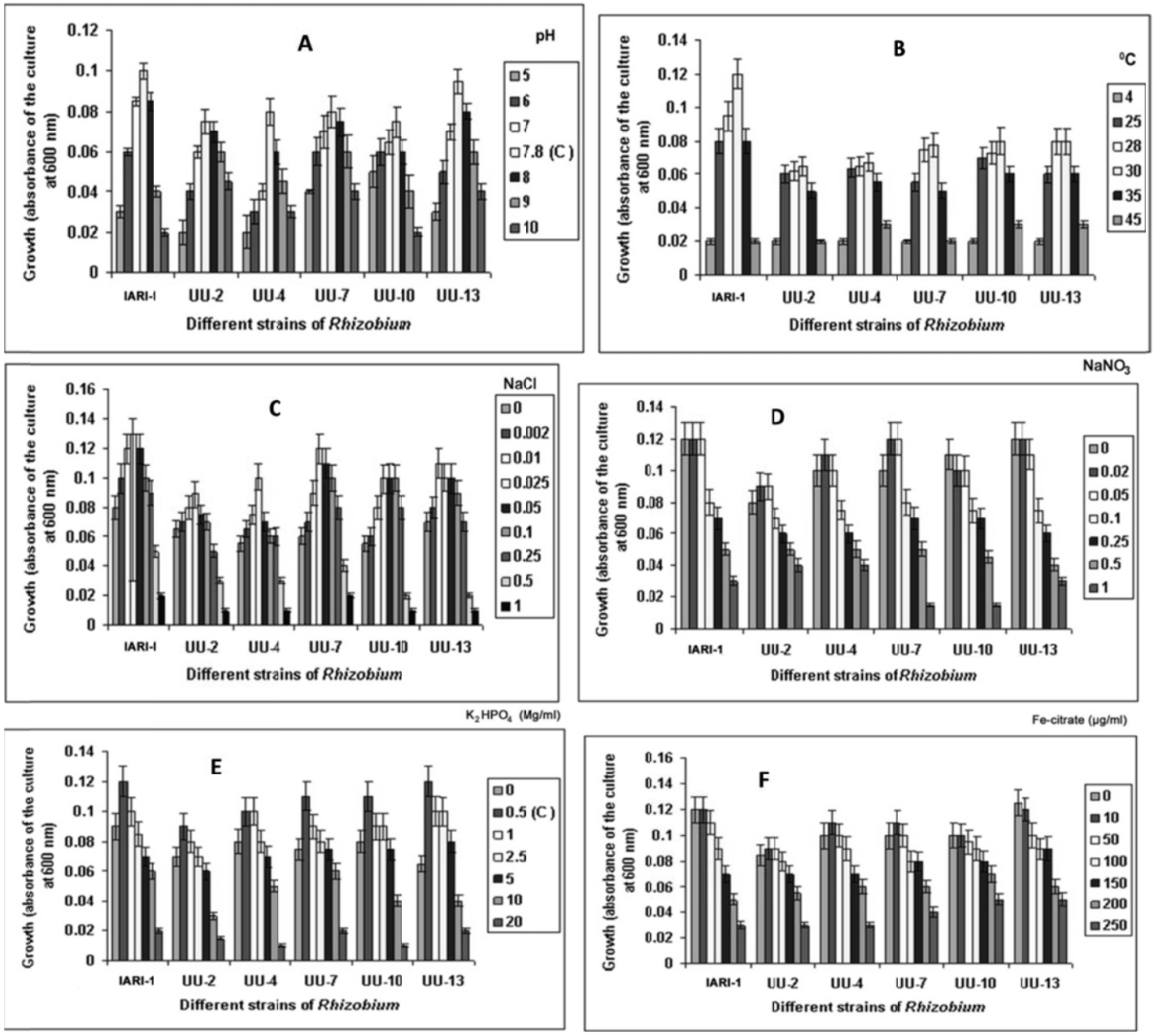

Figure 1. A - F. Growth of different strains of *Rhizobium* isolated from *Vigna radiata* from different temperature (°C), pH, salinity (NaCl), nitrate ($NaNO_3$), iron (Fe-citrate) and phosphate (K_2PO_4). Cultures were incubated for 120 h at 28±2°C. Initial inoculum of the culture suspension at 600 nm was 0.02. IARI-1, IARI culture strain Delhi; UU-2, Gobindapur; UU-4, Maniakati-2; UU-7, Paduraisuni; UU-10, Lathipada; UU-13, Asurabandha.

concentration of iron at 250 µg/ml of iron citrate. Growth of UU-19, UU-20, UU-21 and UU-22 was completely ceased at this concentration though IARI-16 and UU-17 growth was inhibited from 51-66% at the same iron concentration.

However, at low concentration of iron (10 µg/ml), growth of IARI-16, UU-21 and UU-22 almost remained static. In all other strains especially in UU-18, growth was inhibited by 21% even in presence of 10 µg/ml of iron citrate (Figure 2E). With increase in concentration of iron, growth of all the strains decreased in presence of 150-200 µg/ml iron, which is the usual iron concentration of most red soils of Odisha (Sahu et al., 1996). Growth of all these strains was inhibited by 33-53% suggesting that iron rich soils of the region are not conducive for the *Rhizobium* of *Arachis hypogea*. It was important to find that in phosphorous deficient media, growth of all these strains of rhizobia from *Arachis hypogea* was decreased

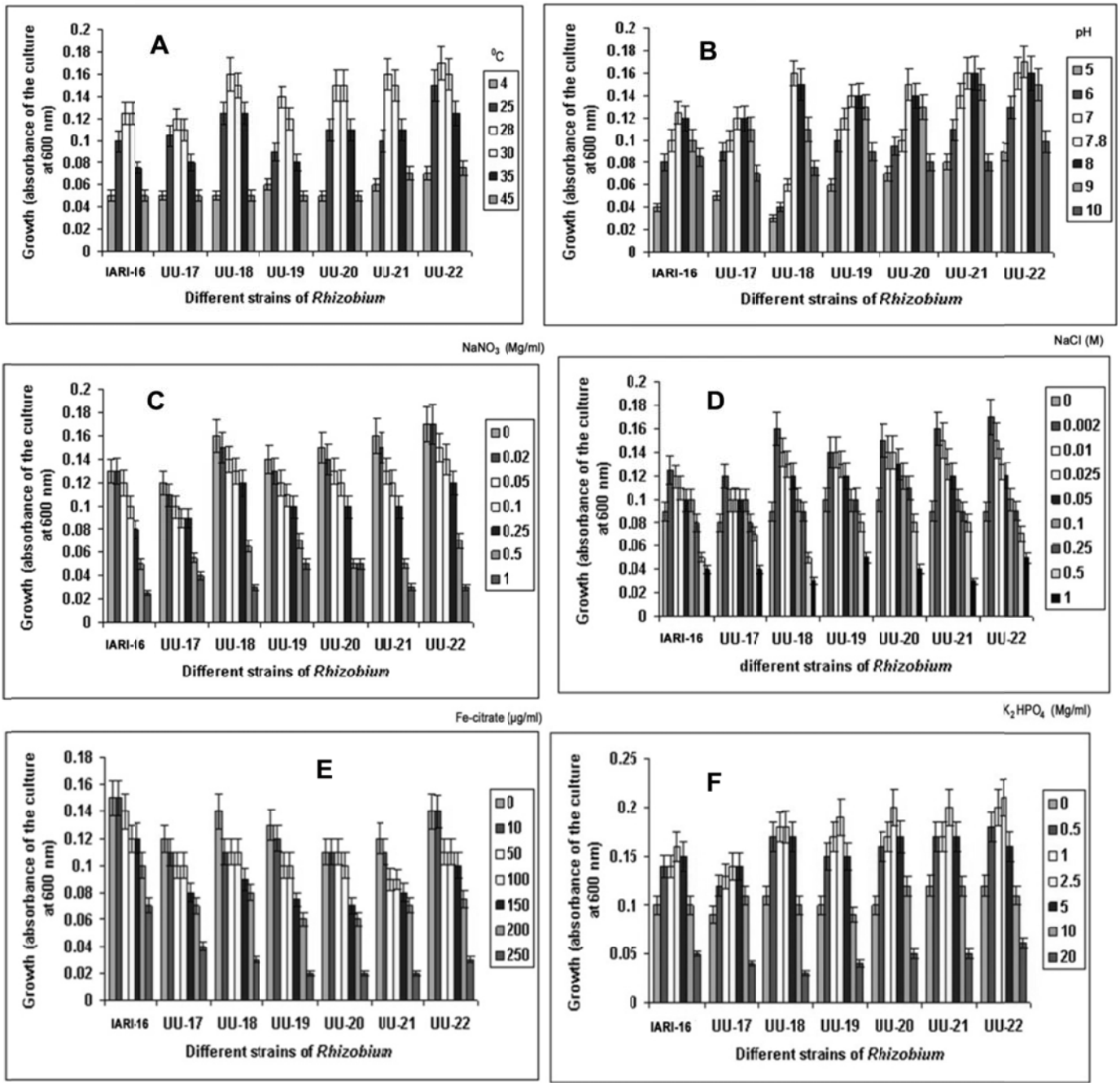

Figure 2. A - F. Growth of different strains of *Rhizobium* isolated from *Arachis hypogea* from different temperature (°C), pH, salinity (NaCl), nitrate ($NaNO_3$), iron (Fe-citrate) and phosphate (K_2PO_4). Cultures were incubated for 120 h at 28±2°C. Initial inoculum of the culture suspension at 600 nm was 0.02. IARI-16, IARI culture strain Delhi; UU-17, Maniakati-4; UU-18, Maniakati-5; UU-19, Amrutulu; UU-20, Buguda; UU-21, Khilabadi; UU-22, Surada-1.

by 28-33%.

However, growth was progressively increased with increase of phosphorous as K_2HPO_4 in the media from 0.5 to 2.5 mg/ml; the increase was more prominent in UU-19, which was up to 26% followed by the strain IARI-16, UU-17, UU-18, UU-20 and UU-22. Growth of most strains except UU-22 either remained unchanged or increased up to 12% in the presence of 5 mg/ml phosphate, but with further increase of the nutrient, growth was adversely affected; the decrease of the growth was up to 64 to 82% in these strains in the presence of 20 mg/ml of K_2HPO_4 (Figure 2F).

Comparative analysis of the growth pattern of severa strains of *Rhizobium* from *V. radiata* and *A. hypogea* to

Table 1. Comparative study of growth response of several strains of *Rhizobium* species isolated from *Vignaradiata* to low and high pH, temperature, salinity, iron, phosphate and nitrate.

Parameter		Strain
Temperature	Low (25°C)	UU-7< UU-13< IARI-1< UU-10< UU-2< UU-4
	High (45°C)	IARI-1< UU-7< UU-2< UU-13< UU-10< UU-4
pH	Low pH (6)	UU-4< UU-13< UU-2< IARI-1< UU-7< UU-10
	High pH (9)	IARI-1< UU-10< UU-4< UU-13< UU-2< UU-7
Salinity	Zero	IARI-1< UU-4< UU-7< UU-13< UU-10< UU-2
	High (0.25 M)	UU-10< UU-2< UU-7< UU-13< IARI-1< UU-4
Nitrate	Low (50 µg/Ml)	UU-13< UU-7< UU-4< IARI-1< UU-2< UU-10
	High (200 µg/Ml)	UU-13< IARI-1< UU-7< UU-10< UU-4< UU-2
Phosphate	Zero	UU-13< UU-7< UU-10< IARI-1< UU-2< UU-4
	High (2.5 Mg/Ml)	IARI-1< UU-7< UU-2< UU-4< UU-10< UU-13
Iron	Low (0.05 Mg/Ml)	UU-7< UU-2< IARI-1< UU-4< UU-10< UU-13
	High (0.25 Mg/Ml)	UU-13< IARI-1< UU-4< UU-10< UU-7< UU-2

Table 2. Comparative study of growth response of several strains of *Rhizobium* species isolated from *Arachis hypogea* to low and high pH, temperature, salinity, iron, phosphate and nitrate.

Parameter		Strain
Temperature	Low (25°C)	UU-21< UU-19< UU-20<UU-18< IARI-16< UU-17< UU-22
	High (45°C)	UU-18< UU-20< UU-19< IARI-16< UU-17< UU-21< UU-22
pH	Low pH (6)	UU-20< IARI -16< UU-21< UU-19< UU-17< UU-22< UU-18
	High pH (9)	UU-18< IARI -16< UU-20< UU-22< UU-17< UU-19< UU-21
Salinity	Zero	UU-22< UU21< UU-18< UU-20< UU-17< UU-19< IARI -16
	High (0.25 M)	UU-22< UU-21< UU-18< IARI -16< UU-17< UU-19< UU-20
Nitrate	Low (50 µg/ml)	UU-21< UU-19< UU-22< UU-18< UU-17< UU-20< IARI -16
	High (200 µg/ml)	UU-19< UU-22< UU-20< UU-18< UU-21< UU-17< IARI -16
Phosphate	Zero	UU-20< UU22< UU-19< UU-21< UU-18< IARI -16< UU-17
	High (2.5 mg/ml)	UU-19< UU-20< UU-21< UU-22< IARI -16< UU-17< UU-18
Iron	Low (0.05 mg/ml)	UU-21< UU-17< UU-19< UU-20< UU-18< UU-22< IARI -16
	High (0.25 mg/ml)	IARI -16< UU-21< UU-20< UU-22< UU-19< UU-18< UU-17

lower and higher pH (6 and 9), temperature (25 and 45°C), salinity (0 and 0.25 M NaCl), iron (0.05 and 0.25 mg/ml of Fe-citrate), phosphate (0 and 2.5 mg/ml) and nitrate (50 and 200 µg/ml) was analyzed and given in Tables 1 and 2. It was found that considerable variation exists between these organisms on the basis of their resistance to several of these environmental variables. UU-4 from *V. radiata* was found to be most tolerant species to high and low temperature, high salinity, phosphate deficiency, relatively higher phosphate and higher nitrate concentrations, but was sensitive to lower pH.

Next to this, UU-2 from the same host tolerated maximum to lower temperature, alkaline pH, phosphate deficiency and to higher concentration of iron, but was very sensitive to growth at little increase in salinity. UU-10 was tolerant to lower pH, low nitrate, relatively higher phosphate in non-saline conditions and in low iron concentration in the media. UU-13 grew well only in the presence

of higher phosphate and low iron, and was sensitive to lower temperature, acidic pH, high iron concentration, phosphate deficiency and to presence of nitrate in the media. IARI-1 was most sensitive to higher temperature, alkaline pH, non-saline condition, higher concentration of phosphorous and nitrate, and was tolerant to higher level of salinity (NaCl).

DISCUSSION

These results show that the same organism did not grow well or were tolerant to either all the stresses or even low and high value of a particular environmental stress that might be occurring in the crop fields. Physico-chemical characteristics of different agro-climatic regions of Odisha showed wide variation in the pH, iron, phosphate, nitrate, conductivity as well as salinity of these soils (Sahu et al., 1996). Hence, it is essential to select a particular strain from the desired crop suitable to its capability to grow under these variables of the crop fields of a particular region so that the inoculated strain can establish and perform leading to higher productivity. Based on the results above on the tolerance of six strains of *Rhizobium* from *V. radiata* and seven strains from *A. hypogea,* three strains each, IARI-1, UU-4 and UU-10 from the former and UU-20, UU-21 and UU-22 from the later host species were selected and changes in their protein profile in response to various environmental stresses was analyzed.

Maximum growth of *Rhizobium* isolated from *V. radiata* and *A. hypogea* was obtained at 28°C and with little increase or decrease of temperature, they had a significant effect on their growth. Maximum soil temperature in the tropics usually exceeds 45°C at 5 cm and 50°C in 1 cm depth (Lal, 1993), and can limit nodulation in relation to rhizobial growth. Upper limit ranges between 32 and 47°C, although tolerance varies among species and strains because high temperature decreases rhizobial survival and establishment in tropical soils.

Hence, repeated and higher rates of inoculation may frequently be needed. The alternative is inoculated strains capable of surviving at the higher temperature of tropics so as to make the inoculation successful. There have been number of investigations on the effect of temperature on infection process of temperate species of *Rhizobium* in environmental growth chamber. The results show that below 10°C, root hair infection by *Rhizobium* is retarded whereas at 24°C and above; the rate of infection is enhanced. However, these results are dependent on variation between *Rhizobium* strains and host cultivars.

The same is true in tropical climatic regime with a higher temperature limit. Rhizobia are known to survive in stored dried soil for several years (Sen and Sen, 1958) and could tolerate 45°C and produce nodules on roots of *Vigna mungo*. Further testing under field condition revealed that this heat tolerant strain of *Rhizobium* significantly increased grain yield of *V. mungo* (Subbarao, 1982).

Rhizobium from both crops grew well at near neutral pH and with variation of the pH to acidic or alkaline pH, their growth were affected though there were minor deviations among the strains to tolerate higher and lower pH levels. The optimum pH for rhizobial growth has been found between pH 6 to pH 7 (Jordan, 1984) with relatively few rhizobia growth in acidic pH (Graham et al., 1994). Intrinsic tolerance cannot be predicted from the pH at the site of isolation because when fast growing rhizobial strains were isolated from nodules that have been inoculated with soil from certain sites where the pH ranges from 3 to 5, only 37% were able to grow in buffered medium at pH 4 and 60% grew at pH 9.5 (Hungria and Vargas, 1996).

A large proportion of tropical soils have developed from old geological formation. This combined with climatic conditions has resulted in highly weathered soils containing predominantly low activity clays. These are usually acidic and infertile, and frequently contain toxic chemicals. Such acid soil conditions pose problems for plants, the bacteria and the symbiosis (Giller and Wilson 1993). The microsymbiont is usually more sensitive to pH. Some rhizobial species can tolerate acidity better than others, however, similar results that the tolerance may vary among strains within a species has been reported earlier (Brockwell et al., 1995, Hungria et al., 1997)

Different species of rhizobia withstand different levels of NaCl, which was invariably higher than the host plant (Subbarao, 1974). Further degree of salinity/alkalinity conducive for good nodulation was different from the limits of tolerance of *Rhizobium* and the host to the salt. Of these, growth responses of several strains of *Rhizobium* from *V. radiata* and *A. hypogea* to different concentration of NaCl ranging from 0.002 to 1 M, showed wide variation in the capabilities of these strains to tolerate the salt.

There are reports that salt tolerant strains significantly enhance their capacity to oxidize carbon sources by increasing growth rate and EPS production that involve in adhesion resulting in a greater adapting capacity to colonize on favorable saline environment (Barboza et al., 2000). Lippi et al. (2000) has studied the effect of salinity on growth, starvation, survival and recovery from salt stress of a *Rhizobium* isolated from nodules of *Acacia*. The results show that survival capacity of starved cultures depended on previous growth condition and culturability subjected to double stress starvation and salinity was reduced considerably. All the starved cultures were capable of regrowth when nutrients became available thus showing
that the strain can withstand long periods of nutrient deprivation in soil while maintaining the capacity for an active metabolism and a potential infectiousness to the host.

All the *Rhizobium* species isolated from *V. radiata* and

A. hypogea grew well in presence of up to a tolerant limit of $NaNo_3$, though *Rhizobium* from *V. radiata* were invariably more tolerant to nitrate than those isolated from *A. hypogea.* There are reports that legume can use nitrogenous fertilizer and grow well but application of such fertilizers, especially at higher doses inhibit nodule number, efficiency of fixation, bacteroids and membrane envelope formation showing that it diminishes all attributes of symbiosis (Subbarao, 1974). Similarly, another soil nutrient phosphate though is essential for growth of all the rhizobia, the ones from *V. radiate* required comparatively less phosphorous than from the other host to grow. Earlier reports showed that application of phosphate to leguminous crops enhances the number of nodules, the nitrogen content and growth of plants (Vyas and Desai, 1953). Acharya et al. (1953) have shown that rotation of crops and phosphate manure enhances soil nutrient content. The results of the present investigation together with the earlier reports show that these two nutrients, nitrate and phosphate are essential at certain concentration for the growth of rhizobia in the soil but the critical concentration as per the requirement varies from species to species.

Although iron is abundant in soil (1 to 6%) and it ranks 4th among all elements on surface of earth, it is often unavailable to the microbes and plants because of its solubility, which is dependent on pH. Under aerobic soil conditions, most iron exists in the insoluble ferric form (Dudeja et al., 1997). It is a component of the cell and its deficiency causes growth inhibition and can also change the cell morphology. To meet the requirement of iron, the organisms evolve a specific high affinity mechanism and when the medium and the soil is low in soluble iron, this mechanism becomes operative, and this happens with involvement of siderophores, which are low molecular weight iron chelators (Dudeja et al., 1997). Iron plays special role in root nodules for the symbiotic nitrogen fixation as this is required for leghaemoglobin, nitrogenase and cytochrome synthesis within the bacteroids in the nodules. Research have shown that presence of active nodules indicate iron deficient stress response in soybean (Dudeja et al., 1997). Odisha soils are rich with iron, which varies from 8 to 376 ppm (Sahu et al., 1990). The locations where the field experiments for the present work were conducted are rich with iron exceeding 100 µg/g soils. Growth response of these strains to various iron concentrations is a critical factor for their establishment after inoculation to make the biofertilizer programme successful. Hence, selection of iron tolerance strains and those grown at comparatively higher iron concentrations were specially taken care for selection of strains for further experiments.

Conclusion

The above experimental results show that *Rhizobium* from both the crops *A. hypogea* and *V. radiata* in response to the same stress was quite different. This shows that there may exists a genetic variability among the rhizobial strains from the same host and also from different host plant to cope with the stress factors prevailing in a specific location. The results clearly demonstrated that rhizobium isolated from the local environments are more tolerant to these environmental stresses than strains collected from IARI, New Delhi, India which belongs to different agro-climatic condition. Hence, it can be concluded that the host as well as the region specific rhizobium isolates is more important for making a biofertilizer programme successful.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Antimicrobial activity of *Streptomyces* sp. isolated from the gulf of Aqaba-Jordan and screening for NRPS, PKS-I, and PKS-II genes

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Forty-nine *Streptomyces* isolates were recovered from sediment samples in the gulf of Aqaba/Jordan. All isolates were tested for antimicrobial activity against Gram positive bacteria, Gram negative bacteria, and yeast. Twenty eight *Streptomyces* isolates were active against at least one of the tested strains. The majority of the isolates showed activity against Gram positive bacteria: *Streptomyces aureus* (89%), *Streptomyces epidermidis* (64%) and *Bacillus Subtilis* (50 %). Lower activity was observed toward Gram negative bacteria with only 25% active against *Pseudomonas aeruginosa*, whereas only 17% were active against the yeast *Candida albicans*. Isolate S34 showed best activity. It produced heat stable antimicrobial activity at both acidic and alkaline pH (5 to 5.5 and 8 to 9.5). S34 was found to be related to *Streptomyces rochei*. Forty-nine *Streptomyces* isolates were screened for genes encoding non ribosomal peptide synthetases (NRPS) and polyketides synthases (PKS; types I and II). NRPS sequences were widely distributed and detected in 81% of *Streptomyces* isolates. PKS types I and II were detected in 63.2 and 65.3% of isolates, respectively. Additionally, the relationship between the occurrences of biosynthetic gene sequences (NPRS and PKS sequences) and the production of antimicrobial activities was determined. The above results reveal that the marine *Streptomycetes* are a promising source of novel and unique products.

Key words: Marine *Streptomyces*, antimicrobial activity, non ribosomal peptide synthetases (NRPS), polyketides synthases (PKS), enzymes, gulf of Aqaba, Jordan.

INTRODUCTION

Streptomyces is the largest genus of *Actinobacteria* with over 500 species been reported. Identification of *Streptomyces* and definition of the species is not easy, due to their variety of morphological, physiological and

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Abbreviations: NRPS, Non ribosomal peptide synthetases; **PKS,** polyketides synthases; **A,** Adenylation; **T,** thiolation; **C,** condensation; **ISP,** international *Streptomyces* project.

biochemical characteristics. The methods used for characterization are based largely on morphological observations, subsequent classifications based on numerical taxonomic analyses of standardized sets of phenoltypic characters and, the use of molecular phylogenetic analyses of gene sequences (Labeda et al., 2012). Members of the genus have high Guanine and Cytosine content in their DNA and aerial mycelia (Anderson and Wellington, 2001). They are considered as one of the most important sources of antibiotics (Dharmaraj, 2010; Ayari et al., 2012; Sirisha et al., 2013). They produce about two thirds of the clinically useful antibiotics that are natural in origin (Jensen et al., 2005a) including streptomycin, erythromycin, tetracycline and neomycin. Indeed *Streptomyces* genus in the marine environment is largely unexplored, although true indigenous marine *Streptomyces* species have been described (Bull et al., 2005), suggesting a promising source of novel and unique bioactive metabolites (Maldonado et al., 2005; Moore et al., 2005; Dharmaraj, 2010; Ayari et al., 2012). Increasing number of novel metabolites of commercial interest was isolated from marine *Streptomyces* (Lam, 2006, Wu et al., 2006; Dharmaraj, 2010; Jayaprakashvel, 2012). Potent and diverse bioactivities were reported, they included antibacterial, antifungal, antitumor, and anticancer activities (Newman and Cragg, 2007; Olano et al., 2009).

Large number of bioactive products, with medicinal and agricultural application, are synthesized by non ribosomal peptides synthetases (NRPS) and polyketides synthases (PKS type I and II) (Ayuso-sacido and Genolloud, 2005; Savic and Vasiljevic, 2006). Polyketides synthases are multienzyme complexes that synthesize polyketides by sequential decarboxylative condensation of acyl coenzyme A units (Hopwood, 1997). NRPSs are multifunctional enzyme complexes organized into modules. Each module contains three essential domains: Adenylation (A), thiolation (T), and condensation (C). Evaluation of the biosynthetic potential, expressed in gene detection, has been extensively described in terrestrial *Streptomycetes* (Metsa-Ketela et al., 1999); but very little is known in marine counterparts. The presence of highly conserved sequences in PKSs, and NRPS systems among terrestrial and marine organisms have been used to design PCR primers, targeting ketosynthase (KS) and malonyl transferase in PKS-I, ketoacylsynthase (KS_{α}) in PKS-II and adenylation domains in NRPS (Ayuso-sacido and Genilloud, 2005; Pathom-aree et al., 2006).

Streptomyces have been isolated from different parts of Jordan, including hot spring areas (Abussaud et al., 2013), arid habitats (Saadoun et al., 2008), forest (Saadoun et al., 2007), and soil (Saadoun and Gharaibeh, 2002; Saadoun et al., 1999). Since marine environments, which constitute a rich source of novel and bioactive marine microorganisms is attracting a major focus of many natural products research efforts, and since the Gulf of Aqaba represents the only marine access of Jordan, we chose this site for our study. Gulf of Aqaba environment is unique in terms of its special marine life, represented mostly by intensive coral reef ecosystems and sea grass meadows; it is a narrow deep basin with an average width of 14 km and a total length of 180 km located in the northernmost part of the Red Sea.

As far as we know, this is the first report for the isolation of marine *Streptomyces* from the Gulf of Aqaba, Jordan. Therefore, this study was initiated to evaluate the bioactivity of *Streptomyces* isolates from the Gulf of Aqaba-Jordan; and to screen for the presence of PKS /NRPS genes associated with bioactivity.

MATERIALS AND METHODS

Isolation and characterization of *Streptomyces*

A total of 295 sediment samples were collected from the Gulf of Aqaba. Samples were obtained at different depths (1 to 40 m), they were placed in sterile universal bottles, and immediately processed in the laboratory, according to the following methods (Mincer et al., 2002; Jensen et al., 2005b): Method 1 (dilution), 1 g of wet sediment was added to 4 ml sterile seawater, heated for 6 min at 55°C to reduce non spore forming bacteria. Aliquots of the sample were spread onto the isolation media. Plates were incubated at 30°C for 7 to 45 days. Method 2 (dry / stamp): 1 g of sediment was dried overnight in laminar hood, then ground lightly. Serial dilutions were made by pressing autoclaved foam-plug onto the sediment, then repeatedly onto the surface of isolation media. The plates were incubated at 30°C for 7 to 45 days. Method 3 (dilute / heat): 1 g of dried sediment was added to 3 ml of sterile seawater, then heated to 55°C for 6 min. 50 µl aliquots of the suspension were inoculated onto the isolation media, plates were incubated at 30°C for 7 to 45 days. Method 4 (dry / stamp+ dilute/ heat): tThe dried sediment was processed using method 2, then as in method 3 before inoculation. Plates were incubated at 30°C for 7 to 45 days.

Each sample was incubated into each of four media: Starch-yeast extract agar medium (SYB; Soluble starch 10 g/l, yeast extract 4.0 g/l, peptone 2.0 g/l, agar 18 g/l); Starch- casein agar medium [SCA; Soluble starch 10 g/l, casein (dissolved in 0.3 M NaOH) 1.0 g/l, agar 15 g/l)]; Starch- nitrate broth medium (SNB; Starch 20 g/l, KNO_3 2 g/l, $K_2HPO_4.3H_2O$ 1 g/l, $MgSO_4.7H_2O$ 0.5 g/l, NaCl 0.5 g/l, $CaCO_3$ 3.0 g/l, Trace salt solution 1.0 ml); and Oatmeal agar (OA; Oat meal 20 g/l, trace salt solution 1.0 ml, Agar 20 g/l). Isolation media were supplemented with 100 µg/ml of cycloheximide and 50 µg/ml of nalidixic acid to inhibit the growth of yeasts, fungi and bacteria. All samples were processed in triplicates. Suspected *Streptomyces* colonies were purified on starch casein agar. Pure cultures were maintained on starch casein agar slants at 4°C. They were sub-cultured every three months. For long term storage, isolates were stored in 20% glycerol at -20°C.

Cultural, morphological and physiological characteristics

Isolates were characterized to the genus level according to the International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966) and Bergey's manual of Determinative Bacteriology (Buchanan and Gibbons, 2002). For cultural and morphological characteristics of the colonies and the ability to produce soluble pigments, the isolates were inoculated onto the media described by Shirling and Gottlieb (1966), and included inorganic salt-starch

Table 1. Primer sequences used for the detection of NRPS, PKSI, and PKSII genes from *Streptomyces* isolates.

Target Gene	Primer Name	Oligonucleotide sequences (5`-3`)	Product Size(bp)	References
NRPS	A3F	GCSTACSYSATSTACACSTCSGG	700-800	Ayuso-Sacido and Genilloud (2005)
	A7R	SASGTCVCCSGTSCGGTAS		
PKS-I	K1F	TSAAGTCSAACATCGGBCA	1200-1400	Ayuso-Sacido and Genilloud (2005)
	M6R	CGCAGGTTSCSGTACCAGTA		
PKS-II	KS_{α}	TSG CST GCT TGG AYG CSA TC	613	Mesta Ketela et al. (2002)
	KSβ	TGG AAN CCG CCG AAB CCG CT		

agar, oatmeal agar, yeast extract-malt extract agar, and Czapek-Dox agar. The plates were incubated at 30°C in darkness and examined after 7, 14, and 21 days of incubation. The production of melanin pigment, in different media, was determined according to the methods of ISP. The morphology of aerial mycelia was described following Bergey's Manual (Buchanan and Gibbons, 2002).

Carbohydrate utilization was determined by growing isolates on basal mineral salts agar medium supplemented with 1% carbon source at 28°C (Pridham and Gottlieb, 1948; Benedict et al., 1955). Tolerance to NaCl was studied using 4, 7, 10, and 13% NaCl concentration in starch casein agar medium [starch (10 g/l), casein (1 g/l), and agar (15 g/l0].

Screening for antimicrobial activity of *Streptomyces*

Antimicrobial activity was determined using agar well diffusion method (Augustine et al., 2005a). *Streptomyces* isolates were inoculated in starch casein broth medium prepared with 75% seawater. After incubation for 7 days at 30°C with shaking (150 rpm), the supernatants were tested against Gram-positive bacteria: *Bacillus subtilis* ATCC66 33, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* clinical isolate*, Micrococcus luteus* ATCC 10260, *β-hemolytic streptococci* clinical isolate. Gram-negative test strains included: *Escherichia coli* clinical isolate, *Pseudomonas aeruginosa* clinical isolate, *Bordetella bronchiseptica* ATCC 19395, *Klebsiella sp.* clinical isolate, plus the yeast *Candida albicans* ATCC 10231. Antimicrobial activity was expressed as the diameter of the inhibition zones (Laidi et al., 2006). Clinical isolates were obtained from the central laboratory of the ministry of health, Amman, Jordan. Test microorganisms were stored on slants at 4°C, and subcultures monthly. *Streptomyces* isolates (S34) showed the highest activity, and was selected for further studies.

Detection of NRPS, PKS-I, and PKS-П genes

In order to evaluate the biosynthetic potential of bioactive compounds from *Streptomyces* isolates, degenerate primers: A3F/A7R, K1F/M6R and $K_{\alpha}F/K_{\beta}R$ were used (Alpha DNA / Montreal) to detect the presence of NRPS, PSK-I and PKS-II genes in all *Streptomyces* isolates obtained from sediment samples from the Gulf of Aqaba.

DNA extraction

Streptomyces isolates were inoculated in Tryptic Soy broth (Sigma) prepared with 70% seawater, and incubated at 30°C for 48 h with shaking (150 rpm). Genomic DNA was extracted using Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer instructions.

PCR primers

The oligonucleotide primers used for detection of NRPS, PKS-I, and PKS-II NRPS genes were obtained from Alpha DNA (Quebec) (Table 1).

PCR amplification

PCR amplification of NPRS, PKS-I, and PKS-II genes were performed on My Cycler (Bio-Rad, USA) in a final volume reaction of 50 µl, containing 25 µl master mix (Promega, USA), 2 ml of each primer and 5 ml of the extracted DNA. NPRS and PKS-I were amplified with primers A3F/A7R and K1F/M6R, respectively. They were performed as recommended by Ayuso-sacido and Genilloud (2005) and Ayuso et al. (2005) using the following programs: 5 min at 95°C and 35 cycles of denaturizing for 30 s at 95°C, annealing for 2 min at 55°C for K1F/M6R and 59°C for A3F/A7R, and extension for 4 min at 72°C, followed by final extension for 10 min at 72°C whereas, the amplification of PKS-II with primer KS_{α}/KS_{β} was performed using the following temperatures: 2 min at 95°C, 30 cycles of denaturizing of 1 min at 96°C, annealing of 1 min at 64°C, 1.5 min at 73°C and final extension of 8.5 min at 73°C (Pathom-aree et al., 2006).

Gel electrophoresis

PCR products were analyzed using agarose gel electrophoresis by loading 10 µl of each PCR sample and 100 bp DNA Ladder into 1% agarose gels (Promega, USA). The electrophoresis gel was run with 100 V for 1 h, then examined and photographed using gel documentation system.

Identification of *Streptomyces* sp. S34

Isolate S34 was identified according to the description of the *Streptomyces* species recorded in Bergey's Manual and International *Streptomyces* Project (Buchanan and Gibbons, 2002).

Antimicrobial bioassay of isolate S34

Antimicrobial activity of isolate S34 was evaluated in Starch casein broth medium by agar diffusion method against Gram-positive bacteria: *S. aureus* ATCC 6538, Gram-negative bacteria: *E. coli* and

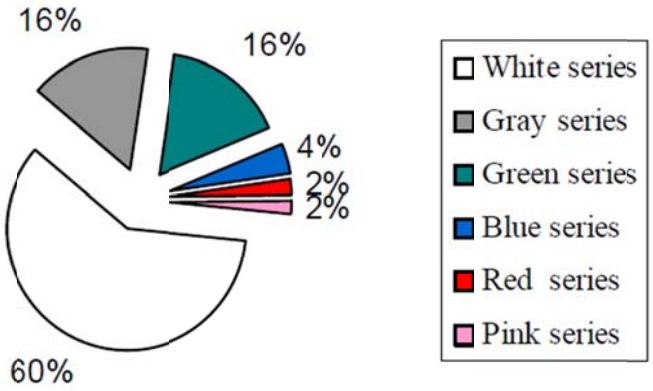


Figure 1. Percentage of *Streptomyces* color series isolated from Aqaba Gulf.

and the yeast *C. albicans* ATCC 10231.

Optimization of antimicrobial compounds production from *Streptomyces* S34

Cell free supernatants of isolate S34 showed significant activity against test microorganisms, thus they were chosen to determine the optimal conditions for bioactivity. Each of the following parameters was optimized: seawater content, effect of medium components, incubation period, pH, temperature, and agitation rate. The bioactivity of S34 was monitored for 14 days (2, 3, 4, 5, 6, 8, 10, 12, and 14 days). The optimal pH and temperature were separately determined by growing the isolate at pH range 3 to 12 with 0.5 differences, and at temperature range of 20 to 50°C with 5°C variance, and agitation rates of 0, 50,100,150, 200 and 250 rpm. All experiments were performed in duplicates. The antimicrobial activity was determined by agar well diffusion assay using nutrient agar medium for *S. aureus* and *E. coli* and Sabouraud agar medium for *C. albicans*.

Thermal stability and the effect of proteolytic enzymes

To study the effect of temperature on the bioactivity of isolate S34, cell free supernatant was heated to 100°C for different time intervals: 5, 15, 30 min, and 1 h. After each interval the supernatant was cooled to room temperature before measuring the residual antimicrobial activity. Supernatants without treatment were used as control (Augustine et al., 2005a). The effect of proteolytic enzymes on the activity was determined by incubating culture supernatant with pepsin and trypsin (Fluka, Germany) at final concentrations of 50 and 100 mg/ml, respectively. Supernatants were then incubated for 1 h at 30°C. The supernatant without any enzyme served as negative control. The residual antimicrobial activity was tested using agar well diffusion assay.

RESULTS

Isolation and characterization of *Streptomyces*

The characterization of *Streptomyces* isolates were performed according to the methods recommended by Bergey's Manual (Buchanan and Gibbons, 2002) and the International *Streptomyces* Project (ISP) as recommended by Shirling and Gottlieb (1966). A total of 49 *Streptomyces* isolates were recovered from 295 sediment samples collected from the Gulf of Aqaba, Red Sea/ Jordan. Among the four methods used to isolate *Streptomyces* (dilution, dry/ stamp, dilute/ heat, dry/ stamp+ dilute/ heat methods), method 4 (dry/stamp+ dilute/heat) yielded the highest rate of *Streptomyces* recovery (69.4%), method 3 (dilute/heat) yielded a relatively good percentage (40.8%), whereas method 1 was the least effective (20.4%). Thus method 4 was selected for the rest of experiments.

Cultural, morphological and physiological characteristics

Based on microscopic and cultural examination, the isolates, were grouped into six series based on the color of aerial mycelia; most of them belonged to the white series (60%), followed by grey and green series (16% each) (Figure 1). Most of the isolates had spiral (S) sporophore morphology (69.4%); the remaining isolates had spores in the straight or flexuous chain. Physiological data indicated that 24% of *Streptomyces* (12 isolates) produced melanin on peptone yeast extract iron agar medium, and tyrosine agar medium (Shirling and Gottlieb, 1966); only 12%(6) produced soluble pigment. Most of isolates were able to utilize D- glucose (48 out of 49 isolates), D-xylose (36), L-arabinose (29), L-rhamnose (32); D-fructose (43), D-galactose (38), D-mannitol (31) and salicin (26); whereas utilization of I-inositol (21), raffinose (5) and sucrose (18) was limited to certain isolates. For NaCl tolerance of *Streptomyces* isolates; it was found that 2% of isolates could tolerate a maximum of 4% NaCl; 24% tolerated 7% NaCl, about half of the isolates (49%) tolerated 10% NaCl, and 24% tolerated 13% NaCl.

Screening for antimicrobial activity of *Streptomyces*

Marine *Streptomyces* isolates, inoculated in starch- yeast extract- peptone broth medium, prepared with 75% seawater and incubated for 7 days, were screened for their antimicrobial activities against 10 test microorganisms using agar well diffusion method. Antimicrobial activity was determined in terms of diameter of inhibition zone surrounding the well (the size of the well was 7 mm). Inhibition zones ranged from 10 to 30 mm except for S4 isolate that gave the largest zone of inhibition against *B. subtilis* (46 mm). Results are summarized in Table 2. Among 49 *Streptomyces* isolates tested, 28 (57%) showed activity against at least one of the test microorganisms (Table 2). Among these isolates, 5 were only

Table 2. Antimicrobial activity of different color series of *Streptomyces* against test microorganisms.

Test microorganism	*Streptomyces* color series						Total number of positive isolates (percentage)
	White	Grey	Green	Blue	Red	Pink	
S. aureus	13	3	7	1	1	0	25 (89.2)
P. aeruginosa	3	1	0	1	0	0	5 (17.8)
M .luteus	9	4	4	1	0	0	18(64.0)
S. epidermidis	10	3	3	1	1	1	18(64.0)
β. hemolytic *Streptococcus*	3	1	2	0	1	0	7 (25.0)
Klebsiella	3	3	0	1	1	0	8 (28.5)
E. coli	8	2	6	1	0	1	18 (64.0)
B. subtilis	8	3	1	1	1	0	14 (50.0)
Bordetella bronchiseptica	4	1	1	0	1	0	7 (25.0)
C. albicans	4	2	0	0	1	0	7 (25.0)

Table 3. PCR detection of NRPS, PKS-I and PKS-II biosynthetic systems in the *Streptomyces* isolates.

Isolate	Active Isolates	NRPS	PKS-I	PKS-II	Inactive Isolates	NRPS	PKS-I	PKS-II
		A3F/A7R Positive	K1F/M6R positive	KSα/KSβ positive		A3F/A7R positive	K1F/M6R Positive	KSα/KSβ positive
No. of *Streptomyces* isolates	29	21	25	17	20	19	6	15

active against Gram-positive bacteria; one isolate was active against Gram- negative bacteria. Only 15 isolates showed inhibitory activity against both Gram- positive and Gram-negative bacteria, whereas 5 isolates inhibited both Gram-positive, Gram- negative and *C. albicans*. Out of the 28 isolates that exhibited antimicrobial activity, 25 isolates were active against *S. aureus*, 18 against *S. epidermidis*, 18 isolates against *Micrococcus luteus*, 17 against *E. coli ,*14 against *B. subtilis*, 8 against *Klebsiella sp*, 7 against *C. albicans*, 7 against *Bordetella bronchiseptica*, 7 against *B-hemolytic Streptococci,* and 5 against *P. aeruginosa. Streptomyces* isolate S34, showed very good activity with a wide spectrum, and thus was chosen for further studies. Furthermore, the antimicrobial activity was stable in all media (that is, starch casein nitrate broth, starch nitrate broth, and Sabouraud broth).

Detection of NRPS, PKS-I, and PKS-П genes

Amplification of NRPS, PKS-I, and PKS-II genes, using A3F/A7R, K1F/M6R and $K_{\alpha}F/K_{\beta}R$, was performed with all *Streptomyces* isolates. The prevalence of these genes is summarized in Table 3.

Identification of *Streptomyces* isolates S34

According to the description of the *Streptomyces* species recorded in Bergey's manual (2002) and International *Streptomyces* Project (Shirling and Gotlieb, 1966), isolate S34 appeared to be highly related to *S. rochei*, but requires further identification (Table 4).

Optimization of antimicrobial compounds production from *Streptomyces* S34

For the optimal production of antimicrobial activity, the following factors were optimized: Seawater content, type of medium, incubation time, pH, incubation temperature, carbon, and nitrogen sources. Results are summarized in Table 5 and Figure 2

Thermal stability and the effect of proteolytic enzymes on the antimicrobial activity of strain S34

Cell free supernatant of isolate S34 was heated to 100°C for 5, 15, 30 and 60 min. Results show that the activity of supernatant was retained during heat treatments even at 100°C for 1 h. The sensitivity of antimicrobial activity to proteolytic enzymes was tested at 37°C; the activity was stable after incubation with pepsin and trypsin for 1 h. These results suggested non proteinaceous nature of the antimicrobial compound(s) produced by isolate S34.

DISCUSSION

Several studies dealing with bioactive compounds from

Table 4 Identification of *Streptomyces* isolates S34.

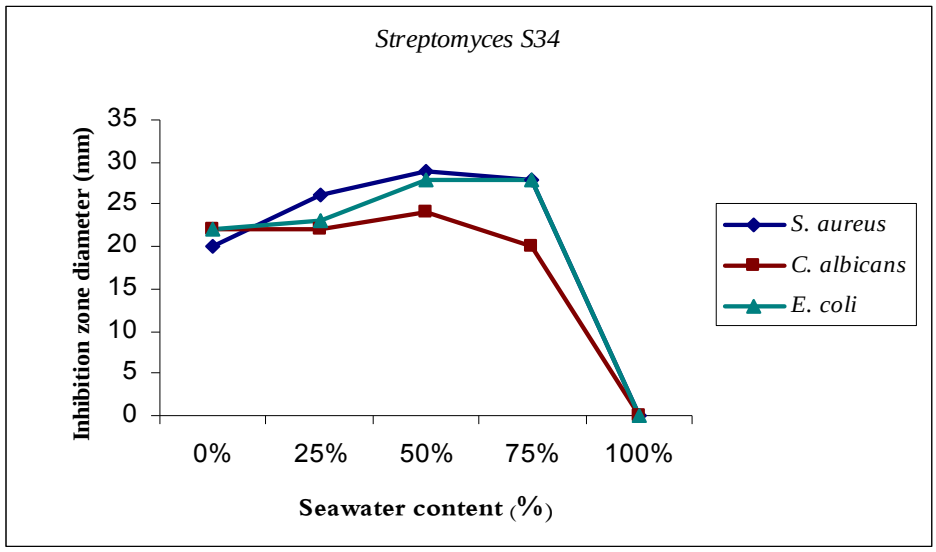
Character	*Streptomyces* S34	*Streptomyces rochei*
Gram stain	Positive	Positive
Cell shape	Filamentous	Filamentous
Color of aerial mycelium	Gray	Gray
Spore chain morphology	Spiral	Spiral
Melanoid pigment	Positive	Positive
Diffusible pigment	Negative	Negative
Growth on Czapek's medium	Good	Moderate
Carbon utilization:		
No carbon	-	-
D-Glucose	+	+
D-Xylose	+	+
L-Arabinose	+	+
L-Rhamnose	+	+
D-Fructose	+	+
D-Galactose	+	+
Raffinose	-	-
D-Mannitol	+	+
I-Inositol	+	+
Salicin	+	+
Sucrose	-	-
Antagonistic activity	Antibacterial and Antifungal	Antibacterial and Antifungal

Table 5. Optimization of antimicrobial compounds production from *Streptomyces* S34.

Parameter under optimization	Variation of the tested parameter	Optimum antimicrobial activity
Sea water content	0, 25, 50,75, and 100%	50%
Medium component	NB,SDB,TSB,SYB, SNB,SCNB,GYMB	SNB
Incubation period	2,3,4,5,6,8,10,12, and 14 days	4-5 days
pH	From 3.0 to 12.0 with 0.5 intervals	5.5 and 8.5-9
Temperature	From 20 to 50°C with 5 intervals	30°C
Agitation rate	From 0 to 250 with 50 differences	150-200 rpm

the genus *Streptomyces* isolated from different habitats in marine environments (sediments, invertebrates, and coral reefs) have been reported. Members of *Streptomyces*, like terrestrial counterparts, are promising source for production of bioactive compounds (Maldonado et al., 2005; Moore et al., 2005; Parthasarathi et al., 2012a, b; Haritha et al., 2012). Since the marine environment in Jordan is still unexplored and unexploited, this study was performed to isolate *Streptomyces* and investigate their antagonistic properties. *Streptomyces* isolates were identified based on cellular and colony morphology, utilization of carbon, and physiological characteristics (Holt et al., 1994). The observed properties indicated that the isolates belonged to the genus *Streptomyces*. Most of the isolates (59%) belonged to white color series, followed by grey and green color series. Dominance of white and grey color series was reported in several studies (Saadoun and Gharaibeh, 2002; Parthasarathi et al., 2012a; b).

Preliminary screening of antimicrobial activity of *Streptomyces* isolates showed that more than half of our isolates (57%) were active against at least one of the test microorganisms. Similarly, the majority of *Streptomyces* isolated from soils in Jordan showed antimicrobial activity (Saadoun et al., 1999). The proportion of active isolates depends on the methods of preliminary screening and on the type of culture used (broth or agar) (Augustine et al.,



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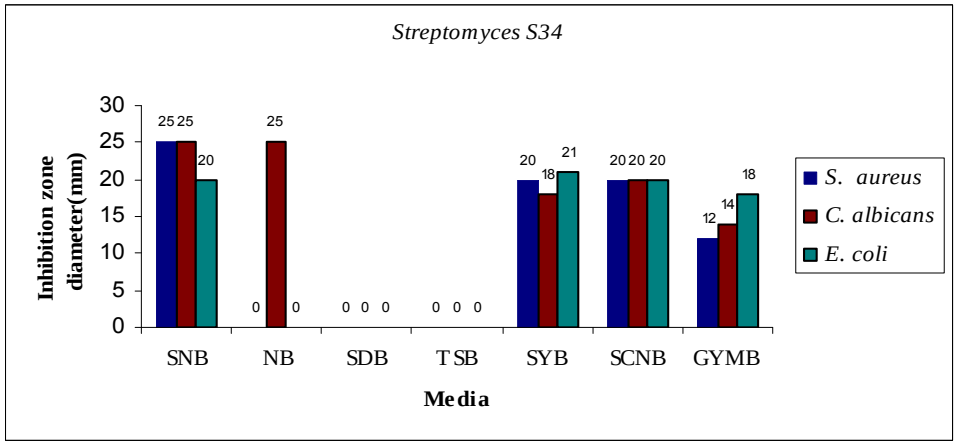

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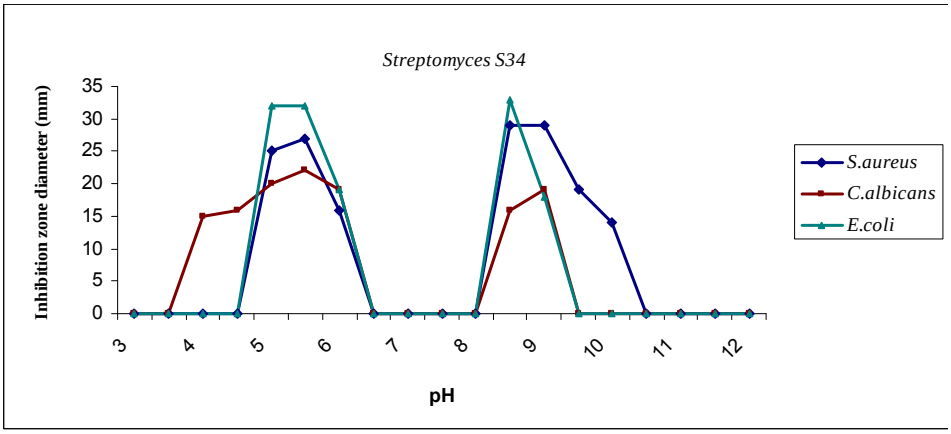

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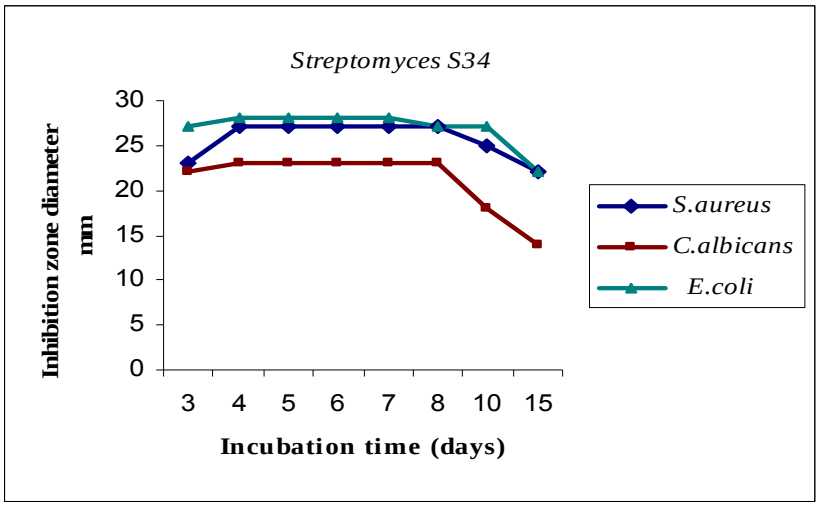
Figure 2. Optimum conditions for the production of antibacterial metabolites from *Streptomyces* S34: Sea water content (1), medium component (2), incubation period (3), pH (4), temperature (5), agitation rate (6).



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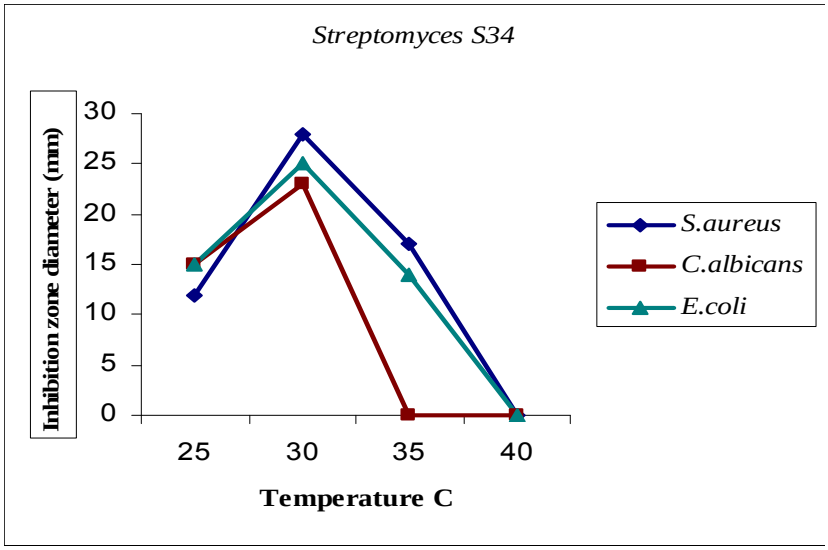

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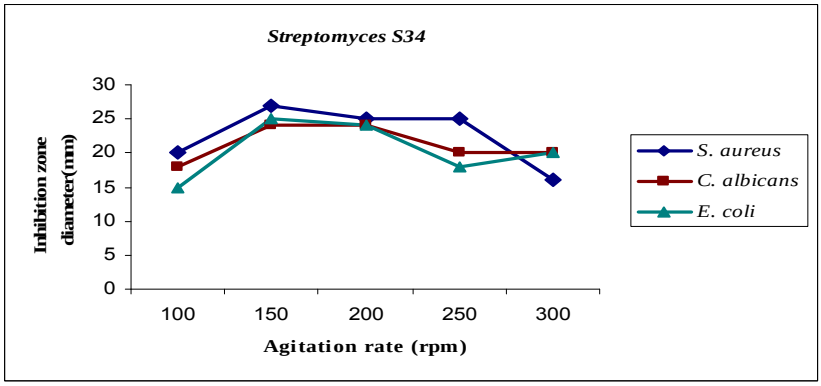

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Figure 2. Contd.

2005a, b). During screening, *Streptomyces* isolates were subjected to the same growth and incubation conditions; it appeared that each isolate required specific growth and antimicrobial production conditions (medium, temperature, pH, and agitation). In addition, the size of sample, stability of antibiotic, bioassay method and test microorganisms appear to affect the number of active isolates (Srivibool and Sukchotiratana, 2006). It was reported that *Streptomyces* isolates were more active against Gram-positive bacteria than Gram-negative bacteria (Silambarasan et al*.,* 2012; Valli et al., 2012). In this study also *Streptomyces* isolates showed a significant antimicrobial activity against *S. aureus*, *S. epidermidis*, and *B. subtilis*, than Gram-negative *P. aeruginosa*. Difference in sensitive between Gram -positive and Gram-negative bacteria might be due to the cell wall structure; the outer polysaccharide membrane present in Gram- negative bacteria which acts as lipopolysaccharide barrier; the lack of this barrier in Gram -positive bacteria makes the cell wall more susceptible (Silambarasan et al*.,* 2012; Valli et al., 2012). For this reason, the amount of antibiotic required for inhibition of Gram-positive bacteria was more than that required for Gram-negative inhibition (Selvin et al., 2004; Sahin, 2005; Srivibool and Sukchotiratana, 2006).

Screening study of the occurrence of biosynthetic pathways of metabolites is of great value to understanding the ecological impact of organisms and fitness of populations (Ehrenreich et al., 2005). Several previous studies assessed the biosynthetic potential of soil *Streptomycetes* were performed (Metsa-Ketela et al., 2002). In the present study, PCR screening of NRPS (700 bp), PKS-I (1400 bp) and PKS-II (613 bp) genes in marine *Streptomycetes* using degenerate primers revealed that NRPS genes were detected in the majority of isolates (81.6%). PKS-I and PKS-II sequences were also detected in most of the isolates tested, but with relatively lower percentage (63.2 and 65.3%, respec-tively). High prevalence of NRPS genes (68%) as well as PKS-I sequences were reported in most of the *Actinomycetes* isolated from marine sediments, of the deepest site of Mariana Trench in the western Pacific Ocean; whereas PKS-I sequences were identified in only 13% of the strains (Pathom-aree et al., 2006). Addi-tionally, NPRS and PKS genes were reported with high frequency in other marine organisms including marine and fresh water cyanobacteria (Ehrenreich et al., 2005) and from marine dinoflagellates (Snyder et al., 2005). Similarly, a study of Ayuso-Sacido and Genilloud (2005) revealed that the NRPS sequences were widely distributed in soil *Actinomycetes* (79.5%), but PKS-I was identified only in 56.7%; whereas among *Streptomyces* isolates, NPRS and PKS-I genes were detected in most of the isolates with higher frequency 97 and 79%, respectively (Ayuso-Sacido and Genilloud 2005). Also, NPRS, PKS- I and PKS-II sequences showed high occurrence in *Streptomyces* isolated from tropical soil samples (60.0, 72.4 and 69.2%, respectively) (Ayuso et al., 2005). Upon comparing the *Streptomyces* local isolates, with and without antimicrobial activity, we observed that higher detection percenttages were obtained for the PKS- I in the group of active isolates than in the group of inactive isolates (Table 4). This relationship between the occurrences of biosynthetic gene sequences and the production of antimicrobial activities was not observed for the NPRS and PKS-II sequences (Table 4). Our results differed from that obtained by Ayuso et al. (2005) who reported that the percentages of positive NRPS and PKS-I amplifications (except for PKS-II sequences) were almost two-fold higher in the active compared with the inactive group.

Ayuso-Sacido and Genilloud (2005) reported that the NPRS primers (A3F/A7R), PKS-I primers (K1F/M6R), and PKS-II primers (KSα/KSβ) amplified the highly conserved sequences of adenylation domains associated with NRPSs and ketosynthase (KS) domains associated with type I PKS. The lack of amplification of these genes in some isolates might indicate their absence or that they were less conserved, hence low homology with the primers. On the other hand, some isolates obtained in this study were negative for NPRS and PKS genes, but they showed bioactivity against test microorganisms, these results suggested that the activities detected were produced by systems other than PKS and NRPS genes, such as aminoglycoside resistance gene (Ayuso et al., 2005). Other isolates did not show any antimicrobial activity in spite of the occurrence of NPRS and PKS systems. It is possible that these detected genes may be silent (nonfunctional) (Hutchinson, 1999, 2003). Studies of sequenced genomes of *Streptomyces coelicolor* and *Streptomyces avermitilis* have demonstrated numerous silent pathways (Challis and Hopwood, 2003; Knight et al., 2003), or that the products of these genes may be involved in primary metabolism (Pathom-aree et al., 2006), or that fermentation conditions used were not optimal for antibiotic production. In fact, the genome of *Streptomyces* contained several gene clusters of NPRS and PKS genes; Pathom-aree et al., (2006) reported that the genome of *S. coelicor* contained five NPRS and three PKS-I clusters, and only four NPRS clusters have known to be involved in the synthesis of known compounds. This may indicate that a huge number of bioactive compounds are still unidentified. Of the 49 *Streptomyces* isolates, S34 showed high antimicrobial activity against test microorganisms. The isolate was identified based on the morphological and cultural characteristics. Isolate produced powdered colony on the surface of agar plate, it is Gram positive and filamentous in nature, belonged to grey color series. S34 showed similar characteristic as that of *S. rochei*.

Isolate S34 was selected to optimize the production of active metabolites. Production of antimicrobial metabolites was significantly influenced by cultural and environ-

mental factors. Influence of these factors has been evaluated in marine *Streptomyces* by several workers (Saha et al., 2005; Narayana and Vijayalakshmi, 2008; Sunga et al., 2008; Arasu et al., 2009; Singh et al., 2009; Thakur et al., 2009). In this study, isolate S34 produced heat stable non proteinaceous metabolites that have broad spectrum and high activity against pathogenic bacteria and yeast tested.

Conclusions

Marine *Streptomyces* species, isolated from the Gulf of Aqaba/Jordan, was found to be highly diverse and produced wide spectrum antimicrobial agents. The optimal medium, nutrients, pH, temperature, and other culture conditions promoted the effectiveness of the antimicrobial agents. The majority of the isolates showed activity against Gram positive bacteria, lower activity was observed toward Gram negative bacteria and yeast. *Streptomyces sp*. S34 had wide spectrum activity (it inhibited Gram-positive, Gram-negative bacteria, and yeast), strong activity, which was determined by largest inhibition zone diameter (30 mm), and antimicrobial activity at both acidic and alkaline pH (5 to 5.5 and 8 to 9.5). Furthermore, antimicrobial activity showed temperature stability. Isolate S34 produced non proteinaceus heat stable antimicrobial metabolites. It can be concluded that marine *Streptomyces* strains isolated from the Gulf of Aqaba have a great potential as a source of secondary metabolites with antibacterial activity. However, further investigation is needed to isolate and characterize the active secondary metabolites.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Moringa oleifera leaf extract potentiates anti-pseudomonal activity of ciprofloxacin

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The aim of this study was to evaluate the *in vitro* antimicrobial interaction between the ethanol leaf extract of *Moringa oleifera* (MO), which is used in Nigeria as a dietary supplement, and ciprofloxacin (Cp), a flouroquinolone antibiotic. Preliminary antimicrobial screening of the ethanol extract of *M. oleifera* and ciprofloxacin was determined *in vitro* using the agar dilution method. The antimicrobial interaction between these agents was evaluated by the Checkerboard technique using *Staphylococcus aureus* and *Pseudomonas aeruginosa* as test organisms. The minimum inhibitory concentration (MIC) values of the extract against *S. aureus* and *P. aeruginosa* were 25.0 and 50.0 mg/mL, respectively, while that of ciprofloxacin were 0.00062 and 0.0005 mg/mL against *S. aureus* and *P. aeruginosa*, respectively. The antibacterial interaction studies indicated that the combinations predominantly showed additive effects at Cp : MO ratios of 8:2, 7:3, 6:4 and 5:5 against *S. aureus* while Cp : MO ratios of 9:1, 8:2, 7:3 and 6:4 yielded predominantly synergistic effect against *P. aeruginosa*. Other combination ratios had no MIC, hence no observed effect. This study has demonstrated that the ethanol leaf extract of *M. oleifera* possesses potent antibacterial effect against *S. aureus* and *P. aeruginosa*. Overall, the combined antimicrobial effect of the interaction between the extract and ciprofloxacin was predominantly synergistic against *P. aeruginosa*. Regarding its relevance, this study has provided a preliminary evidence of some kind of antibacterial interaction between ethanol extract of *M. oleifera* leaf and ciprofloxacin against *P. aeruginosa* and has established that the use of *M. oleifera* concurrently with ciprofloxacin would yield greater effectiveness in the treatment of infections in which *P. aeruginosa* is implicated than when either ciprofloxacin or the extract is used alone.

Key words: *Moringa oleifera* leaf, antibacterial interaction, checkerboard technique, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, ciprofloxacin.

INTRODUCTION

In tropical countries, infectious diseases account for approximately one half of all deaths and are considered major threats to human health due to unavailability of vaccines or limited chemotherapy. They continue to be a

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growing public health concern and have become the third leading cause of death since 1992, with an increase of 58% (Iwu et al., 1999; Kone et al., 2006; Pinner et al., 1996). Unfortunately, most of the current antibiotics have considerable limitations in terms of antimicrobial spectrum, side effects and their widespread overuse has led to increasing clinical resistance of previously sensitive micro-organisms and to the occurrence of uncommon infections (Cos et al., 2006; Ofokansi et al., 2013). The upsurge in side effects of many synthetic and semisynthetic anti-microbial agents in addition to multidrug resistant bacteria has spurred scientists on the research for plant-based antimicrobials of therapeutic potential (Betoni et al., 2006; Lewis and Ausubel, 2006; Lee et al., 2007).

The primary benefit of using plant-derived medicine is that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatments (Ajali and Okoye, 2009). In recent times, focus on plant research has increased all over the world and a lot of evidence has been collected to show immense potential of medicinal plants used in various traditional systems (Dahanuka et al., 2002). Plants may become the bases for the development of a new medicine or they may be used as phyto-medicine for the treatment of disease (Iwu et al., 1999). It is estimated that today, plant materials are present in, or have provided the models for 50% Western drugs (Giridhari et al., 2011). The study of antibacterial activity of medicinal plants is based on the investigation of active principles such as alkaloids, saponins, tannins, flavonoids, glycosides, vitamins and volatile oils (Iwu, 1993; Trease and Evans, 2002; Sofowora, 2008; Okore, 2009). These active principles reside in parts of plants such as the leaves, stems, barks, roots, fruits, seeds and flowers. However, certain substances (lignin, starch, cellulose and chitin) could modify or inhibit these activities of medicinal plants making it imperative to carry out extraction, characterization and identification of active principles as well as *in vitro* antimicrobial activity before proceeding to an *in vivo* trial (Okore et al., 2009; Kone et al., 2006; Cos et al., 2006).

Moringa oleifera Linn. (Family Moringaceae), also known as the horse-radish tree or drumstick tree, a rapidly-growing tree, native to Indian sub-continent, is now widely cultivated and has become naturalized in many locations in the tropics (Alam et al., 2011). The plant is rich in vitamins (A, B and C), minerals (such as calcium, potassium and iron), highly digestible proteins and carotenoids (including β-carotene or pro-vitamin A) (Fahey, 2000; Mensah et al., 2012; Dolly et al., 2009). Almost all parts of the plant have dietary as well as medicinal properties owing to its phytoconstituents. In particular, the iron content of the leaves is very good and prescribed for anaemia in the Northern Nigeria and the Philippines. The leaves are excellent sources of proteins and sulphur-containing amino-acids: methionine and cystine which are often in short supply in the plant kingdom (Mensah et al., 2012; Fozia et al., 2012). The leaf of the plant is widely used in folkloric medicine owing to its anti-tumor, hypotensive, anti-oxidant, radio-protective, anti-inflammatory and diuretic properties. *M. oleifera* has antibiotic, anti-tryponosomal, hypotensive, hypoglycemic, anti-diabetic and anti-inflammatory activities (Giridhari et al., 2011; Mensah et al., 2012). Specific phytochemicals of the plant that have been reported to possess hypotensive, anticancer and antibacterial activities include 4- (4'-O-acetyl-α-L-rhamnopyranosyloxy) benzyl isothiocy- anate, 4-(α-L-rhamnopyranosyloxy) benzyl isothiocy-anate, niazimicin, pterygospermin, benzyl isothiocyanate and 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate (Fahey, 2000; Akhtar and Ahmad, 1995; Anwar and Bhanger, 2003; Asres, 1995).

Ciprofloxacin is a synthetic broad spectrum fluoro-quinolone (Ofokansi et al., 2013). It has *in vitro* and *in vivo* activities against a wide range of Gram-negative and positive aerobic and anaerobic microorganisms, including *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Chambers, 2004). Ciprofloxacin inhibits bacterial deoxyri-bonucleic acid (DNA) gyrase and topoisomerase IV, enzymes essential for bacterial replication. Inhibition of topoisomerase IV interferes with separation of the replicated chromosomal DNA into the respective daughter cells during cell division whereas inhibition of DNA gyrase prevents the relaxation of positively supercoiled DNA that is required for normal transcription and replication (Radberg et al., 1990). Concurrent use of orthodox and herbal medicines is practiced in many urban and rural communities in Africa and Asia including many communities and cities in Nigeria. It is likely that certain interactions may be taking place, without detection, in persons who have this habit of concomitant use of orthodox medicines and herbal drugs. Such interactions may result in synergistic, antagonistic, indifferent or additive effects (Ofokansi et al., 2008, 2012; Esimone et al., 2002).

A lot of work has been carried out regarding the interaction of herbal extracts and ciprofloxacin (Ofokansi et al., 2013; Esimone et al., 2002; Ofokansi et al., 2012). The interest in the present study is being spurred by our observation, over the years, that a large number of people habitually use *M. oleifera* as a dietary supplement and a good number of these people usually continue in this habit unsuspectingly even when they are placed on one kind of drug or the other including antibiotics such as ciprofloxacin. To the best of our knowledge, there has not been any reported work on the interaction between ciprofloxacin and *M. oleifera* ethanolic leaf extract. Consequently, the objective of this study was to investigate, *in vitro*, the interaction of crude ethanol leaf extract of *M. oleifera* and ciprofloxacin and their effect, in combination, on isolates of *S. aureus* and *P. aeruginosa* using the Checkerboard method. The result obtained would help to a great extent in designing a highly effective antibiotic combination against infections caused by these bacteria.

MATERIALS AND METHODS

Analytical grades of ethanol 99% (Fluka, Germany) and dimethyl-sulphoxide, DMSO (Merck, Germany) were used for extraction and dilution respectively of the *M. oleifera* leaf extract. Distilled water was collected from an all-glass still. Nutrient agar (Fluka, Germany), Mueller Hinton agar (Oxoid, England) and Nutrient broth (Biotech, Germany) were used as media for the study. Ciprofloxacin pure powder (Juhel Nig. Ltd., Nigeria) was used as synthetic antibiotic.
Cultures of *S. aureus* ATCC 1370 and *P. aeruginosa* ATCC 9648 were obtained from stock cultures in the Pharmaceutical Microbiology Laboratory, Department of Pharmaceutics, University of Nigeria, Nsukka.

Collection and identification of plant material

Fresh leaves of *M. oleifera* were obtained in June, 2012 from Akokwa, in Isikwuato Local Governement Area of Imo State, Nigeria. Their botanical identities were determined and authenticated by Mr. A. Ozioko, a taxonomist with the International Centre for Ethnomedicine and Drug Development (INTERCEDD), Nsukka. The voucher specimen was deposited at the centre for future references.

Preparation of the *M. oleifera* leaf ethanol extract

The *M. oleifera* leaves were air dried under shade for two consecutive days and then pulverized using electric blender at the Soil Science Department of the University of Nigeria, Nsukka. Approximately 500 g of the fine powder was extracted with 2 L of ethanol (90% v/v) using a Soxhlet apparatus. The extract was further filtered, allowed to evaporate to a semi-solid residue and stored at 25°C until required for use.

Preparation of culture media and standardization of stock microbial cultures

All culture media were prepared according to the manufacturer's specification. Appropriate quantity of the media as calculated was dissolved in the required amount of solvent (distilled water). Heat was applied to aid dissolution. They were then dispensed into bijou bottles and sterilized in the autoclave at 121°C for 15 min.
The stock microbial cultures were maintained on nutrient agar slants at 4°C. For each round of experiment, the isolates were activated, by sub-culturing into 5 mL sterile nutrient broth and incubated at 37°C for 18 - 24 h. The isolates were standardized by dilution (1:100) using sterile distilled water which was a modification of the method employed by Grierson and Afolayan (1999).

Preliminary antimicrobial screening

Preliminary antimicrobial screening of the *M. oleifera* leaf extract was carried out using the agar dilution method (Ofokansi et al., 2008; Esimone et al., 2002; Ofokansi et al., 2012). Molten Mueller-Hinton agar (19 mL) in a sterile Petri dish (a plate for each dilution) was seeded with 1 mL of each of the two-fold dilution of the extract in DMSO (100, 50, 25, 12.5, 6.25 and 3.125 mg/mL) and thoroughly mixed. The agar plates were allowed to set and thereafter the plates were dried at 37°C for 1 h and a loopful of *S. aureus* broth culture was inoculated on the agar surface. The incubation was done at 37°C for 24 h and thereafter the plates were observed for growth. The experiment was repeated for *P. aeruginosa*. A control experiment was also set up against each test organism using DMSO as a control diluent. The whole experiment was similarly repeated for 100 mg/mL of ciprofloxacin using sterile distilled water as the solvent for dilution.

Determination of the minimum inhibitory concentration (MIC)

The MIC of the *M. oleifera* leaf extract was obtained using the agar dilution technique (Ofokansi et al., 2008). A stock solution of the extract (2 g/mL) was prepared by dissolving 10 g of the extract in 5 mL of 50% DMSO (one part of DMSO in one part of water). Then two-fold serial dilutions were made with sterile distilled water to obtain concentrations down to 62.5 mg/mL. A volume of each of the concentrations equal to 1 mL was transferred into an agar plate and made up to 20 mL with Mueller-Hinton agar and then allowed to set. The surface of the agar was then dried and streaked with isolates. An over-night (24 h) broth culture was used for this experiment. The same procedure was repeated with ciprofloxacin but in this case a stock solution of 100 mg/mL was prepared and the final concentrations obtained in agar plates ranged from 100 to 0.0001 mg/mL. Control plate having 5 mL of 50 % DMSO in 15 mL of molten agar was prepared for *M. oleifera* leaf ethanol extract. The plates were then incubated at 37°C for 24 h. The MIC was taken to be the lowest concentration which showed no visible growth of each of the test isolate on the agar surface.

Evaluation of the interaction between *M. oleifera* leaf extract and ciprofloxacin

Two stock solutions of ciprofloxacin and *M. oleifera* leaf ethanolic extract were prepared for evaluation of their combined effect on *S. aureus* and *P. aeruginosa.* Ciprofloxacin and *M. oleifera* ethanol leaf extract solutions were prepared with DMSO in sterile test tubes, each containing twice their individual MICs (32 and 10,000 µg/mL respectively against *P. aeruginosa* and 1 and 10,000 µg/mL respectively against *S. aureus*). The two agents were mixed in varying ratios of 0:10, 1:9, 2:8……. to 10:0 of *M. oleifera* leaf extract and ciprofloxacin in accordance with the continuous variation Checkerboard technique (Esimone et al., 2002; Ofokansi et al., 2012). Each of the eleven combinations of these two antimicrobial agents was serially diluted (2-fold) in 3 mL of DMSO into eight places. A 2 mL volume of each of the dilutions of the stock mixtures was seeded into 18 mL of molten Mueller-Hinton agar. After setting, the surface of the agar was then streaked with the test microorganisms. The streaked agar plates were then incubated at 37°C for 24 h. The combined effect of the antimicrobials on the test microorganisms was determined and recorded from the fractional inhibitory concentration (FIC) index. The FIC index was calculated as follows (Ofokansi et al., 2013):

$$FIC_{Index} = FIC_{Cp} + FIC_{ML} \quad (1)$$

$$FIC_{Cp} = \frac{\text{MIC of ciprofloxacin in combination with Moringa oleifera leaf extract}}{\text{MIC of ciprofloxacin alone}} \quad (2)$$

$$FIC_{ML} = \frac{\text{MIC of Moringa oleifera leaf extract in combination with ciprofloxacin}}{\text{MIC of Moringa oleifera leaf extract alone}} \quad (3)$$

Where Cp is the drug ciprofloxacin, M is *M. oleifera* ethanol leaf extract, FIC_{Cp} is the fractional inhibitory concentration of ciprofloxacin and FIC_{ML} is fractional inhibitory concentration of *M. oleifera* leaf extract.

RESULTS AND DISCUSSION

The MIC values of the ethanol extract of *M. oleifera* leaf

Table 1. The combined antibacterial effect of the ethanol extract of *M. oleifera* leaf and ciprofloxacin against *S. aureus*.

Drug combination ratio (Cp : MO)	MIC of Cp (µg/mL)	MIC of MO (µg/mL)	FIC of Cp	FIC of MO	FIC Index	Effect
10:0	-	-	-	-	-	-
9:1	-	-	-	-	-	-
8:2	0.0500	5000	0.80	0.20	1.00	Add
7:3	0.0438	7500	0.70	0.30	1.00	Add
6:4	0.0375	10000	0.60	0.40	1.00	Add
5:5	0.3125	12500	0.50	0.50	1.00	Add
4:6	-	-	-	-	-	-
3:7	-	-	-	-	-	-
2:8	-	-	-	-	-	-
1:9	-	-	-	-	-	-
0:10	-	-	-	-	-	-

Add = Additivity; MIC of Cp and MO evaluated from agar dilution method against *S. aureus* were 0.00062 ± 0.0001 and 25.0 ± 0.3 mg/mL, respectively.

Table 2. The combined antibacterial effect of the ethanol extract of *Moringa oleifera* leaf and Ciprofloxacin against *P. aeruginosa*.

Drug combination ratio (Cp : MO)	MIC of Cp (µg/mL)	MIC of MO (µg/mL)	FIC of Cp	FIC of MO	FIC Index	Effect
10:0	0.5000	0.0000	1.00	0.00	1.00	Add
9:1	0.2250	2500	0.45	0.05	0.50	Syn
8:2	0.2000	5000	0.40	0.10	0.50	Syn
7:3	0.1750	7500	0.35	0.15	0.50	Syn
6:4	0.1500	10000	0.30	0.20	0.50	Syn
5:5	-	-	-	-	-	-
4:6	-	-	-	-	-	-
3:7	-	-	-	-	-	-
2:8	-	-	-	-	-	-
1:9	-	-	-	-	-	-
0:10	-	-	-	-	-	-

Syn = Synergism; Add = Additivity; MIC of Cp and MO evaluated from agar dilution method against *P. aeruginosa* were 0.0005 and 50.0 mg/mL, respectively.

against *S. aureus* and *P. aeruginosa* were determined to be 25.0 and 50.0 mg/mL respectively while that of ciprofloxacin was calculated to be 0.00062 and 0.0005 mg/mL against *S. aureus* and *P. aeruginosa*, respectively. Tables 1 and 2 show the results of the combined antimicrobial effect of the ethanol extract of *M. oleifera* leaf and ciprofloxacin against the test microorganisms.

Table 1 shows the combined activity of ethanol extract of *M. oleifera* leaf and ciprofloxacin against *S. aureus*. The combinations predominantly showed additive effects at Cp : MO ratios of 8:2, 7:3, 6:4 and 5:5. In Table 2, additivity was observed in the combination ratio of 10:0 (Cp/MO) while other combinations (9:1, 8:2, 7:3 and 6:4) yielded predominantly synergistic effect against *P. aeruginosa*. Other combining ratios could show antagonism or even indifference against the test organisms but this was not observed since no MIC was obtained from such combination ratios.

Antimicrobial substances are desirable tools in the control of undesirable microorganisms especially in the treatment of infections and in preservation of food. The active components usually interfere with the growth or metabolism of microorganisms in a negative manner (Ofokansi et al., 2013).

The preliminary sensitivity screening shows that the ethanol extract of *M. oleifera* leaf possesses activity against *S. aureus* (a Gram positive bacterium) and *P. aeruginosa* (a Gram negative bacterium) (Chambers, 2004). The effect produced by the ethanol extract of *M. oleifera* leaf is however lower than that of the standard drug (ciprofloxacin). This suggests that higher concentrations of the extract could produce comparable antimicrobial results. The antimicrobial activity of an agent is usually quantified by determining the MIC values which serve as a guide for treatment of most infections (Ofokansi et al., 2012). Thus, the result of the preliminary antimicrobial screening was further supported by the MICs of the extract which were 25.0 and 50.0 mg/ml against *S. aureus* and *P. aeruginosa*, respectively. This shows that the active principles of the *M. oleifera* leaf are

active against the test organisms, consistent with previous studies (Abdulmoneim and Abu, 2011; Karthy et al., 2009; Suarez et al., 2005; Fisch, 2004). The MIC results indicate that unlike the ethanol extract of *M. oleifera* leaf, which showed only a marginal activity against *S. aureus* and *P. aeruginosa*, ciprofloxacin showed very high activities against the two organisms as expected, being a broad-spectrum highly active fluoroquinolone (Chambers, 2004; Radberg et al., 1990; Esimone et al., 2002; Ofokansi et al., 2012).

The problem of antimicrobial resistance has considerably reduced since the inception of combined antimicrobial chemotherapy; hence, the combination of two or more antimicrobials has for many years, been recognized as an important method for preventing or at least delaying bacterial resistance (Ofokansi et al., 2013, 2012). In this study, the Checkerboard method was adopted for the evaluation of the antibacterial effects of ciprofloxacin and ethanolic leaf extract of *M. oleifera* and fractional inhibitory concentration (FIC) index was used to assess the nature of the observed effects. The FIC index is interpreted as synergism if its value is less than 1.0, additivity if it is equal to 1.0, indifference if it is more than 1.0 but less than 2.0 and antagonism if it is more than 2.0 (Ofokansi et al., 2008; Esimone et al., 2002). A more critical look at Tables 1 and 2 would reveal that the combined effect of the two antimicrobial agents depends on the type of the test microorganism employed as exemplified by *P. aeruginosa* and *S. aureus*. It is clear from Tables 1 and 2 that, while the combined antimicrobial effect against *S. aureus* was predominantly additive, synergism was recorded in most of the Cp : MO combinations against *P. aeruginosa*. It was equally observed that the synergy and additivity recorded for combinations of ciprofloxacin and *M. oleifera* leaf ethanol extract against *P. aeruginosa* and *S. aureus* respectively was independent of the ratios of the combination. However, it is discernible from Table 2 that the highest potency of *M. oleifera* ethanol leaf extract in combination was found at MIC combinations of 250:0.225 (*Moringa* : ciprofloxacin), where the MIC of the extract was reduced by 200-fold. This implies that *M. oleifera* ethanol leaf extract is most active at this concentration against *P. aeruginosa*. For this plant extract, it is possible that the antibacterial principles reside within the secondary metabolites and the effects are more pronounced when used together than when used singly. A probable explanation of the enhanced activity in combination of CP : MO, particularly the potentiation of the effect of ciprofloxacin on *Pseudomonas aeruginosa* by *M. oleifera* is that the ciprofloxacin and the antimicrobial principles in ethanol extract of *M. oleifera* leaf may possibly have same mechanism of action or may be inhibiting a common step in the same biosynthetic pathway of the organism resulting in an overall synergy at certain combinations. Ciprofloxacin is known to act by preventing bacterial replication through inhibition of DNA gyrase. Although the mechanism of action of *M. oleifera* leaf extract is yet to be completely elucidated, pterygospermin, the main active constituent of *M. oleifera* has severally been reported to be responsible for its antimicrobial activity (Giridhari et al., 2011; Fahey, 2000; Mensah et al., 2012; Fozia et al., 2012; Anwar and Bhanger, 2003). More so, it has been documented that *M. oleifera* inhibits transaminase, an important enzyme in bacterial protein synthesis (Abduhnoneim and Abu, 2011; Karthy et al., 2009; Suarez et al., 2005; Fisch, 2004). Since both drugs target cellular activity, synergism or at least additivity is expected. The accumulation of both drugs in the cell could be responsible for the synergistic/additive effect observed at certain combination ratios. However, it has been noted that two antimicrobial agents may interact antagonistically if one is bacteriostatic and the other is bactericidal (Betoni et al., 2006).

Moreover, synergy was observed in most of the combination ratios with *M. oleifera* ethanol leaf extract against *P. aeruginosa* indicating that the organism is more sensitive than *S. aureus* to the leaf extract of *M. oelifera*. This could be seen to mean a potentiation of the effect of ciprofloxacin against *P. aeruginosa* in the presence of ethanol extract of *M. oleifera* leaf. The results suggest that it could be more therapeutically beneficial to use the combined extract and ciprofloxacin against infec-tions caused by *P. aeruginosa*, an opportunistic, nocosomial pathogen of immuno-compromised individuals, which not only colonizes medical devices (e.g., catheters) and infects the pulmonary tract, urinary tract, burns, wounds but also causes blood infections, infections of burn injuries and of the outer ear (otitis externa) (Ofokansi et al., 2013; Abduhnoneim and Abu, 2011; Karthy et al., 2009; Suarez et al., 2005; Fisch, 2004). In that case, greater antibacterial effect could be obtained at lower doses of each agent thereby minimizing their possible adverse effects and resistance of *P. aeruginosa* to these agents.

Conclusions

In conclusion, combination chemotherapy is clinically adopted to achieve a broad-spectrum coverage of invading organisms and to prevent the emergence of resistant organisms. Owing to the variability in the characteristics of microorganisms to antimicrobial agents, and their combinations, the clinical application of any combination requires the prior *in vitro* determination of the usefulness of the combination in any particular disease state. This study has provided a preliminary evidence of some kind of antibacterial interaction between ethanol extract of *M. oleifera* leaf and ciprofloxacin against *P.aeruginosa* and has established that the use of *M. oleifera* concurrently with ciprofloxacin would yield greater effectiveness in the treatment of infections in which *P. aeruginosa* is implicated than when either ciprofloxacin or the extract is used

alone. The combined effect of the interaction against *S. aureus* may not be highly significant at some ratios of combination of ciprofloxacin and the ethanol extract of *M. oleifera* leaf. Further *in vivo* studies would be required to assess the potential usefulness of these preliminary results in real infectious states when *P. aeruginosa* or *S. aureus* is the invading bacterium.

Conflict of Interests

The author(s) have not declared any conflict of interests

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Full Length Research Paper

Two-dimensional profiling of *Xanthomonas campestris* pv. *viticola* proteins extracted by four different methods

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An efficient method for protein extraction is a prerequisite for the successful implementation of proteomics, which is being used as a tool in the study of the interaction between plants and phytopathogens. With the objective to optimize a method of protein extraction for proteomic analysis of the phytobacterium *Xanthomonas campestris* pv. *viticola*, the efficiency of four methodologies were compared, based on the two-dimensional gel electrophoresis profile (2D-PAGE). Trizol®, phenol, centrifugation and lysis methods were tested and through quantitative and qualitative analysis, the most suitable method to obtain high-quality protein was selected. All methodologies enabled the extraction of a significant amount of proteins; nevertheless, the centrifugation method allowed obtaining the highest concentration of solubilized proteins. However, the analysis of the 2D-PAGE gel images revealed a larger number of spots in the lysis method when compared to the others. Taking into consideration the quality of the results and the practical advantages of the lysis method, this is recommended as the best option for total protein extraction of *X. campestris* pv. *viticola* for proteomic studies.

Key words: Bacterial canker, *Vitis vinifera*, proteomics, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional gel electrophoresis (2D-PAGE).

INTRODUCTION

Xanthomonas campestris pv. *viticola* (*Xcv*) (Nayudu) Dye is the causal agent of bacterial canker, one of the most important grapevine (*Vitis vinifera* L.) diseases, responsible for severe damage and representing a serious potential risk to Brazilian viticulture (Silva et al., 2012). Outside of Brazil, this disease occurs only in India (Jambenal et al., 2011) and Thailand (Buensanteai, 2004), where, anyway, severe losses have not yet been

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recorded. The characteristic symptoms of the disease are cankers in the branches, petioles and stems. Small dark and angular lesions are observed in the leaves; these lesions necrose large areas of the leaf when they coalesce. The veins become necrosed, particularly on the lower surface of the leaf blade (Nayudu, 1972; Trindade et al., 2007). Vein necrosis is an important symptom for diagnosis of the disease when the leaf lesions are atypical and cankers are absent. The berries of infected plants are non-uniform in size and color and may exhibit necrotic lesions (Rodrigues et al., 2011). For the successful control of bacterial canker of grapevine, it is necessary to understand the characteristics of *X. campestris* pv. *viticola* and the pathogenesis mechanisms involved in this plant-pathogen interaction, which have not yet been fully clarified (Tostes et al., 2014). Thus, proteomics technologies can integrate the basic knowledge necessary for the understanding of the mechanisms that phytobacteria use to cause diseases in their host (Norbeck et al., 2006).

Proteomics is defined as the analysis of proteins expressed by a cell or any biological sample at a given time and under specific conditions (Dierick et al., 2002). Proteins are functional molecules that play key roles in cells (Görg et al., 1995), being important for comprehensive understanding of any biological system (Beranova-Giorgianni, 2003). In comparison with genomic studies, investigations of the proteome provide detailed information, such as the abundance of proteins and post-translational modifications (Galdos-Riveros et al., 2010).

Among the various technologies used for the investigation of protein expression on a large scale, two-dimensional gel electrophoresis (2D-PAGE) stands out. This method separates proteins using a relative isoelectric point and molecular weight on its mobile base in a polyacrylamide gel matrix (Kim et al., 2007). The spots generated are used to create databases. However, it is necessary to obtain high quality protein samples, that is, free from contaminants (high levels of salts, nucleic acids, polysaccharides, phenolic compounds, pigments and other compounds) that can interfere with 2D-PAGE (Chan et al., 2002, 2004a, 2004b). Thus, an efficient method of extraction is a prerequisite for the successful implementation of proteomics (Mehmeti et al., 2011) for studies of the plant-pathogen interaction and continues to be a challenge for scientists (Natarajan et al., 2005). In this context, four methodologies to extract proteins from the phytobacterium *X. campestris* pv. *viticola* were tested, in order to optimize the sample preparation for two-dimensional electrophoresis.

MATERIALS AND METHODS

Culture conditions

The isolate of *X. campestris* pv. *viticola* (*Xcv* 137) used in the experiments was obtained from the Culture Collection of the Phytobacteriology Laboratory of the Federal Rural University of Pernambuco (Universidade Federal Rural de Pernambuco), Brazil. It was grown in 20 ml of NYD liquid medium (10 g/l dextrose, 5 g/l peptone, 5 g/l yeast extract and 3 g/l meat extract) for 24 h at 28°C under shaking (150 rpm) to obtain the pre-inoculate. The concentrations of the bacterial suspensions were adjusted to A570 = 0.4 (10^8 CFU/ml) using a spectrophotometer (Analyser 500 M, São Paulo, Brazil). Following this, 180 ml of the same NYD medium were added and the culture maintained under the same growth conditions for 24 h.

Protein extraction

In this study, four different extraction methods (Trizol®, phenol, centrifugation and lysis) were used to extract protein from a suspension of bacterial cells grown in NYD medium. The bacterial suspensions were then centrifuged at 10 000 x *g* for 5 min (CENTRIFUGE MCD-2000, Shanghai, China) and washed three times with saline solution (0.9% NaCl). The pellets were stored at 20°C and used in each method. Three biological replicates (independent cultures) were performed for each method.

Trizol method

The protocol was carried out according to manufacturer's instructions of Trizol® (Invitrogen®, Carlsbad, USA), modifying only the protein resolubilization step by using 0.5 ml of rehydration buffer without the bromophenol blue (7 M urea, 2 M thiourea, 4% CHAPS) instead of washing solution (0.3 M guanidine hypochlorite in 95% ethanol).

Phenol method

The bacterial pellet was washed in phosphate buffer (1.24 g/l K_2HPO_4, 0.39 g/l KH_2PO_4, 8.8 g/l NaCl, pH 7.2) and 0.75 ml of extraction buffer (0.7 M sucrose, 0.5 M Tris-HCl, 30 mM HCl, 50 mM EDTA, 0.1 M KCl and 40 mM DTT) was added, followed by incubation for 15 min at 28°C. The same volume of phenol was added, and after 15 min of agitation in a vortex, the suspension was centrifuged at 14 000 × *g* for 6 min at 4°C and the phenolic phase was recovered. This procedure was repeated two more times. Proteins were precipitated with the addition of five volumes of 0.1 M ammonium acetate in methanol (Mehta and Rosato, 2003). The precipitate was washed with 1 ml of 80% acetone and resolubilized as described in the previous paragraph.

Centrifugation method

Resuspension of the bacterial pellet was performed in 500 µl of extraction buffer (0.3% SDS, 200 mM DTT, 28 mM Tris-HCl and 22 mM Tris). Subsequently, the Eppendorf tube containing the cell suspension was gently agitated for 10 min at 4°C. Afterward, the sample was centrifuged at 14 000 × *g* for 10 min at 4°C, incubated at 100°C for 5 min and then cooled on ice. Next, 24 µl of assay buffer (24 mM Tris, 476 mM Tris-HCl, 50 mM $MgCl_2$, 1 mg/ml DNAse I and 0.25 mg/ml RNAse A) were added, and the sample incubated on ice for 15 min. The reaction was stopped by the addition of four volumes of ice cold acetone and precipitation of proteins was left to occur on ice for 20 min. Cell debris were removed by centrifugation at 14 000 × g for 10 min at 4°C (Giard et al., 2001). The pellet was dissolved by using 0.5 ml of rehydration buffer (7 M urea; 2 M thiourea; 4% CHAPS), and incubated at 50°C for 2 h.

Table 1. Quantification of proteins of *Xanthomonas campestris* pv. *viticola* obtained by four different methods of extraction.

Method	Concentration (µg/µl)
Centrifugation	9.1a ± 0.17
Trizol®	8.6b ± 0.17
Lysis	7.8c ± 0.17
Phenol	7.2d ± 0.15

Values are means ± standard deviation (SD) of three technical replicates. Low case letters a, b, c, d indicate significant differences using Tukey's test ($p < 0.05$).

Lysis method

Bacterial pellet was resuspended in 0.5 ml rehydration buffer (7 M urea; 2 M thiourea; 4% CHAPS), homogenized in a vortex for 5 min and centrifuged at 10 000 x *g* at 4°C for 30 min. The supernatant was then transferred to a new 1.5 ml tube (Jangpromma et al., 2007).

Quantification of proteins

The concentration of total cellular proteins obtained with each extraction method was determined by the 2-D Quant Kit, according to the manufacturer's instructions (GE Healthcare®, Piscataway, NJ, USA). Bovine serum albumin (BSA) was used as standard and the assay was performed by measuring the absorbance at 480 nm. This kit was selected as it does not interfere or interact with any chemicals used during the extractions and is therefore compatible with isoelectric focusing (IEF). The samples and the standards were read in triplicate.

One-dimensional gels (SDS-PAGE)

For the preparation of the SDS-PAGE gel the methodology of Laemmli (Laemmli, 1970) was used, which involved a 15% polyacrylamide separation gel and a 4% concentration standard molecular weight marker (High-Range Amersham™ Rainbow™) from GE Healthcare®. In each well, 30 µg of protein were loaded. Electrophoresis ran at 40 mA for 15 min and then at 100 mA for 2 h, in a vertical Owl P10DS cube (Thermo Scientific®, Hudson, New Hampshire, USA). The gels were stained using the reagent Coomassie brilliant blue (Coomassie Brilliant Blue G250) (Candiano et al., 2004) and bleached in a solution of 7.5% methanol and 5% glacial acetic acid until complete visualization of bands.

Two-dimensional gel (2D-PAGE)

The two-dimensional electrophoresis was performed in two stages according to the 2-D electrophoresis instructions of GE Healthcare®. In the first step, isoelectric focusing (IEF) was done, in which proteins were resuspended in rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS (w/v), 2 mM DTT, 1% IPG buffer (w/v) and 0.2% bromophenol blue).

The IEF was conducted using Ettan IPGphor 3 (GE Healthcare®) in 7 cm strips of immobilized pH gradient (IPG) ranging from 3 to 10 (Amersham Bioscience AB, Uppsala, Sweden) which were loaded with 150 µg of protein. Subsequently, the strips were balanced in reducing solutions of disulfide bridges containing DTT (dithiothreitol) and iodoacetamide (Görg et al., 1995). In the second step, 2D-PAGE electrophoresis was performed using a 15% polyacrylamide gel in an initial run of 15 mA for 20 min per gel, increasing to 45 mA per gel for about 3 h. The gels were stained as in the SDS-PAGE until complete visualization of spots.

Image analysis of gels

After staining, the gels were scanned using Image Scanner software (Amersham Biosciences) in transparency mode with a resolution of 300 dpi (dots per inch). The images of 2D-PAGE gels were analyzed using Image Master 2D-Platinum software, version 7.0 (Amersham Biosciences). The program provided the number of protein spots from each of the gels which was validated by visual inspection. For each biological replicate three technical replicates were made to confirm the reproducibility of the results.

The efficiency of the methodologies used in this study was evaluated by the qualitative parameters (resolution and intensity of bands) for SDS-PAGE and for both quantitative (amount of proteins and number of spots) and qualitative (resolution and intensity of spots, and reproducibility) parameters for 2D-PAGE.

Statistical analysis

Statistical analysis was made using the Statistix® software (version 9.0, Analytical Software, Tallahassee, USA). Data were analyzed by one way analysis of variance (ANOVA) followed by Tukey's test. In all statistical analyses, $p < 0.05$ was taken as the level of significance.

RESULTS AND DISCUSSION

For both SDS-PAGE and 2D-PAGE, which are techniques commonly used in proteomics, thorough and careful sample preparation is very important for the quantification and high resolution of proteins. Due to the different physical and chemical properties of proteins, an appropriate and standardized bioassay of a given sample, including protein extraction with different methods, favors their identification (Mehmeti et al., 2011).

In this study, four different extraction methods (Trizol®, phenol, centrifugation and lysis) were compared to determine which of them increase the solubilization of proteins of the *X. campestris* pv. *viticola*. All methodologies tested proved to be efficient in detecting a large and different ($p < 0.05$) amount of proteins (Table 1). According to Shi et al. (2013), complete solubilization of samples is the best way to achieve the goal of standardizing the recovery of proteins. The highest protein yield was obtained by the centrifugation method. The potential reasons for that may be the use of SDS in the centrifugation solution and the high temperature heating of 100°C, both recognized as critical in protein extraction (Shi et al., 2006). In the SDS-PAGE gel image analysis, the protein bands were sharp, well defined and without presenting characteristics of degradation (Figure 1).

The results of the two-dimensional gels were different

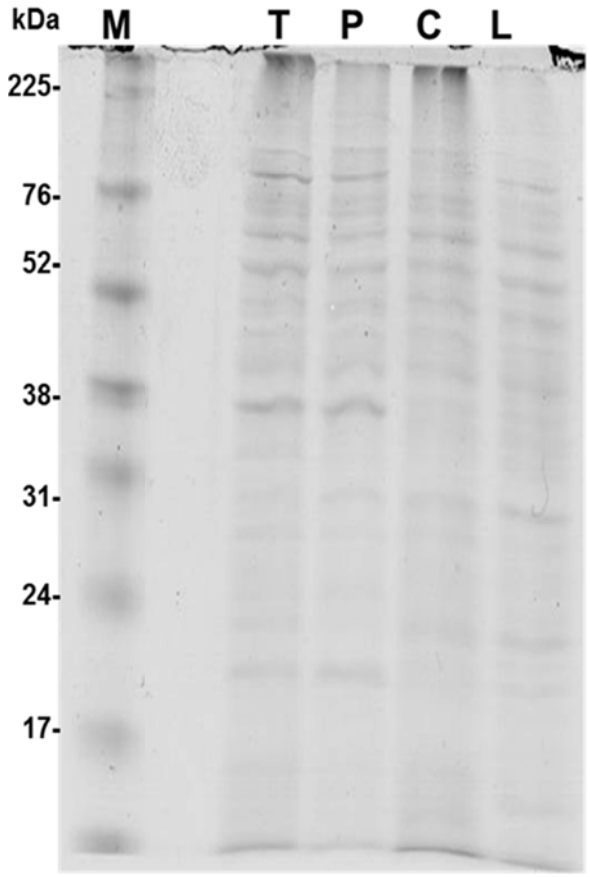

Figure 1. Representation of SDS-PAGE gel (15%) of proteins of *Xanthomonas campestris* pv. *viticola* extracted by four extraction methodologies: Trizol® (T), Phenol (P), Centrifugation (C) and Lysis (L); kDa marker (M).

in relation to the quality of the sample and numbers of spots obtained from the four methods studied (Figure 2). In the Trizol® method (Figure 2A) most of the rare and sparse spots were distributed throughout the pH range of acid and were between 76 to 24 kDa. The phenol method (Figure 2B) did not allow for a good quality sample, making focusing impossible; several horizontal stripes were observed in the gel, whose proteins were separated only by molecular weight.

According to Saravanan and Rose (2004), the presence of non-protein impurities can critically affect the quality of 2D-PAGE separation, resulting in the formation of spots and/or horizontal and vertical striations, and a notable decrease in the number of spots. In the centrifugation and lysis methods (Figure 2 C; D), the spots were more concentrated in the range of 225 to 24 kDa. The three technical replicates showed similar results for the number of spots. The lysis method presented the largest number of spots and differs significantly ($p < 0.05$) from the other extraction methods (Figure 3). Therefore, lysis method was defined as the best among the tested methods, since it allowed obtaining the highest number of spots with the best definition in 2D-PAGE gel.

Despite the lysis method did not provide the greatest concentration of proteins, as verified for centrifugation (Table 1), a high quality profile of proteins was observed in terms of resolution, number and intensity of the spots (Figure 2D). This result indicates that proteins with low abundance and high molecular weight were clearly revealed and detected in the 2D-PAGE gel by this method. Furthermore, it was observed that different sets of proteins were detected by the presence of bright spots with good resolution. The lysis method stands out not only for its greater representativeness of spots, but also for being a fast (about 1 h) and practical method, accessible to any laboratory. The most interfering materials (non-protein components) are effectively removed; the proteins are protected against degradation by proteases, thus not requiring the use of protease inhibitors. In addition, reagents used are of low cost and toxicity, when compared with other methodologies (Tan et al., 2011). This method greatly reduces the extraction time, which in turn, improves the quality of the sample.

A large amount of proteins of *X. campestris* pv. *viticola* was extracted by the phenol method (Table 1), however, the unsatisfactory results obtained from the analysis of 2D-PAGE (Figure 2B) indicated absence of different protein concentrations or inefficiency of the staining method. These two aspects are cited by Görg et al. (2004) as being more important than the concentration of protein for the success of 2D-PAGE analysis. Phenol is an effective solvent of proteins that can greatly reduce molecular interactions between proteins and other compounds that inhibit electrophoresis (Wang et al., 2007). This method has been successfully employed for protein extraction of *X. citri* subsp. *citri* (Mehta and Rosato, 2003; Soares et al., 2010), but its effectiveness was not proven under the extraction conditions of *X. campestris* pv. *viticola*.

Many techniques including physical methods and those based on detergents are available for cell disruption and protein extraction (Grabskia, 2009). These techniques can vary widely in reproducibility and in representation of the proteome and, thus, need to be adapted to the phytobacteria. Using various extraction methods, proteomic studies have been conducted with *Xanthomonas* spp. like *X. campestris* pv. *campestris* (Villeth et al., 2009), *X. oryzae* pv. *oryzicola* (Zhao et al., 2011), *X. oryzae* pv. *oryzae* (González et al., 2012), *X. axonopodis* pv. *passiflorae* (Tahara et al., 2003), *X. axonopodis* pv. *citri* (Zimaro et al., 2013) and *X. citri* subsp. *malvacearum* (Razaghi et al., 2012).

Conclusion

The results obtained in this work were satisfactory taking

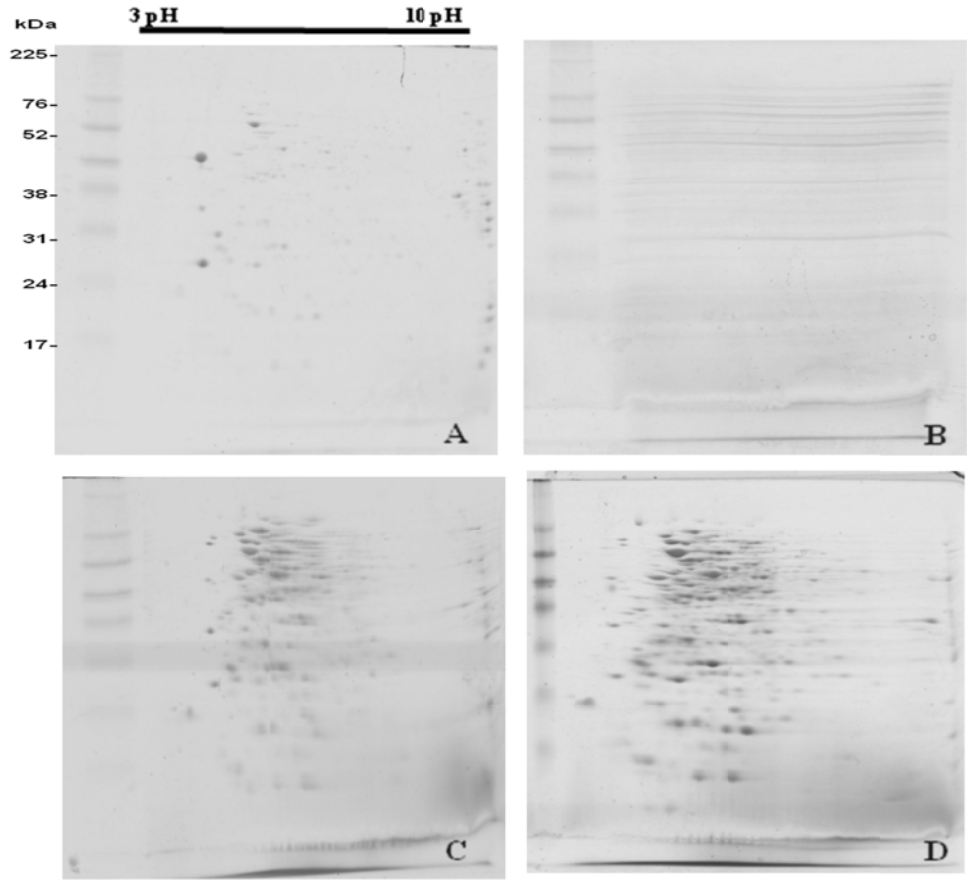

Figure 2. Representation of the 2D-PAGE gel of total proteins of *Xanthomonas campestris* pv. *viticola*, focused on strips of 7 cm pH 3-10, extracted by four methodologies: Trizol® (A), Phenol (B), Centrifugation (C) and Lysis (D).

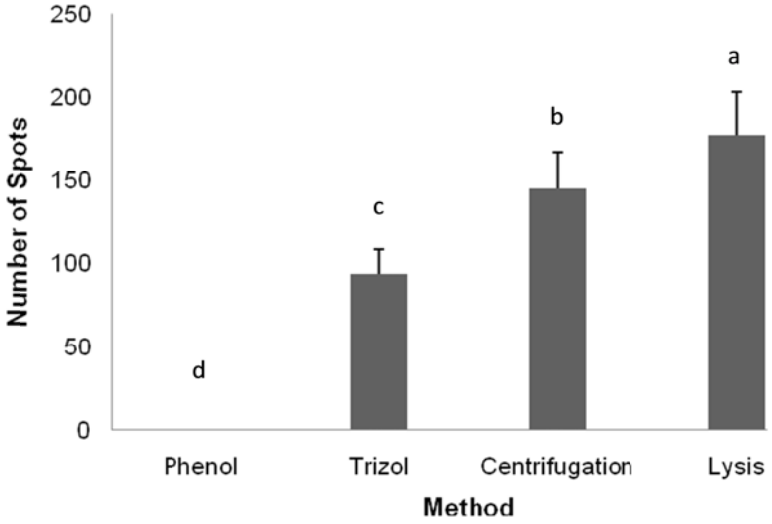

Figure 3. Number of spots identified by the Image Master 2D Platinum program in four different protein extraction methodologies of *Xanthomonas campestris* pv. *viticola*. Values are means ± standard deviation (SD) of three technical replicates. Low case letters a, b, c, d indicate significant differences using Tukey's test ($p < 0.05$).

into consideration that, in the literature consulted, no results were found of a single or a combination of methods developed for protein extraction of *X. campestris* pv. *viticola*, making this study probably the first. Therefore, considering the excellent profile of proteins obtained in 2D-PAGE analysis by the lysis method, this is recommended as the best option for total protein extraction of *X. campestris* pv. *viticola*. This extraction method can be used in proteomic research with this phytobacterium in order to study population diversity based on protein profile, detection of pathogenesis-related proteins, and biofilm formation, among others. This is an excellent opportunity to make great progresses in the understanding of plant-pathogen interaction, aiming at establishing efficient management measures of bacterial canker of grapevine.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Multidrug-resistant hepatocellular carcinoma cells are enriched for CD133$^+$ subpopulation through activation of TGF-β1/Smad3 pathway

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Chemotherapy is a main treatment for cancer, while multidrug-resistance is the main reason for chemotherapy failure, and tumor relapse and metastasis. Cancer stem cells or cancer stem-like cells (CSCs) are a small subset of cancer cells, which may be inherently resistant to the cytotoxic effect of chemotherapy. Some studies suggest that CSCs could be enriched by chemotherapy. However, the mechanism of chemotherapy regulating CSCs remains unknown. Therefore, we investigated whether drug treatment could enrich CSCs in hepatocellular carcinoma (HCC) cells and the molecular mechanism of chemotherapy regulating the expression of CSCs markers. In the present study, a multidrug-resistant (MDR) human HCC subline, Huh7.5.1/PTX, was developed by exposing parental cells to paclitaxel (PTX) repeatedly at a single high concentration. The cell counting kit-8 (CCK-8) assay was used to determine cellular sensitivity of various anticancer drugs. Flow cytometry (FCM) was used to analyze the CSCs markers expression level. Western blotting (WB) was used to analyze the changes of TGF-β1/Smads signaling. Our results show that PTX treatment of HCC cells *in vitro* resulted in a development of subline six months later, and Huh7.5.1/PTX, with stable MDR phenotype. Huh7.5.1/PTX cells enriched CSCs fraction and strongly activated the TGF-β1/Smad3 signaling. Activation of TGF-β1/Smad3 signaling resulted in enrichment of the CSCs population (CD133$^+$ cells), while inhibition of this pathway activity attenuated the percentage of these cells. Taken together, our results suggest that MDR HCC cells are enriched with CSCs, which is partially dependent on TGF-β1/Smad3 pathway. Inhibition of TGF-β1/Smad3 pathway may be useful for targeting CSCs to develop more effective treatments for HCC.

Key words: Hepatocellular carcinoma (HCC) CD133, chemotherapy, cancer stem cells, TGF-β1, Smad3.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world, the third leading cause of

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Abbreviations: CSCs, Cancer stem cells or cancer stem-like cells; **HCC,** hepatocellular carcinoma; **MDR**, multidrug-resistant or multidrug-resistance; **PTX**, paclitaxel.

cancer- related deaths and an aggressive tumor with a poor prognosis (Ferlay et al., 2010). Current curative treatments such as liver resection and transplantation are limited to the early disease stage. Chemotherapy has generally not improved overall mortality in HCC except for a recent report using sorafenib, which improved advance stage mortality by less than 3 months (Thomas et al., 2010). Therapeutic strategies against this disease target mostly rapidly growing differentiated tumor cells. However, the result is often dismal because of the chemo-resistant nature (Thomas et al., 2008).

Recent research efforts on stem cells and cancer biology have shed light on new directions for the eradication of CSCs in HCC (Zou, 2010a). The CSCs theory has been proposed to explain the tumor heterogeneity and the carcinogenesis (Reya et al., 2001). According to this model, tumor can be viewed as a result of abnormal organogenesis driven by CSCs, defined as self-renewing tumor cells able to initiate and maintain the tumor and to produce the heterogeneous lineages of cancer cells that consist of the tumor (Clarke et al., 2006). The existence of CSCs was first proven in acute myeloid leukemia (Lapidot et al., 1994), and more recently in many solid tumors including breast (Ponti et al., 2005), brain (Singh et al., 2003), prostate (Collins et al., 2005; Patrawala et al., 2006), pancreatic (Li et al., 2007), colon cancer (Ricci-Vitiani et al., 2007) and melanoma (Schatton et al., 2008). To date, it has been shown that CSCs in HCC can be identified by several cell surface markers, such as CD133 (Ma et al., 2007; Suetsugu et al., 2006; Yin et al., 2007; Zhu et al., 2010) and epithelial cell adhesion molecule (EpCAM) (Terris et al., 2010; Yamashita et al., 2009).

Chemotherapy is a main treatment for cancer, while MDR is the main reason for chemotherapy failure and tumor relapse (Zhou et al., 2009). Cancer often recurs after treatment and this can be attributed to the presence of CSCs. CSCs are a subpopulation of cancer cells, which may be inherently resistant to chemotherapy because of their low proliferation rate and resistance mechanisms, such as the expression of multidrug transporters of the ATP-binding cassette (ABC) superfamily (Dean et al., 2005). Some studies have suggested that chemotherapy has no effect on CSCs and can enrich CSCs (Bertolini et al., 2009; Dylla et al., 2008; Levina et al., 2008; Yu et al., 2007). Two recent reports suggested that pancreatic cancer cells resistant to chemoradiotherapy rich in stem-cell-like tumor cells (Du et al., 2011) and CSCs can be isolated with drug selection in human ovarian cancer cell line SKOV3 (Ma et al., 2010).

TGF-β1 (transforming growth factor- beta1) is a multipotent cytokine that plays an important biological effect on tissue and organ development, cellular proliferation, differentiation, survival, apoptosis and fibrosis (Ikushima and Miyazono, 2010; Kelly and Morris, 2010). In the liver, TGF-β1 is hypothesized to serve as an important link between chronic injury, cirrhosis, and HCC (Matsuzaki, 2009). Previous reports indicate that TGF-β1 expression is decreased in early-stage HCC and increased in late-stage HCC (Abou-Shady et al., 1999; Matsuzaki et al., 2000). A recent report indicated that dysregulation of the TGFβ pathway leads to HCC through disruption of normal liver stem cell development (Tang et al., 2008). Two more recent studies reported that the percentage of SP (side population) cells, a potent marker of stem cell, and CD133+ cells are increased by TGF-β treatment (Nishimura et al., 2009; You et al., 2010). Furthermore, their results suggested that the phenotypic change with increased aggressiveness in HCC cells caused by TGF-β stimulation may be relevant to the kinetics of CSCs (Nishimura et al., 2009; You et al., 2010).

It is believed that CSCs resist the radiotherapy and the cytotoxic effect of chemotherapy (Dean et al., 2005; Zhou et al., 2009). However, the relationship between chemotherapy and CSCs is not clear and needs to be further elucidated. Based on the potential role of TGFβ1 in liver cancer progression and the importance of CSCs in HCC, we hypothesized that chemotherapy can enrich liver CSCs through constituted activation of TGF-β1 pathway. Using Huh7.5.1 HCC cells and PTX, we developed a MDR HCC subline model, Huh7.5.1/PTX. Furthermore, we found that MDR Huh7.5.1/PTX cells showed high percentage of CD133, CD90 and EpCAM positive cells and strongly activated the TGF-β1/Smad3 signaling. Activation of TGF-β1/Smad3 signaling can lead to propagation of $CD133^+$ population, while inhibition of this pathway activity attenuated the percentage of these cells. In summary, our findings propose that CSCs could be enriched in MDR HCC cells, which is partially dependent on TGF-β1/Smad3 pathway.

MATERIALS AND METHODS

Cell line and cell culture

The human hepatocellular carcinoma cell line, Huh7.5.1, was kindly gifted from Dr. Wenyu Lin (Massachusetts General Hospital, Harvard Medical School). Huh7.5.1 cells were cultured in Dulbecco's modified eagle's medium/high glucose (DMEM/H) containing 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), and were incubated at 37°C in a humidified incubator with an atmosphere of 5%CO_2.

Reagents

DMEM/H, FBS and Trypsin-EDTA were purchased from Hyclone (Thermo Scientific). CCK-8) was obtained from Beyotime (Hangzhou, China). Paclitaxel (PTX), Cisplatin (DDP), gemcitabine (GEM), 5-fluorouracil (5-Fu), doxorubicin (ADM), and mitomycin (MMC) was obtained Shanghai Xudong Haipu Pharmaceutical Co. Ltd (Shanghai, China). Fluorochrome-conjugated antibodies against human CD29, CD34, CD44, CD54 and CD105 (ICAM-1), and CD133 and associated isotype control antibodies were from eBioscience, Inc (San Diego, CA USA) and CD90, CD326 (EpCAM), and CD338 (ABCG2) and associated isotype control antibodies were from Biolegend (San Diego, CA USA). Antibodies

against CD133, Smad3, Smad4, and phosphorylated Smad3 (pSmad3) were from Abcam Inc. (Abcam,Cambridge, MA). Cytokine TGF-β1 and antibodies against TGF-β1 and β-actin were from R&D Systems INC. (Minneapolis, MN). SIS3, a specific Inhibitor of Smad3[46], was from Merck (NJ, USA).

Establishment of a PTX-resistant Huh7.5.1 cell line (Huh7.5.1/PTX) *in vitro*

Huh7.5.1/PTX was produced by exposing Huh7.5.1 cells to PTX repeatedly at a single high concentration over a period of 12 h. Briefly, Huh7.5.1/PTX was selected by a procedure consisting of six pulse drug treatments with 5 μg/ml PTX. When Huh7.5.1 cells were growing exponentially, they were exposed to PTX for 12 h. The majority of the cells were dead following 12 h exposure to PTX. The treated cells were then washed with phosphate buffered saline (PBS) and cultured in PTX-free growth medium. After two to three days, the dead cells were washed out with PBS and fresh medium was added again. The resistant subclones were isolated by limiting dilution.

After four weeks' incubation at 37°C in a humidified atmosphere containing 5% CO_2, the cells recovered at an exponential rate and were then subcultured. Once cells reached 80-90% confluence, the cells were preserved and treated again as described above. The PTX-resistant subclone was established 6 months after the treatment was initiated, and the resistant phenotype developed. For maintenance of PTX -resistant cells, the Huh7.5.1/PTX cells were grown in the presence of 0.01 μg/ml PTX. Before experimentation, Huh7.5.1/PTX cells were maintained in a PTX-free culture medium and subcultured at least 3 times.

Detection of cellular sensitivity to anticancer drugs using CCK-8 assay

The MDR characteristics of these Huh7.5.1/PTX cells were tested using various concentrations of anticancer drugs including PTX, DDP, GEM, ADM, MMC and 5-FU. The effects of chemotherapeutic agents on the growth of Huh7.5.1 and Huh7.5.1/PTX cells were evaluated with CCK-8. Cells (5×10^3) were seeded into 96-well plates in 100 μL of DMEM/H with 10% FBS incubated at 37°C in a humidified atmosphere containing 5% mL/L CO_2. After 12 h, the medium was removed, and exchanged with media containing a test chemotherapeutic agent at various concentrations. After incubation for 48 h at 37°C, the drug-containing growth medium was replaced with 110 μL medium containing CCK-8 reagent. After 2 h, the absorbance was read at 450 nm with a reference wavelength at 600 nm. The experiment was replicated at least 3 times. The IC_{50}, defined as the drug concentration required to reduce cell survival to 50%, was calculated by probit regression analysis using SPSS 13.0 statistical software.

FCM analysis of cell surface markers expression levels

FCM was used to measure cell surface markers expression levels (CD11b, CD29, CD34, CD40, CD44, CD45, CD54, CD90, CD105, CD133, EpCAM and ABCG2 in Huh7.5.1 and Huh7.5.1/PTX cells). The cultured Huh7.5.1 and Huh7.5.1/PTX cells with or without SIS3, TGF-β1 and anti-TGF-β1 monoclonal antibody stimulation were collected by trypsinization, washed in ice-cold PBS, and then directly immunostained using fluorochrome- conjugated antibodies described above. The isotype control IgG was evaluated in each experiment to determine the level of background fluorescence of negative cells. Mean fluorescence intensity was determined for positively stained cells. Samples and results were analyzed using a Epics XL flow cytometer and WinMDI 2.9 software.

WB

The cultured Huh7.5.1 and Huh7.5.1/PTX cells with or without stimulation were lysed in radio-immuno-precipitation assay buffer. The samples were incubated for 2 h on ice. Samples were then centrifuged at 12 000 *g* for 15 min and protein concentrations were measured in the supernatants using a BCA protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Cell extracts were denatured in LDS sample buffer for 5 min at 95°C, and electrophoresed on a 10-20% SDS-PAGE and blotted onto PVDF membranes (0.2 μm, Invitrogen). Membranes were blocked with 5% milk or 5% bovine serum albulin (BSA) in TBS-T (TBS containing 0.05% Tween 20) for 1 h at room temperature and were subsequently incubated overnight at 4°C with primary antibodies described above. After incubation with the respective primary antibodies, membranes were washed three times for 5 min in TBS-T, and then incubated with species-specific horseradish peroxidase (HRP)-labeled secondary antibodies at 37°C for 1 h. The membrane was developed using the ECL Plus WB reagent (Biomiga) with visualization on X-ray films. The expression of β-actin was detected as an internal control.

Statistical analysis

All experiments were run at least three times, and the results are given as mean ± SD. Statistical analyses were performed using either a one-way analysis of variance (ANOVA) or Student T test. The difference was considered statistically significant when the P value was less than 0.05. All statistical analyses were carried out with GraphPad Prism 5 software.

RESULTS AND DISCUSSION

Huh7.5.1/PTX cells show higher chemotherapeutic resistance and MDR

To study the enrichment of CSCs in HCC by chemotherapy, we firstly developed a drug-resistant model. We compared the sensitivity of Huh7.5.1 cells to various drugs and found that Huh7.5.1 cells were most sensitive to PTX (Figure 1A). By exposing Huh7.5.1 cells to PTX repeatedly at a single high concentration over a period of 12 h, the PTX-resistant clones was established six months after the treatment was initiated. To test the resistance to anticancer drugs, we used CCK-8 assay to determine the effects of PTX, DDP, GEM, 5-Fu, ADM and MMC on the growth of Huh7.5.1 and Huh7.5.1/PTX cells. We found that besides PTX, Huh7.5.1/PTX cells were also more resistant to some other anticancer drugs including DDP, GEM, 5-Fu, ADM and MMC. Huh7.5.1/PTX cells showed high resistance to PTX and the IC_{50} (50% inhibitory concentration) of these drugs in Huh7.5.1/PTX cells were significantly higher than those in Huh7.5.1 cells (Figure 1B). Huh7.5.1/PTX cell showed MDR and varying degree of drug-resistance, high degree of PTX and DDP, medium degree of 5-Fu and ADM, and low degree of MMC and GEM concerning that RI (resistance index) of Huh7.5.1/PTX cells to PTX, DDP, GEM,5 -Fu, ADM and MMC was 15.70, 11.41, 5.00, 5.29, 2.26 and 2.31, respectively (Figure 1C).

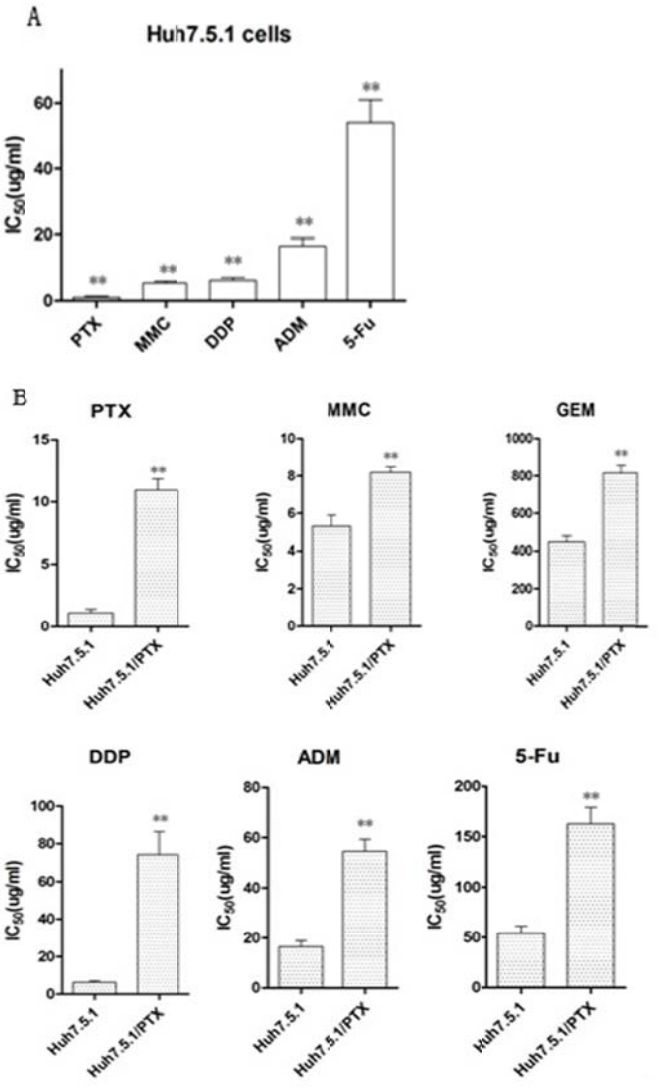
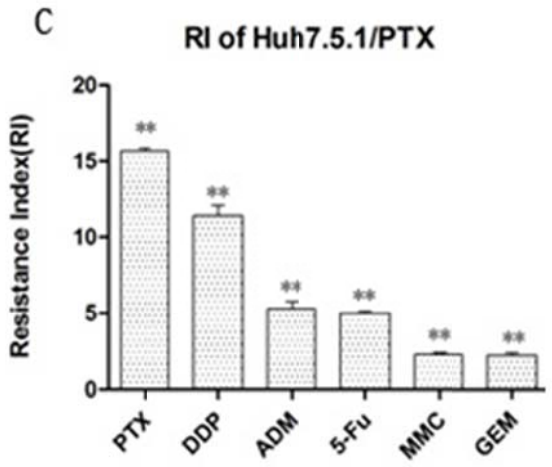


Figure 1. Huh7.5.1/PTX cells show higher chemotherapeutic resistance and have cross-resistance. A. IC_{50} (50% inhibitory concentration) of Huh7.5.1 cells to various drugs (including PTX, MMC, DDP, ADM and 5-Fu). Huh7.5.1 cells are most sensitive to PTX. **p<0.01 (one-way analysis of variance). **B.** Huh7.5.1/PTX cells show more resistance to PTX, DDP, GEM, 5-Fu, ADM and MMC than parental Huh7.5.1 cells. **p<0.01(Student t test). **C.** RI (Resistance Index) of Huh7.5.1/PTX cells to anti-cancer drugs. Huh7.5.1/PTX cells showed highest resistance to PTX and have multi-drug resistance. RI of Huh7.5.1/PTX cells to PTX, DDP, 5-Fu, ADM, GEM and MMC was 15.70, 11.41, 5.00, 5.29, 2.26 and 2.31, respectively. **p<0.01(one-way analysis of variance). Each value represents the mean ± standard deviation for at least three independent experiments.

Huh7.5.1/PTX cells express higher level of CSCs markers

Resistance to chemotherapy distinguishes CSCs from other cancer cells. As mentioned above, Huh7.5.1/PTX cells were resistant to chemotherapy. To examine whether



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chemotherapy might enrich for CSCs, we compared the proportion of CD133, CD90, CD44, CD326, CD338, CD29, CD34, CD54 and CD105 positive cells in Huh7.5.1 and Huh7.5.1/PTX cells by FCM. CD133, CD90, CD44 and CD326 have been reported to isolate stem-like cells from HCC cell lines or HCC patients tissues and they may be the candidate markers of liver CSCs.

Concerning our research results, we found that Huh7.5.1/PTX cells contained high percentage of these markers positive cells: (CD 133 69.9% vs. 19.4%, CD90 6.02% vs. 0.88%, CD44 90.1% vs. 2.57%, CD326 90.7% vs. 24.1%). Besides that, some markers were also expressed by a high level in Huh7.5.1/PTX cells including stemness-and drug-resistance-associated markers: CD338 (81.3% vs. 1.92%), CD34 (23.8% vs. 0.47%) and CD105 (98.7% vs. 3.61%). Huh7.5.1/PTX cells expressed low level of CD54 (64.8% vs. 94.3%) and did not express CD11b and CD45 (data not shown). CD133, CD90, CD44, CD326, CD338, CD34, CD54 and CD105 expression are statistically significant and there is no significant expression of CD29 in Huh7.5.1 and Huh7.5.1/PTX cells (Figure 2).

TGF-β1/Smad3 pathway is activated in MDR Huh7.5.1/PTX cells

To determine the activity of TGF-β1/Smad3 signaling, we compared the protein expression level of TGF-β1, Smad3, Smad4, pSmad3 and CD133 in parental and resistant cells by WB. Compared to the parental cell line, the protein level of CD133, TGF-β1 and pSmad3 were higher in MDR cells and total Smad3 did not changed (Figure 3). Our results show that MDR Huh7.5.1/PTX cells showed higher activity of TGF-β1/Smad3 signal, and these results are concordant with the results of percentage of CD133, CD90, CD326 and CD44 positive cells in MDR Huh7.5.1/PTX cells.

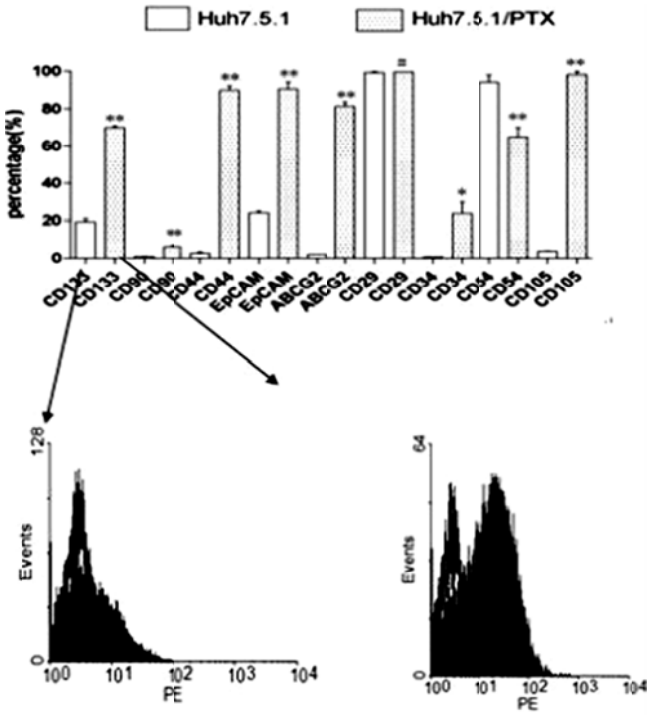


Figure 2. Huh7.5.1/PTX cells are enriched for cancer stem-like cells. The proportion of CD133, CD90, CD44, CD326，CD338, CD29, CD34, CD54 and CD105 positve cells in Huh7.5.1 and Huh7.5.1/PTX cells was examined by FCM.Huh7.5.1/PTX cells contained high percentage of these markers positive cells: (CD133 69.9% vs. 19.4%, CD90 6.02% vs. 0.88%,CD44 90.1% vs. 2.57%,CD326 90.7% vs. 24.1%) and also expressed high level of stemness-and drug-resistance-associated markers including CD338(81.3% vs. 1.92%), CD34 (23.8% vs. 0.47%) and CD105 (98.7% vs. 3.61%). Huh7.5.1/PTX cells expressed low level of CD54 (64.8% vs. 94.3%) and did not express CD11b, CD40 and CD45 (data not sown). There is no significant expression of CD29 (99.5% vs. 99.2%) in Huh7.5.1 and Huh7.5.1/PTX cells. Each value represents the mean ±standard deviation for at least three independent experiments. # p>0.05,*p<0.05, **p<0.01 (Student t test)

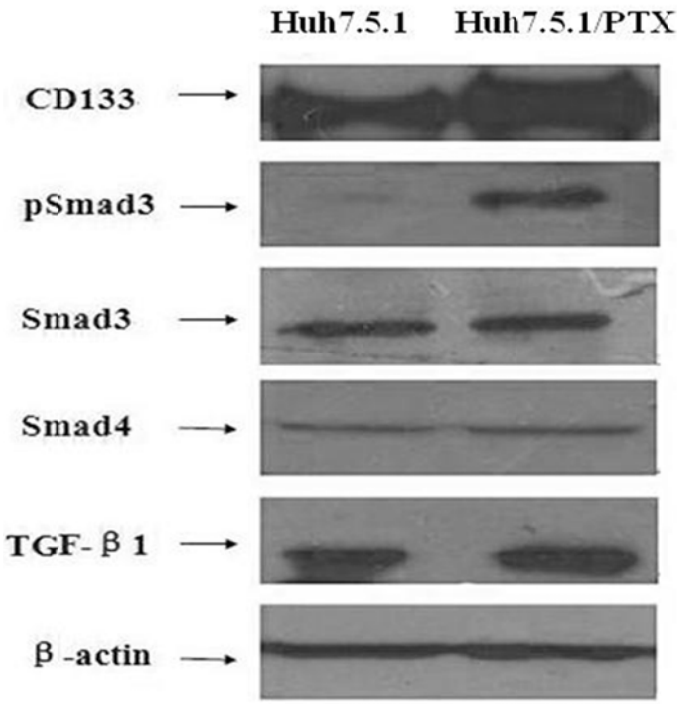


Figure 3. TGF-β1/Smad3 pathway is activated in chemo-resistant Huh7.5.1/PTX cells. The protein level of TGF-β1, Smad4, Smad3, pSmad3 and CD133 was compared between Huh7.5.1/PTX and Huh7.5.1 cells by WB. Huh7.5.1/PTX cells showed elevated expression level of CD133, TGF-β1, and phosphorylated Smad3 compared to Huh7.5.1 cells. Elevated expression of phosphorylated Smad3 in Huh7.5.1/PTX cells was not a result of an increase in total Smad3 protein level. β-Actin was used as a control for equal loading.

CSCs marker-CD133 expression is regulated by TGF-β1/Smad3 pathway

Based on the fact that MDR Huh7.5.1/PTX cells show both high percentage of CSCs and higher activity of TGF-β1/Smad3 signaling, we hypothesized that MDR Huh7.5.1/PTX cells may enrich these cells through activation of TGF-β1/Smad3 pathway. In order to assess whether TGF-β1/Smad3 signaling regulates the expression of CSCs markers, reagents including TGF-β1 and SIS3, were added to the medium in serum-free cultured Huh7.5.1 cells for 48 h.

In cultured Huh7.5.1 cells, CD133 expression was reduced with SIS3 (3 ug/ml) alone stimulation (11.9% vs. 18.6%). CD133 expression was reduced with SIS3 (3 ug/ml) and TGF-β1 (10 ng/ml) co-stimulaton (15.5%) compared with the TGF-β1 alone stimulation (35.3%).

We also found that high concentration of TGF-β1 (10 ng/ml) could up-regulate the percentage of $CD133^+$ cells in Huh7.5.1 cells (35.3% vs. 18.6%) (Figure 4A). Based on this finding, we presumed that CD133 expression was partially dependent on the TGF-β1/Smad3 pathway.

For Huh7.5.1/PTX cells, reagents including monocotlonal anti-TGF-β1 neutralization antibody (TGF-β1 mAb) and SIS3 were added to the medium in normal cultured conditions. FCM analysis showed decreased CD133 expression with SIS3 (3 ug/ml) stimulation (29.9%) compared with CD133 expression of control group cells(69.9%), and reduce expression of CD133 with TGF-β1 mAb (10 ug/ml) stimulation (39.5%) compared to the control group (53.6%), respectively (Figure 5A). Other liver CSCs candidated markers (including CD90, CD44, CD326) have no significant changes with the changes of TGF-β1/Smad3 pathway activity (data not shown). All the FCM results were also demonstrated by WB (Figures 4B and 5B).

Here, we report that CSCs could be propagated by chemotherapy in HCC cell line Huh7.5.1，which may be partially dependent on the activity of TGF-β1/Smad3 pathway.

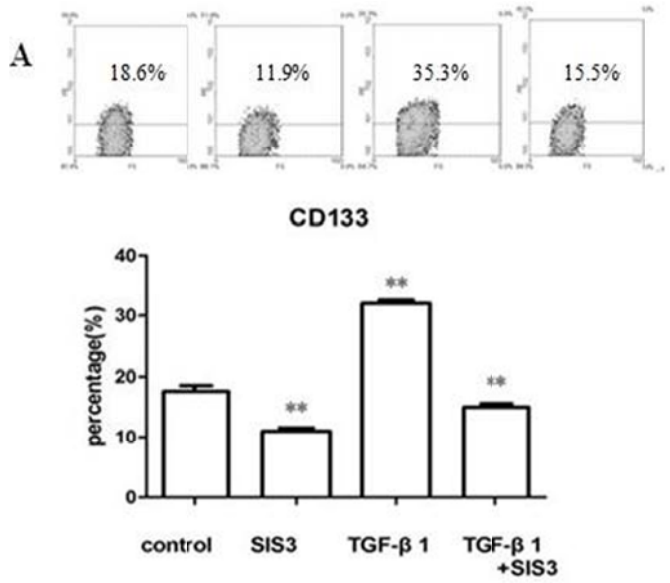
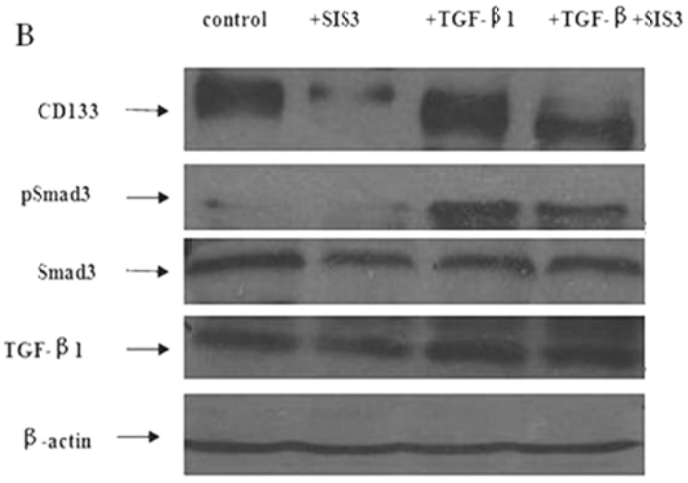


Figure 4. CD133 expression is regulated by TGF-β1/Smad3 pathway in Huh7.5.1 cells. **A.** CD133 expression was reduced with SIS3(3 ug/ml) alone stimulation(11.9%) and increased with TGF-β1 alone stimulation (35.3%)compared with control group(18.6%). CD133 expression was also decreased with SIS3(3 ug/ml) and TGF-β1(10 ng/ml) co-stimulation(15.5%) compared with the TGF-β1 alone stimulation(35.3%). Each value represents the mean ± standard deviation for at least three independent experiments. ** p<0.01(Student t test) Other liver CSCS candidate markers (including CD90,CD44,CD326) show no significant changes (data not shown) . **B.** The results of WB demonstrated our FCM results. TGF-β1 could activate the phosphorylation of Smad3 and SIS3 could inhibit the phosphorylation of Smad3. CD133 expression changes are concordant with the activity changes of Smad3.



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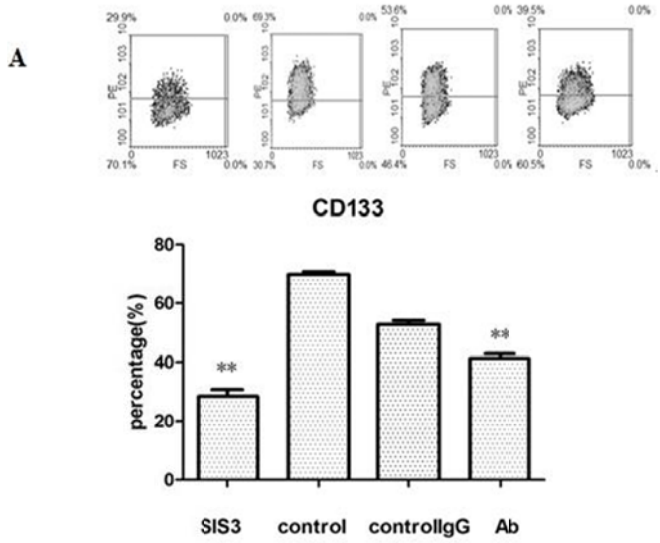
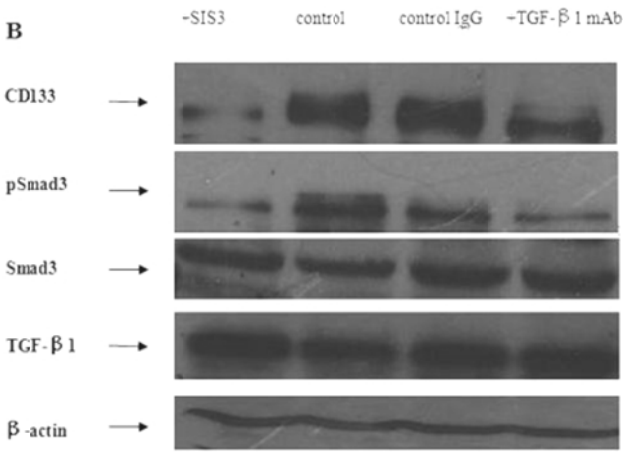


Figure 5. TGF-β1/Smad3 pathway is involved in CD133 expression changes in Huh7.5.1/PTX cells. **A.** CD133 expression was attenuated with SIS3 (3 ug/ml) alone stimulation (29.9%) and TGF-β1 mAb (10 ug/ml) alone stimulation (39.5%) compared with the control group 69.9%, 53.6%, respectively. Each value represents the mean ± standard deviation for at least three independent experiments. **p<0.01 (Student t test). Other markers (including CD90, CD44 and CD326) show no significant changes (data not shown). **B.** The results of WB demonstrated our FCM results. WB results showed that CD133 expression is in conformity with the activity of Smad3.



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The recent discovery of CSCs in solid tumors has played a pivotal role in changing our view of carcinogenesis and chemotherapy (Zou, 2010b). There are two dominant models of carcinogenesis: stochastic organization (clonal evolution model) and hierarchical organization of tumor (CSCs model) (Clarke et al., 2006; Reya et al., 2001). According to the latter, CSCs is at the germinal center of tumor evolution, which is similar to normal adult stem cells and possesses the capacity of self-renewal and differentiation potential (Clarke et al., 2006). Over the past few years, increasing evidence has emerged in

support of the hierarchic cancer model for many solid tumors (Collins et al., 2005; Li et al., 2007; Patrawala et al., 2006; Ponti et al., 2005; Ricci-Vitiani et al., 2007; Schatton et al., 2008; Singh et al., 2003) including HCC (Ma et al., 2007; Suetsugu et al., 2006; Thomas et al., 2010; Yamashita et al., 2009; Yang et al., 2008a; Yang et al., 2008b; Yin et al., 2007; Zhu et al., 2010). The CSCs are posited to be responsible not only for tumor initiation but also for the generation of distant
metastasis and relapse after therapy (Zhou et al., 2009). CSCs are responsible for the formation and growth of neoplastic tissue and are naturally resistant to chemotherapy, explaining why traditional chemotherapy can initially shrink a tumor but fails to eradicate it in full, allowing eventual recurrence (Dean et al., 2005).

Chemotherapy is used to treat unresectable liver cancer with limited efficacy, which might result from HCC cells with stem-like properties and chemo-resistant characteristics (Dean et al., 2005; Zhou et al., 2009; Zou, 2010b). However, the molecular mechanism by which CSCs escape conventional therapies remains unknown. Therefore, investigating the possible molecular mechanism of chemotherapy regulating the expression of CSCs markers is very significant. Some studies have suggested that chemotherapy could enrich CSCs (Bertolini et al., 2009; Du et al., 2011; Dylla et al., 2008; Levina et al., 2008; Ma et al., 2010; Yu et al., 2007). However, in the context of HCC, the relationship between chemotherapy and CSCs remains unclear and the molecular mechanism is unknown. Therefore, we investigated whether drug treatment could enrich CSCs in HCC cells and the possible potential molecular mechanism of chemotherapy regulating the expression of CSCs markers.

Firstly, to test our hypothesis, we established a MDR cell model, Huh7.5.1/PTX. The reasons why we used Huh7.5.1 cells are as follows: (1) There's a moderate percentage of $CD133^+$ cells (19.4% of $CD133^+$) compared to some others HCC cell line in Huh7.5.1 cells (including HepG2, Bel-7402, SMMC-7721, Huh7 and MHCC97-H) (data not shown); (2) If there's a lower or higher percenttage of $CD133^+$ cells in HCC cells, they may not be suitable for enrichment of CSCs. For example, there are almost no $CD133^+$ cells in HepG2 and we found that chemotherapy did not affect the percentage of them (data not shown). Huh7 cells contained high percentage of $CD133^+$ cells (data not shown)and we found that low concentration of chemotherapeutic drugs almost have no effect on this cell, while use of high concentration of drugs in experiments, especially in clinical patients, is no account. Concerning the percentage of $CD133^+$ cells and the sensitivity of cells to drugs, we therefore selected Huh7.5.1cells that contained moderate percentage and PTX to carry out our experiments. The reasons why we used PTX are as follows: (1) Huh7.5.1 cells showed higher sensitivity to PTX at a low concentration (Figure 1A); (2) CSCs are mainly shown in the cell cycle of G0/G1 phase (Kamohara et al., 2008) and PTX mainly kill cells that are in the G2/M phase (Jin et al., 2010). As a result, we selected PTX so that we can kill non-stem cells in cancer to enrich the stem-like cells in HCC cells. Besides that, there are two methods of establishment of drug-resistant model including gradually increasing concentrations of drugs and intermittent administration of high-dose of drugs (Zhang et al., 2010a; Zhang et al., 2010b; Zhou et al., 2010). Concerning the latter, it mimicked the clinical regimen that patients with cancers would receive. As a result, we selected this method to establish our MDR model, which ensured that more than 90% of cells underwent apoptosis or senescence or necrosis with the cells eventually dying, thereby selecting the most resistant clones. Eventually, it took us six months to establish the chemo-resistant model-Huh7.5.1/PTX.

Secondly, to test whether our model is available, we tested the drug sensitivity of Huh7.5.1/PTX. Results demonstrated the availability of the Huh7.5.1/PTX. Huh7.5.1/PTX cells showed high resistance to PTX and had various degree of resistance to other chemotherapeutic drugs. Recent studies have started to link CSCs to chemo-resistance (Dean et al., 2005; Zhou et al., 2009). Therefore, we next compared parental and chemo-resistant Huh7.5.1 cells for cell surface stem cell markers, including CD133, CD90, EpCAM and other stemness-associated markers including (CD29, CD34, CD105, CD308 etc.). We found that MDR Huh7.5.1 cells showed elevated expression of known CSCs markers such as CD90, CD133, and EpCAM in HCC. Recently, the cell surface marker CD133 identifies cancer-initiating cells in a number of malignancies and it has also been used to isolate stem-like cells from HCC cells (Ma et al., 2007; Suetsugu et al., 2006; Yin et al., 2007; Zhu et al., 2010). In summary, these data suggest that chemo-resistant cells derived from cancer cell lines are enriched for CSCs.

Thirdly, we found that chemotherapy can enrich the percentage of CSCs. However, the mechanism of this phenomenon is unknown. Some other reports also suggested that chemotherapy could enrich stem-like cells in breast (Yu et al., 2007), lung (Bertolini et al., 2009; Levina et al., 2008), colorectal (Dylla et al., 2008), pancreatic (Du et al., 2011), and ovarian (Ma et al., 2010) cancer. To the best of our knowledge，the mechanism study of chemotherapy regulating the CSCs is not researched so far. Therefore, we next investigated the potential mechanism of this enrichment. TGF-β1 pathway plays an important role in cell proliferation, apoptosis, and tumorigenesis (Ikushima and Miyazono, 2010; Kelly and Morris, 2010). Recently, a report suggested that $CD133^+$ liver CSCs exhibited relative resistance to TGF-β1-induced apoptosis (Ding et al., 2009). Cells through epithelial-mesenchymal transition by TGF-β could acquire the features of stem cells (Mani et al., 2008; Singh and Settleman, 2010). A recent research reports that dysregulation of the TGFβ pathway leads to HCC through

disruption of normal liver stem cell development (Tang et al., 2008). Two more recent studies reported that the percentage of SP and CD133+ cells were increased by TGF-β treatment in HCC cells (Nishimura et al., 2009; You et al., 2010). Based on the potential role of TGFβ in HCC and CSCs, we hypothesized that chemotherapy resistant cells may have constituted activation of TGF-β1 pathway activity. To validate our hypothesis, we compared the activity of TGF-β/Smad3 pathway in Huh7.5.1 and MDR Huh7.5.1/PTX cells. Our results demonstrate the higher activity of TGF-β/Smad3 pathway in Huh7.5.1/PTX cells.

Eventually, now that MDR Huh7.5.1/PTX cells showed both high percentage of CSCs and higher activity of TGF-β1/Smad3 signaling, we hypothesized that MDRHuh7.5.1/PTX cells may enrich these cells through activation of TGF-β1/Smad3 pathway. In order to assess whether TGF-β1/Smad3 signaling regulates the expression of CSCs markers, we investigated the association of cancer stem markers expression changes and activity of TGF-β1/Smad3 signal. Through activation and inhibition of TGF-β1/Smad3 pathway, we found that CD133 expression was decreased when inhibition and elevated when activation of TGF-β1 pathway. Besides that, we also analyzed other cell surface marker expression such as CD90 and CD326; our results show that there were no significant changes via inhibition or activation of TGF-β1 signal (data not shown). Perhaps, there are other mechanisms involved in regulation of CD90 and CD326 (reported as liver CSCs candidated markers) in MDR Huh7.5.1/PTX cells. We will investigate the possible mechanism in future.

In conclusion, we are the first to report on the mechanism of chemotherapy regulating the expression of $CD133^+$ CSCs in HCC, which is involved in TGF-β1/Smad3 pathway. Taken together, our results suggest that MDR HCC cells are enriched for CSCs, which is partially dependent on TGF-β1/Smad3 pathway. These findings could provide some insight into novel therapy via inhibition of TGF-β1/Smad3 pathway, which may be useful for targeting CSCs to develop more effective treatments for HCC.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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