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

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

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Full Length Research Paper

Optimization of fermentation conditions for the anti-cyanobacterial substances production by *Streptomyces* sp. HJC-D1 using response surface methodology

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To investigate the influence of fermentation conditions such as temperature, initial pH, volume and agitation rate on anti-cyanobacterial active substances production, response surface methodology (RSM) was carried out to optimize the fermentation conditions of an anti-cyanobacterium *Streptomyces* sp. HJC-D1, and the anti-cyanobacterial effect was evaluated. Most common and widespread bloom-forming cyanobacterium *Microcystis aeruginosa* that is associated with microcystic toxins secretion was used as indicator cyanobacterium. The central composite design (CCD) was applied to evaluate the combined effects of the four factors, that is, temperature, initial pH, volume and agitation rate. Based on the analysis of 30 performed experiments, the best optimum level of operating parameters was 33.1°C for temperature, 11.8 for initial pH, 91.2 mL for volume and 337.5 rpm for agitation rate. Additionally, the maximum removal efficiency of chlorophyll *a* under the optimized culture conditions in flask cultures was 93.7%. It is noteworthy that the yield of the anti-cyanobacterial active substances produced by *Streptomyces* sp. HJC-D1 was significantly improved using response surface methodology and suggested the potential to develop a commercial biological control agent against *M. aeruginosa*.

Key words: Response surface methodology, optimization, fermentation conditions, anti-cyanobacterial effect, eutrophication control.

INTRODUCTION

Eutrophication has caused a series of problems such as odor and microcystins (MC) pollution in recent years and damages to ecological systems and threats to human health (Davis and Koop, 2006; Hitzfeld et al., 2000; Qu

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and Fan, 2010). Biological methods of eutrophication control such as anti-cyanobacterial compounds have received increased scientific and technological interest because the microbial-produced anti-cyanobacterial active substances are biodegradable and nontoxic and their degradation intermediates are not secondary pollutants (Qin et al., 2006; Qu and Fan, 2010). Microorganisms such as viruses (Yoshida et al., 2006), bacteria (Kim et al., 2008b; Lovejoy et al., 1998; Shi et al., 2006; Yoshida et al., 2006; Zhang et al., 2011) and golden alga (Zhang et al., 2009) are of particular interest for cyanobacteria control (Kim et al., 2008a; Qin et al., 2006). However, the anti-cyanobacterial bacteria are far from being applied for eutrophication control as the anti-cyanobacterial active substances are so limited in quantity, moreover, the aquatic environment conditions are not the optimal conditions for the growth of anti-cyanobacterial bacteria. Thus, we aim to improve the anti-cyanobacterial active substances production by optimizing the fermentation conditions of the anti-cyanobacterial bacteria.

In recent years, a lot of studies on the influencing factors and inhibiting mechanism by microbes have been published (Kim et al., 2008a; Lovejoy et al., 1998; Uribe and Espejo, 2003; Yoshida et al., 2006; Zhang et al., 2011). Previous studies indicated that the production of antimicrobial compounds by microbial cells were influenced by the composition of the medium, such as carbon sources, nitrogen sources and inorganic salts (Fu et al., 2009; Kong et al., 2014b; Mao et al., 2007; Purama and Goyal, 2008; Rao et al., 2007). In addition, the environmental conditions including temperature, initial pH, volume and agitation rate also had an effect on the production of antimicrobial compounds (Fu et al., 2009; Liu et al., 2011; Mao et al., 2007; Purama and Goyal, 2008; Rao et al., 2007; Sen et al., 2009; Song et al., 2007). Considering the significance of fermentation conditions and the interacting effects of the influencing factors, response surface methodology (RSM) is a useful mathematical and statistical technique for searching the optimal conditions as it could provide statistical models and help in designing experiments for revealing the interactions among the different factors (Bankar and Singhal, 2010; Gao et al., 2009; He et al., 2009), Furthermore, the optimal value of each parameter could be calculated according to the statistical models. Therefore, RSM has been widely used for improving the product yield, reducing the development time and the overall process costs of the fermentation.

It has been shown that microorganisms belonging to *Streptomyces* sp., which are common bacteria found in eutrophication ponds and soils, have been identified as producers of a wide range of anti-cyanobacterial active substances (Choi et al., 2005; Kong et al., 2013, 2014a; Luo et al., 2013; Zheng et al., 2013). In previous study, we isolated a strain of *Streptomyces* sp. HJC-D1 producing anti-cyanobacterial active substances which were efficient for inhibiting the growth of *Microcystis aeruginosa* (Kong et al., 2013, 2014a). The results obtained from preliminary research demonstrated that the optimal medium composition for the growth and anti-cyanobacterial substances production of strain HJC-D1 was 22.7 gL^{-1} sucrose, 0.96 gL^{-1} KNO_3 and at an initial pH of 8.8 (Kong et al., 2014b).

In the present paper, we tested the influence of other factors which may be taken into account to achieve a comprehensive optimization of the fermentation conditions. To optimize the fermentation conditions, the effects of four factors, including temperature, initial pH, volume and agitation rate on the production of anti-cyanobacterial compounds that inhibit the growth of *M. aeruginosa* were studied using RSM.

A full factorial design with relevant statistical analysis has also been investigated to predict the optimal operating parameters of the fermentation for attaining a higher anti-cyanobacterial activity.

MATERIALS AND METHODS

Microorganism

The strain *Streptomyces* sp. HJC-D1 used in this study was originally isolated from an eutrophication pond and was shown to have an anti-cyanobacterial effect on *M. aeruginosa* (Kong et al., 2013, 2014a). *M. aeruginosa* FACHB-905 was purchased from the Freshwater Algae Culture Collection of Institute of Hydrobiology (FACHB), Chinese Academy of Sciences (Wuhan, China).

Culture conditions

Preculturing of *Streptomyces* sp. HJC-D1 was carried out in a 250 mL Erlenmeyer flask, which contained 100 mL of preculture medium and inoculated with a loopful of the bacterium, and incubated at 30°C on a rotary shaker at 150 rpm for 72 h. The seed culture was then transferred to a 250 mL Erlenmeyer flask containing 100 mL Gause's synthetic medium (Kong et al., 2014b) using 5% inoculum, and incubated at 30°C on a rotary shaker at 150 rpm for 72 h.

M. aeruginosa FACHB-905 was cultured under standard conditions: sterilized BG11 medium (Rippka et al., 1979), 2000 lux white light, light:dark = 14:10 h, 25°C, for seven days to reach the log phase before using as inoculants (Kong et al., 2013).

Cyanobacterial inhibition bioassay

The cell-free filtrate of *Streptomyces* sp. HJC-D1 was obtained according to the method described in previous studies (Kong et al., 2013, 2014a, b). The anti-cyanobacterial effects were studied by adding 5 mL *Streptomyces* sp. HJC-D1 cell-free filtrate into 95 mL *M. aeruginosa* culture with the initial chlorophyll *a* (Chl *a*) concentration of 62.7 ± 7.4 μg L^{-1}. For the control group, the cell-free filtrate was the Gause's synthetic medium. Both control and treatment groups were replicated three times and incubated in 250 mL sterilized conical beaker at conditions described above.

Determination of anti-cyanobacterial activity

After incubating for 4 days, the Chl*a* concentrations of both control

Table 1. Values of experimental variables for the application of CCD.

X	Factor	Level				
		-2	-1	0	+1	+2
X_1	Temperature (°C)	20	25	30	35	40
X_2	Initial pH	4.0	6.0	8.0	10.0	12.0
X_3	Volume (mL)	40	80	120	160	200
X_4	Agitation rate(rpm)	0	75	150	225	300

Table 2. Experimental design and results of CCD.

Run	Code				Removal efficiency (%)	
	X_1	X_2	X_3	X_4	Actual value	Predicted value
1	-1	-1	-1	-1	78.4	78.1
2	+1	-1	-1	-1	88.1	86.6
3	-1	+1	-1	-1	80.8	79.9
4	+1	+1	-1	-1	91.3	89.5
5	-1	-1	+1	-1	89.3	86.1
6	+1	-1	+1	-1	82.0	81.9
7	-1	+1	+1	-1	83.5	82.9
8	+1	+1	+1	-1	81.0	79.8
9	-1	-1	-1	+1	79.7	78.9
10	+1	-1	-1	+1	87.4	86.6
11	-1	+1	-1	+1	87.4	86.1
12	+1	+1	-1	+1	93.7	94.9
13	-1	-1	+1	+1	85.2	85.5
14	+1	-1	+1	+1	81.7	80.6
15	-1	+1	+1	+1	88.2	87.7
16	+1	+1	+1	+1	85.2	84.0
17	-2	0	0	0	79.2	81.1
18	+2	0	0	0	84.2	85.8
19	0	-2	0	0	82.3	84.3
20	0	+2	0	0	88.1	89.5
21	0	0	-2	0	92.5	93.8
22	0	0	+2	0	88.6	90.7
23	0	0	0	-2	69.2	72.3
24	0	0	0	+2	76.8	77.1
25	0	0	0	0	90.5	90.3
26	0	0	0	0	90.2	90.3
27	0	0	0	0	90.4	90.3
28	0	0	0	0	90.2	90.3
29	0	0	0	0	90.2	90.3
30	0	0	0	0	90.1	90.3

and treatment groups were determined by spectrophotometric method (APHA, 1998). The removal efficiency of Chl *a* was calculated according to the following equation:

$$\text{Removal efficiency} = (1 - C_t / C_0) \times 100\% \quad (1)$$

Where, C_0 is the Chl*a* concentration at time *t* in the control group and C_t is the Chl *a* concentration at time *t* in the test group (Kong et al., 2014b).

Table 3. ANOVA for the response surface quadratic model.

Source	D.F.	S.S.	M.S.	*F*-value	*P>F*
Model	9	834.52	59.61	16.34	<0.0001
Residual (error)	15	54.70	3.65		
Lack of Fit	10	54.57	5.46	210.09	<0.0001
Pure Error	5	0.13	0.026		
Total	29	889.23			

D.F., degrees of freedom; S.S., sum of squares; M.S., mean square.Std. Dev. = 1.91; R^2 = 0.9385; C.V.= 2.23%; Adj. R^2 = 0.8811.

Experimental design and data analysis

On the basis of our previous studies, the fermentation condition for anti-cyanobacterial activity production by *Streptomyces* sp. HJC-D1 was optimized by central composite experimental design (CCD) (Kong et al., 2014b). The four factors (temperature, initial pH, volume and agitation rate) and respective code and actual levels are given in Table 1. A 30-run experiment generated by Design Expert 7.0 (Stat-Ease, Minneapolis, MN, USA) were carried out with 16 factorial points, 8 axial points and 6 trials at the center point (Table 2). In order to correlate the response variable to the independent variables, the removal efficiency of Chl *a* was fitted according to the following second-order polynomial model:

$$Y = b_o + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{i=1}^{k} b_{ij} x_i x_j, \qquad i \neq j \quad (2)$$

Where, Y is the predicted response, X_i and X_j are the coded independent factors, b_0 is a constant; b_i, linear terms coefficients; b_{ii}, quadratic terms coefficients and b_{ij}, interaction coefficients.

The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). This analysis included the Fisher's *F*-test (overall model significance), its associated probability *p*(*F*), correlation coefficient R, determination coefficient R^2 which measured the reliability of the fit of the regression model. It also included the Student's *F*-value for the estimated coefficients and the associated probabilities *p*(*F*). For each variable, the quadratic models were represented as contour plots (3D).

RESULTS

Effects of the four variables, including temperature, initial pH, volume and agitation rate on the removal efficiency of Chl *a* were investigated. To examine the combined effects of these independent variables, thirty treatments were established using CCD. The results of the second-order response surface models for the Chl *a* removal efficiency in the form of ANOVA were given in Tables 3 and 4, respectively. Using the designed experimental data (Table 2), the following quadratic regression equation was obtained to describe the removal efficiency of Chl *a*:

$$Y = 90.26 + 1.16 X_1 + 1.29 X_2 - 0.76 X_3 + 1.21 X_4 + 0.29 X_1 \times X_2 - 3.16 X_1 \times X_3 - 0.18 X_1 \times X_4 - 1.24 X_2 \times X_3 + 1.35 X_2 \times X_4 - 0.33 X_3 \times X_4 - 1.71 X_1^2 - 0.84 X_2^2 + 0.50 X_3^2 - 3.89 X_4^2 \quad (3)$$

Table 4. Results of regression analysis of CCD.

Parameter	Estimate	Std. Error	*F*-Value	*P*-Value
Intercept	90.26	0.78	16.34	<0.0001
X_1	1.16	0.39	8.92	0.0092
X_2	1.29	0.39	10.98	0.0047
X_3	-0.76	0.39	3.81	0.0699
X_4	1.21	0.39	9.63	0.0073
X_1^2	-1.71	0.36	21.95	0.0003
X_2^2	-0.84	0.36	5.34	0.0355
X_3^2	0.50	0.36	1.86	0.1924
X_4^2	-3.89	0.36	113.87	<0.0001
$X_1 \times X_2$	0.29	0.48	0.37	0.5510
$X_1 \times X_3$	-0.36	0.48	43.88	<0.0001
$X_1 \times X_4$	-0.18	0.48	0.14	0.7114
$X_2 \times X_3$	-1.24	0.48	6.77	0.0200
$X_2 \times X_4$	1.35	0.48	8.00	0.0127
$X_3 \times X_4$	-0.33	0.48	0.47	0.5048

Where, *Y* is the predicted removal efficiency of Chl *a*, X_1, X_2, X_3 and X_4 are the coded values of temperature, initial pH, volume and agitation rate, respectively.

The actual and predicted values of Chl *a* removal efficiency based on CCD experimental design are shown in Table 2. By applying ANOVA (Table 3), the model was found to be significant (P< 0.0001), as is evident from the *F*-value (16.34) with a very low probability value [(P>F)<0.0001]; likewise, the reliability of fit of the model was checked by determination coefficient (R^2), and the determination coefficient of the model was 0.9385, which indicated 93.85% of the variability in the response could be obtained by this model. The 0.8811 value of the adjusted R^2 was also sufficiently good. At the same time, the coefficient of variation (C.V. = 2.23%) demonstrated a good precision of the experiments. Nevertheless, the predicted R^2 value of 0.6463 was not as close to the adjusted R^2 value of 0.8811 as it was expected, this was probably due to a large block effect.

The interactions of the four factors on the Chl *removal* efficiency are illustrated in Figure 1. The Chl *a* removal efficiency exhibited a strong response surface depended on both temperature and initial pH (Figure 1a); the value of removal efficiency changed from about 79.7% (at the temperature of 25°C and pH 6.0) to about 93.7% (at 35°C and pH 10.0). Moreover, a good system behavior was consistent with the removal efficiency of 88%, which was obtained at 32°C and pH 6.0. The response surface versus temperature and volume is presented in Figure 1b. It is evident that a relatively weak effect of volume and a stronger effect of temperature could be noted, and the optimal temperature for the Chl *removal* efficiency was 35°C, while the worst conditions were achieved at 25°C with the volume of 100 mL.

Figure 1c shows the effects of temperature and agitation rate on the Chl *a* removal efficiency. It was obvious that the effect of temperature on Chl *a* removal efficiency became less significant as the agitation rate increased to nearly the middle range. Therefore, the maximum removal efficiency of Chl *a* is around the middle range of the corresponding variables. Figure 1d indicates that the removal efficiency is concerned with both initial pH and volume. At the same temperature of 30°C and stirring rate of 175 rpm, the removal efficiency of Chl *a* is dependent on initial pH, which is varied from about 86 to about 93% as the initial pH was increased from 6.0 to 10.0.

Figure 1e depicts the response surface of the effects of two factors, namely, initial pH and agitation rate. It is evident that the interaction between the two factors was significant (P < 0.05). The Chl *a* removal efficiency increases with the increase of agitation rate from 100 to 180 rpm, however, a further increase in the agitation rate leads to the decrease of removal efficiency. As agitation rate is fixed for the fermentation of microorganisms, volume becomes the important factor for microorganisms obtaining dissolved oxygen. Figure 1f shows the response surface of the effect of volume and agitation rate on the removal efficiency of Chl *a*. It is obvious that the Chl *a* removal efficiency was increased rapidly with the increase of agitation rate from 100 to 175 rpm.

After having accomplished the ANOVA test on the complete quadratic model, all the negligible effects were eliminated in order to improve the model predictive performance. The best optimum level of operating parameters to operate the fermenter was found to be 33.1°C, 11.8, 91.2 mL and 337.5 rpm for temperature, initial pH, volume and agitation rate, respectively. In order to check the agreement between the optimized fermentation conditions and the prediction by the present model (Equation 3), the predicted conditions were performed in triplicate with the batch cultivation. Under the suggested conditions, the mean value of the removal efficiency was 93.7%, which was in agreement with the optimum value predicted by the model. The good correlation between the experimental and predicted results demonstrated that the second-order model was accurate and reliable for predicting the removal efficiency of *M. aeruginosa* by strain *Streptomyces* sp. HJC-D1 (Table 2).

DISCUSSION

Fermentation conditions are one of the most important factors affecting biomass production (Song et al., 2007; Yin et al., 2010). Environmental conditions, including temperature, initial pH value, volume, agitation rate, and even medium composition such as carbon and nitrogen sources, could be optimized to increase the yield produced by microorganisms (Liu et al., 2011; Purama and Goyal, 2008; Queiroga et al., 2012; Song et al.,

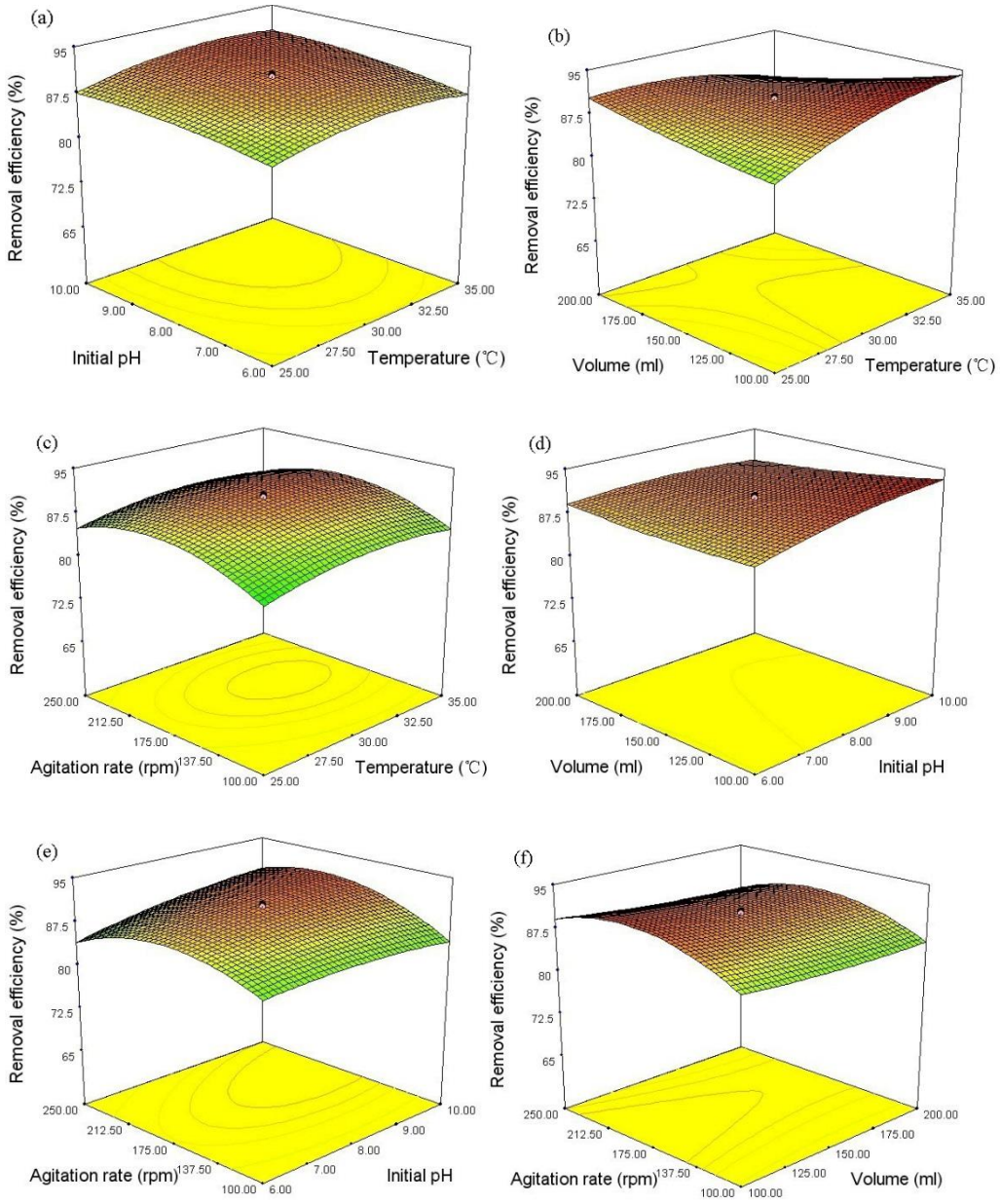

Figure 1. Effect of interaction between different factors on the Chl *a* removal efficiency: (a) initial pH and temperature, (b) volume and temperature, (c) agitation rate and temperature, (d) volume and initial pH, (e) agitation rate and initial pH and (f) agitation rate and volume.

2007). Compared with the traditional method, statistically based experimental design is a much efficient approach to deal with a great number of variables. As a useful statistical technique, RSM has been widely and successfully applied to the optimization of the medium components and culture conditions (Gao et al., 2009; He et al., 2009; Liu et al., 2011).

A previous study demonstrated the influence of the culture conditions on exopolysaccharides (EPS) production from *Zunongwangia profunda* SM-A87, and the optimum incubation temperature of 9.8°C was achieved by RSM (Liu et al., 2011). The reason for the low temperature for EPS production could be that *Z. profunda* was isolated from deep-sea sediment, which was regarded as extreme environments with low nutrient concentration, low temperature and high pressure. With the exception of the influence of a single factor, interactions between the factors should also be considered. It was reported that protease synthesis depended chiefly on temperature and peptone level (Queiroga et al., 2012), and a temperature of 43°C was considered to be the most favorable for protease synthesis

by *Bacillus* sp. HTS102. A previous study also showed that the optimum fermentation conditions for fructo-oligosaccharides production by *Aureobasidium pullulans* were 32°C and 385 rpm (Dominguez et al., 2012), which suggested that temperature and agitation rate were the most significant parameters. In the present study, it was found that the influence of initial pH was greater than the other three variables (Table 4). The temperature and agitation rate optima were 33.1°C and 337.5 rpm for each as expected, moreover, the results were in agreement with another report (Dominguez et al., 2012). On the other hand, the factors such as temperature and volume level were found to be most significant upon Chl *a* removal efficiency ($P < 0.0001$), therefore, they were considered as the main factors which had a significant impact on the production of anti-cyanobacterial active substances (Table 4); surprisingly, the favourable effects of temperature towards volume were too marginal to be classified as statistically significant (Figure 1b), and the highest removal efficiency was obtained at a high level temperature.

In the natural environment, anti-cyanobacterial bacteria play an important role in regulating harmful cyanobacterial biomass (Davis and Koop, 2006; Qin et al., 2006). Previous studies revealed that anti-cyanobacterial bacteria had the ability to biodegrade cyanobacteria (Choi et al., 2005; Kim et al., 2008b; Shi et al., 2006; Yoshida et al., 2006; Zhang et al., 2011; Kong et al., 2013), suggesting that anti-cyanobacterial agents produced by these bacteria were a promising and environment-friendly way for eutrophication control (Qu and Fan, 2010; Luo et al., 2013; Zheng et al., 2013). By now, the harmful cyanobacteria are hard to be controlled by anti-cyanobacterial bacteria as these anti-cyanobacterial bacteria in natural environments were so limited. Therefore, it is particularly important to provide a suitable growing environment for the growth of anti-cyanobacterial bacteria. Although the anti-cyanobacterial bacterium strain *Streptomyces* sp. HJC-D1 selected for this study was isolated from a weak alkaline environment (pH from 9.2 to 10.6), the optimum levels of its anti-cyanobacterial effect were found to be at a higher pH (pH=11.8); in addition, the best optimum levels of temperature was 33.1°C, which was much higher than that in natural environment. It is common that, for a given micro-organism, the optimum culture conditions for growth are different from those required or specific metabolite production (Queiroga et al. 2012). It is noteworthy that strain *Streptomyces* sp. HJC-D1 could produce anti-cyanobacterial active substances with a higher activity after optimization of the fermentation conditions. Given the optimized fermentation conditions, the removal efficiency of Chl *a* was increased to 93.7%. In view of the results above, we consider this study useful for the highly efficient production of anti-cyanobacterial active substances that inhibit the growth of *M. aeruginosa* on a bioreactor scale.

In conclusion, the best optimum level of operating parameters for anti-cyanobacterial active substances was 33.1°C for temperature, 11.8 for initial pH, 91.2 mL for volume and 337.5 rpm for agitation rate, respectively. Furthermore, the maximal removal efficiency of Chl *a* under the optimized culture conditions was 93.7%. It should be noted that this study focused on laboratory research and examined the increase of anti-cyanobacterial active substances production on this scale. We have to point out that we did not test the characteristics (especially ecological safety) of the anti-cyanobacterial substances so they could not be used in nature. Another limitation of this study is that the biodegradation of cyanobacterium *M. aeruginosa* would result in the release of microcystin (Hitzfeld et al., 2000). Notwithstanding the limitations, this study clearly indicates the yield of anti-cyanobacterial active substances was significantly improved using response surface methodology and does suggest the potential to develop a commercial biological control agent against *M. aeruginosa*.

Conflict of Interests

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

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Fungal endophytes of sorghum in Burkina Faso: Occurrence and distribution

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A survey was conducted to assess the natural occurrence and distribution of fungal endophytes in sorghum in relation to plant performance in two distinct agro-ecological zones in Burkina Faso. Sorghum farm-saved seeds were sown in 48 farmers' fields in Sahelian and North Sudanian zones to produce sorghum plants. In each field, leaf samples were collected from five well-developed (performing) and five less-developed (non-performing) plants at 3-5 leaf stage, while at plant maturity leaf, stem and root samples were collected from the same plants and fungal endophytes were isolated. A total of 39 fungal species belonging to 25 genera were isolated. The most represented genera included *Fusarium*, *Leptosphaeria*, *Curvularia*, *Nigrospora* and *Penicillium*. The genera *Fusarium* and *Penicillium* occurred significantly higher in performing plants as compared to non-performing plants while the genera *Colletotrichum* and *Alternaria* were most represented in non-performing plants. Among the *Fusarium* species identified, *Fusarium moniliforme* was the most common fungus isolated from the plants. *Fusarium* spp. and *Penicillium* sp. were significantly present in a higher number of performing plants than in non-performing plants, while *Colletotrichum sublineolum* was more encountered in non-performing plants than in performing plants. Distribution of fungi varied based on the tissue and root accounting for the majority of the fungi isolated. This work represents the first description of the diversity of fungal species and the fungal community in sorghum, and the first report attempts to document endophytic fungal presence in Burkina Faso.

Key words: Endophytes, bio-resource, *Sorghum bicolor*, fungi.

INTRODUCTION

Sorghum (*Sorghum bicolor* L.) is the fifth most important grain crop in the world after maize, rice, wheat and barley and is on average the second most produced grain in the African continent in 2004-2013 (http://faostat.fao.org) (2014). Drought tolerance makes sorghum particularly important in the dry regions of North-East Africa, which is recognized as the centre of diversity of sorghum, where agricultural and environmental conditions are unfavourable for other cereal crops (Paterson et al., 2009). It is the major food crop used in rural populations within the

semi-arid area in Africa, and in 2013 25.7 million tonnes of grain sorghum was produced as compared to 23.5 and 8.8 million tonnes in America and Asia, respectively, making Africa the largest sorghum producer in the world (http://faostat.fao.org) (2014). However, despite high production levels in Africa, average yield is often low (0.957 t/ha) in comparison with average yields in America (3.525 t/ha) which is due to a combination of agronomic and environmental factors as well as the use of inferior sorghum varieties in Africa. In 2013, sorghum production in Burkina Faso was 1.9 million tonnes with an average yield of 1.078 t/ha. Even though total sorghum production has been increasing in recent years, this has only been achieved through cultivation of more land (Belton and Taylor, 2004).

Sorghum production is menaced by abiotic factors such as drought in the semi-arid regions of Africa. Furthermore, biotic factors such as insect pests and pathogenic fungi which are either present in the soil or are transmitted by sorghum seeds, represent other major threats to sorghum production (Chandrashekar and Satyanarayana, 2006). These biotic threats lead to significant crop damage, contributing to the severe yield losses mentioned above. Pathogenic fungi such as *Phoma*, *Curvularia*, *Fusarium* and *Colletotrichum* spp. are known threats to sorghum, and sorghum grains are also susceptible to colonization by *Aspergillus* spp. during wet periods after harvest, which can result in the accumulation of the unwanted mycotoxin, aflaxotin, in the grain (Chandrashekar and Satyanarayana, 2006). Not all species of *Curvularia* and *Fusarium* are pathogenic on sorghum, but those that are pathogenic mainly affect the stem (*Curvularia*) or the stem and leaf (*Fusarium*). Penetration and infection by pathogenic *Phoma* spp. are restricted by the thickness of the mesocarp (Kumari et al., 1992), and the research data accumulated over many years indicate innate differences among sorghum grains in their ability to resist fungal colonization (Chandrashekar and Satyanarayana, 2006). *Colletotrichum sublimeolum* is mainly a pathogen of sorghum leaves, infecting the sorghum seed head, and is the causative agent of sorghum anthracnose disease (Chandrashekar and Satyanarayana, 2006).

Several methods currently exist for the control of pathogenic fungi in sorghum tissues, most notably the use of chemical fungicides. The use of chemical control methods is for many African farmers challenged by the economic cost and the physical unavailability of fungicides. Furthermore, environmental concerns about potential adverse effects from the use of chemical fungicides call for alternative methods to control pathogenic fungi within this area. The use of botanicals against crop pathogenic fungi is a strategy currently under development in many countries. Application of an aqueous extract of *Eclipta alba*, a weed, as seed treatment was reported to inhibit sorghum seed-borne *Leptosphaerella sacchari* (*Phoma sorghina*) and increase yield in Burkina Faso (Zida et al., 2012). One currently unexploited approach towards reducing fungal diseases of sorghum is the potential use of endophytes as biocontrol agents against pathogenic microbes (Clay, 1989; Schardl et al., 2004; Schardl et al., 1991).

An endophyte can be defined as any microorganism, typically bacterial or fungal, that lives within a plant (Clay and Schardl, 2002). There is now a substantial amount of literature regarding beneficial endophytes, mostly related to the ascomycete endophytes of the fungal genera *Neotyhodium* and *Epichloë*, which are associated with temperate grasses (*Poöideae*). There is a well-accepted notion that grass endophytes have mutualistic relationships with their hosts, and this has led to claims that they co-evolve with their hosts (Faeth, 2002; Porras-Alfaro and Bayman, 2011). The growing list of beneficial effects imparted by endophytes to their hosts includes tolerance to drought (Clay and Schardl, 2002; Hahn et al., 2008; Malinowski and Belesky, 2000; Redman et al., 2002; Sherameti et al., 2008), improved salt tolerance (Baltruschat et al., 2008; Redman et al., 2011), enhanced growth (Bae et al., 2009; Mucciarelli et al., 2003; Waller et al., 2005) and increased tolerance to pathogens (Porras-Alfaro and Bayman, 2011). Furthermore, endophytic fungi have been noted for benefits to the consumers of their plant hosts, such as reduction of mycotoxins produced by mycotoxinogenic fungi (Danielsen and Jensen, 1999). Currently, endophytes have a well-recognized potential as biocontrol agents in a wide variety of plants, and the potential for endophytes as biocontrol agents in cereals has recently been reviewed (O'Hanlon et al., 2012). Investigating and harnessing the potential of endophytes expands the possibility for developing biocontrol strategies to control sorghum pathogens as well as enhancing stress tolerance through artificially inoculated stable endophytes. To date, no study has been undertaken on the tissue-specific prevalence of endophytic fungi in sorghum, and therefore it is conceivable that this is a resource which should be explored for its potential use as biocontrol agents to manage fungal diseases as well as to enhance stress tolerance.

The objective of this study was to isolate and identify the endophytic fungal diversity within different tissues of sorghum plants originating from farm-saved sorghum seeds grown in two agroecological zones of Burkina Faso as a starting point for further investigations into the potential of endophytes to control fungal pathogens in this important cereal crop.

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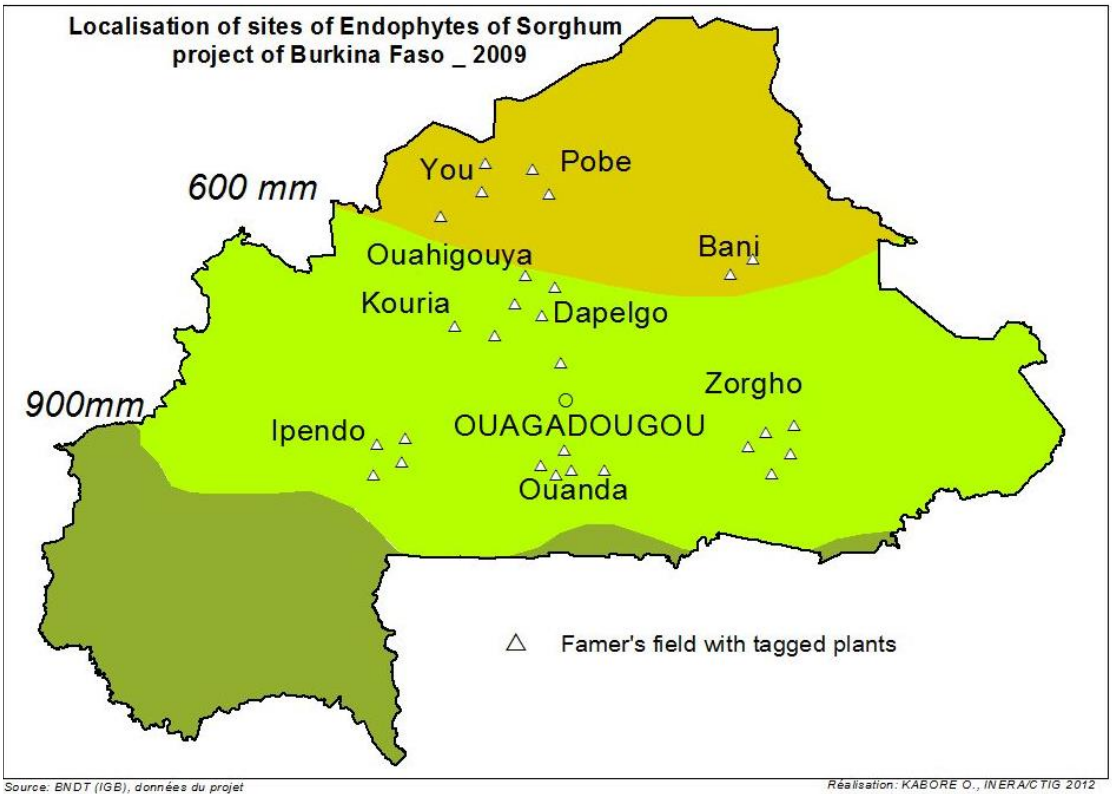


Figure 1. Distribution of sites for collection of endophytic fungi from sorghum plants in Burkina Faso in 2009.

MATERIALS AND METHODS

Sample collection

In general, in sub-Saharan Africa, vegetation, soil, etc. are strongly linked to the annual precipitation. Sorghum plant samples were collected in farmer's fields in two agro-ecological zones during the raining season, 2009: the Sahelian zone with an average annual precipitation ranging from 300 to 600 mm and the North Sudanian zone with 600 to 900 mm precipitation. At the first sampling period, rains were regular and the relative humidity was relatively high (> 70%) and temperature was in the interval of 22-35°C. At plant maturity (second sampling period), rains were rare or completely absent, temperature was high (30-39°C) and relative humidity <50°C. Nine villages (Bani, Pobe, You, Ouahigouya from the Sahelian zone and Kouria, Dapelgo, Ipendo, Ouanda and Zorgho from the North Sudanian zone) were considered as sampling sites (Figure 1). In each village, samples were collected in five fields belonging to farmers who establish their crop from farm-saved seed. The agronomic management of the sampling sites was carried out according to the farmers' capacity (soil cultivation, plant establishment, fertilizer application, etc.). None of the farmers used fungicides. In each field within an area of approximately 3 x 3 m, ten plants (five performing plants (P) (well-growing, vigorous plants) and five neighbouring non-performing plants (NP) (less vigorous, without disease symptoms) were identified and tagged in early summer (Figure 2). Within each field, P and NP plants were at the same developmental stage and either performing or non-performing plants showed disease infection. The first sampling was carried out non-destructively when sorghum plants had 3-5 leaves, while the second sampling was carried out on the same plants at maturity. The first sampling was restricted to the leaves, while during the second sampling leaf, stem and root fragments were collected for endophyte isolation from the tagged plants. Fungal occurrence was subsequently compared within these groups of plants and within plant tissues. A total of 330 from the 450 labelled plants in the two zones were still available for investigation. The difference was due to loss of labels (unintended removals of various kinds) or plants had already been harvested before sampling. At the 3-5 leaf stage, 250 plants (125 P plants and 125 NP plants) were investigated, while at maturity stage 280 plants (140 P and 140 NP plants) were subject to investigation. Sampling and transport to the villages was time consuming and some plants were developed further than the 3-5 leaf stage before sampling. Therefore, the total number of plants sampled was higher in sampling 2 than in sampling 1.

Samples were transported in paper bags to the laboratory and stored in the refrigerator. Within one to two days after collection, samples were surfaced-sterilized and incubated on PDA medium for nine days. Analysis, which primarily involved fungal isolation and identification, was carried out in the Laboratory of Phytopathology of Kamboinsé Research Station in Burkina Faso. Furthermore, agronomic data (stem diameter, plant height, plant weight, panicle length and weight, grain weight and total number of grains) were recorded for these plants. These data are to be presented in a follow-up paper.

Isolation and identification of endophytic fungi

Fungal endophytes were isolated from samples of leaf, stem and root collected from individual field plants according to the protocol described by Petrini (1986) with modifications. Sorghum leaf, stem and root tissues were cut into 12-15 mm pieces prior to sterilization. All fragments were surface-sterilized in 70% (v/v) ethanol for one

Figure 2. Pictures of performing (P) plant (right) and a non-performing (NP) plant (left).

minute followed by immersion in 3% (v/v) sodium hypochlorite (NaOCl) for four minutes and then in 70% (v/v) ethanol for 30 s. Tissue fragments were rinsed three times in sterilized distilled water. The fragments were subsequently plated in Petri dishes containing potato dextrose agar (PDA) (3.9% w/v), which was aseptically supplemented with streptomycin antibiotic (0.2% v/v) to inhibit bacterial growth. Plates were incubated in the dark for nine days at 28°C. All colonies observed were sub-cultured onto fresh PDA without streptomycin and incubated at 24°C for seven days under a cycle of 12 h UV light/12 h darkness. Fungal isolates were primarily identified based on fungal morphology and compared with the current published identification keys (Hyn et al., 2004; Mathur and Kongsdal, 2003; Singh et al., 1991).

Statistical analysis

One-way analysis of variance (ANOVA) and least significant difference (LSD) were performed on the data recorded. Fungal occurrence was compared within the two agro-ecological zones, within the two groups of plants (P and NP plants) and within plant tissues.

RESULTS

Endophytic fungal biodiversity in sorghum in two agro-ecological zones in Burkina Faso

From the potential 450 tagged plants (9 villages x 5 farmers x (5 P + 5 NP)), a total of 330 plants were investigated. In total, 39 fungal species, belonging to 25 genera, were isolated from sorghum plants in the two agro-ecological zones in Burkina Faso during two sampling periods (Table 1). *Fusarium moniliforme*, *Fusarium* spp., *Leptosphaerella sacchari*, *Nigrospora oryzae*, *Curvularia* spp. and *Penicillium* sp. were frequently encountered in plants in both zones. About 15.38% of these fungal species were mainly associated with sorghum plants in the Sahelian zone, while 28.20% were most abundant in the Sudanian zone. All the other fungi were invariably present in each of the two zones.

Occurrence of endophytic fungi in performing and non-performing plants

The major genera identified included *Fusarium*, *Leptosphaeria*, *Curvularia*, *Penicillium, Nigrospora*, *Alternaria*, *Rhizoctonia*, *Colletotrichum* and *Exserohilum*. At both sampling times, the percentages of performing plants colonized by these genera were generally higher than those of non-performing plants, except for *Colletotrichum* (Figure 3). The genus *Colletotrichum* seemed to be most abundant within non-performing plants. Statistical analysis showed that among the major genera, the presence *in plantae* of *Fusarium* spp. ($p = 0.0077$ at first and second samplings) and *Penicillium* spp. ($p = 0.011$ at first sampling) was significantly associated with plant performance.

Among the fungal species identified, only 27 species were isolated from leaf samples at the first sampling (3-5 leaf stage), whereas all of the 39 fungal species were isolated at the second sampling (at maturity) when fungal isolation was performed on leaf, stem and root samples (Table 2). The results showed that at the 3-5 leaf stage, significant differences were observed between performing and non-performing plants colonized by *F. moniliforme* ($p = 0.0099$), *Fusarium* spp. ($p = 0.0102$), *Penicillium* sp. ($p = 0.0116$) and *Colletotrichum sublineolum* ($p = 0.0174$). *Fusarium* sp. *F. moniliforme* and *Penicillium* spp. were significantly associated with

Table 1. Occurrence of fungal endophytes in sorghum plants in two agro-ecological zones in Burkina Faso.

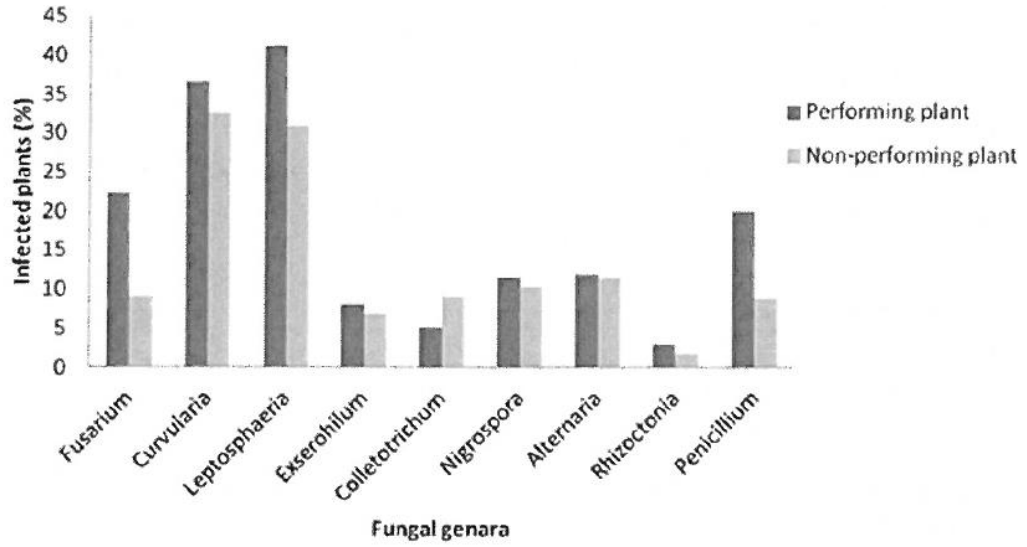
Fungi	Sahelian zone	Sudanian zone	Average	LSD (5%)
Fusarium moniliforme	37.32[a]	18.82[b]	28.50	5.31
Fusarium pallidoroseum	0.35[a]	0.78[a]	0.56	0.90
Fusarium equiseti	1.78[a]	0.19[b]	1.02	1.21
Fusarium culmorum	0.71[a]	0.39[a]	0.56	0.90
Fusarium spp.	13.92[a]	8.43[b]	11.30	3.86
Leptosphaeria sacchari	17.85[b]	32.15[a]	24.67	5.11
Phoma sp.	0.17[a]	0.00[a]	0.09	0.37
Macrophomina phaseolina	0.17[a]	0.39[a]	0.28	0.64
Cladosporium sphaerospermum	1.07[b]	3.52[a]	2.24	1.77
Colletotrichum sublineolum	1.25[b]	8.62[a]	4.76	2.52
Colletotrichum gloerosporioides	0.17[a]	0.98[a]	0.56	0.90
Colletotrichum spp.	0.17[b]	1.56[a]	0.84	1.09
Exserohilum rostratum	4.28[a]	2.15[a]	3.27	2.13
Nigrospora oryzae	17.69[a]	15.88[a]	16.82	4.00
Gloeocercospora sorghi	0.35[b]	1.96[a]	1.12	1.26
Rhizopus sp.	4.10[a]	2.54[a]	3.36	2.17
Curvularia lunata	0.53[b]	4.90[a]	2.61	1.90
Curvularia penniseti	0.00[a]	0.19[a]	0.09	0.37
Curvularia spp.	26.25[a]	22.74[a]	24.57	5.20
Acremonium strictum	1.25[a]	1.96[a]	1.40	1.41
Acremonium sp.	0.89[a]	0.78[a]	0.37	0.73
Penicillium sp.	11.60[b]	31.37[a]	21.02	4.75
Trichothecium sp.	0.71[a]	0.39[a]	0.56	0.90
Epicoccum purpurascens	0.17[a]	0.78[a]	0.74	1.04
Bipolaris spicifera	0.53[a]	0.00[a]	0.28	0.63
Bipolaris sorghicola	0.53[a]	0.39[a]	0.46	0. 82
Bipolaris spp.	1.25[a]	0.58[a]	0.93	1.16
Melanospora zamiae	1.60[a]	0.39[a]	1.02	1.21
Alternaria alternata	0.17[b]	2.94[a]	1.49	1.45
Alternaria longissima	0.71[b]	2.15[a]	1.40	1.41
Alternaria spp.	1.25[a]	0.19[b]	0.74	1.03
Ascochyta sp.	0.17[a]	0.00[a]	0.09	0.37
Botryodiplodia theobromae	1.60[a]	0.19[b]	0.93	1.15
Cercospora sp.	0.17[b]	2.35[a]	1.21	1.31
Rhizoctonia solani	5.00[a]	5.49[a]	5.23	2.68
Myrothecium sp.	0.17[a]	0.00[a]	0.09	0.37
*Diplodia*sp.	0.00[a]	0.58[a]	0.28	0.63
Peronoslerosporasorghi	0.00[a]	0.19[a]	0.09	0.37
Phaeoisariopsis griseola	0.17[a]	0.19[a]	0.18	0.52

Means within the same line followed by the same letter are not significantly different at the level of 5%, according to LSD test.

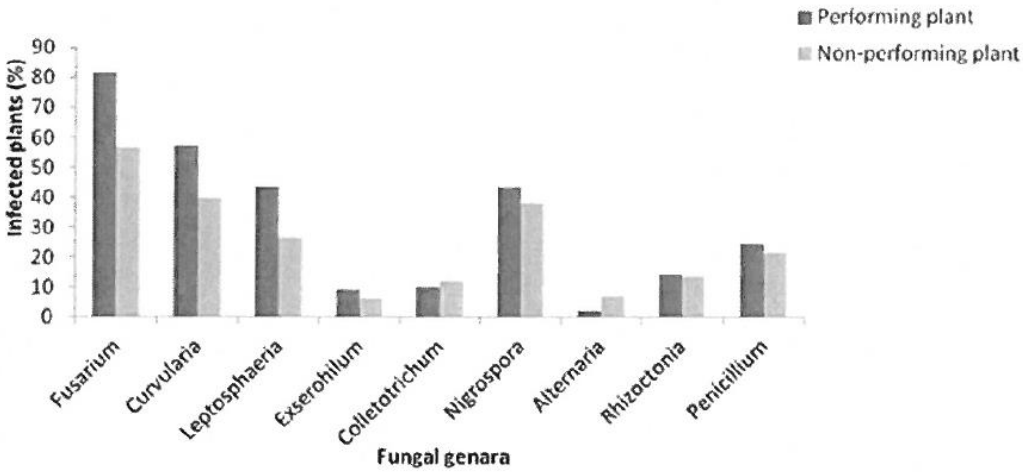
performing plants, while *C. sublineolum* was associated with non-performing plants. At plant maturity and for each fungus, performing and non-performing plants presented similar levels of infection.

Potential beneficial/pathogenic fungal endophytes isolated from sorghum plants in Burkina Faso

Despite the high occurrence of certain fungal species in performing plants, these plants were well developed and looked healthy in comparison with their/the neighbouring non-performing plants. The genus *Fusarium* with five fungal species was the most common fungus associated with sorghum plants. *F. moniliforme* and *L. sacchari* infecting 28.50 and 24.67% respectively of sorghum plants most highly represented fungal species (Table 1). Our observations allow us to identify only one species of *Exserohilium*: *Exserohilum rostratum*.



(a) At 3-5 leaf stage.



(b) At plant maturity stage.

Figure 3. Percentages of performing and non-performing sorghum plants colonized by nine major genera of endophytic fungi in Burkina Faso at 3-5 leaf (a) and maturity (b) stages.

In the Sudanian zone, only *F. moniliforme* significantly colonized a higher number of performing plants than non-performing plants ($p = 0.0085$ and $p = 0.0105$ respectively). In the Sahelian zone, *Fusarium* spp. ($p = 0.0407$), *Nigrospora oryzae* ($p = 0.0407$) and *Penicillium* sp. ($p = 0.0431$) colonization was significantly more abundant in performing plants than in non-performing plants (Table 3). According to the hypothesis that beneficial endophytic fungi could be strongly associated with performing plants, while pathogenic endophytic fungi could be strongly associated with non-performing plants, the fungal species *F. moniliforme*, *Fusarium* spp., *Nigrospora oryzae* and *Penicilliums*p. were considered potential beneficial endophytes (Table 3). In contrast, *C. sublineolum*, occurring in higher numbers of non-performing plants than performing plants in the Sudanian zone ($p = 0.0298$), was considered a potential pathogenic endophyte. Potentially beneficial endophytic *F. moniliforme* mainly occurred in the Sudanian zone, while *Fusarium* spp. and *Penicillium* sp. were most abundant in the Sahelian area. Potentially pathogenic *C. sublineolum* was most encountered in the Sudanian zone.

According to the results presented in Table 3, the potential beneficial/pathogenic endophytes (*F. moniliforme*, *Fusarium* spp., *Nigrospora oryzae*, *C. sublineolum* and *Penicillium* sp.) were isolated from plants at the 3-5 leaf stage, while only *F. moniliforme* was also detected in plants at maturity. At plant maturity and for each fungus, performing and non-performing plants presented similar levels of colonization. These results indicated that plant growth stage might be the best indicator for the isolation of potentially beneficial

Table 2. Occurrence of endophytic fungi within performing and non-performing plants of sorghum at plant growth and plant maturity stages in Burkina Faso.

Fungal species	Plant growth stage		Plant maturity stage	
	Performing plants (%)	Non-performing plants (%)	Performing plants (%)	Non-performing plants (%)
Fusarium moniliforme	14.40*	4.80	12.43	10.40
Fusarium pallidoroseum	2.40	0.80	0.16	0.00
Fusarium equiseti	0.80	0.00	0.48	0.32
Fusarium culmorum	0.00	0.00	0.40	0.08
Fusarium spp.	8.80*	1.60	4.22	4.55
Leptosphaeria sacchari	42.40	32.00	7.15	6.74
Phoma sp.	0.00	0.00	0.00	0.08
Macrophomina phaseolina	0.80	0.80	0.08	0.00
Cladosporium sphaerospermum	3.20	3.20	0.73	0.48
Colletotrichum sublineolum	0.80*	6.40	1.62	1.78
Colletotrichum gloerosporioides	0.80	2.40	0.00	0.16
Colletotrichum spp.	0.80	0.80	0.16	0.40
Exserohilum rostratum	4.00	4.80	1.13	0.81
Nigrospora oryzae	13.60	9.60	6.34	5.93
Gloeocercospora sorghi	0.80	4.00	0.24	0.24
Rhizopus sp.	0.80	0.00	1.46	1.38
Curvularia lunata	4.00	7.20	0.65	0.48
Curvularia penniseti	0.00	0.00	0.08	0.00
Curvularia spp.	28.80	30.40	7.64	7.80
Acremonium strictum	0.00	0.00	0.89	0.32
Acremonium sp.	0.00	0.00	0.16	0.16
Penicillium sp.	20.00*	8.80	8.13	7.23
Trichothecium sp.	0.80	0.00	0.24	0.16
Epicoccum purpurascens	0.80	0.80	0.24	0.24
Bipolaris spicifera	0.00	0.00	0.16	0.08
Bipolaris sorghicola	0.80	0.00	0.16	0.16
Bipolaris spp.	0.80	0.00	0.32	0.40
Melanospora zamiae	0.80	0.00	0.56	0.24
Alternaria alternata	5.60	4.80	0.16	0.08
Alternaria longissima	3.20	4.80	0.24	0.16
Alternaria spp.	0.00	0.00	0.08	0.56
Ascochyta sp.	0.00	0.00	0.24	0.00
Botryodiplodia theobromae	0.00	0.00	0.16	0.65
Cercospora sp.	3.20	0.80	2.27	2.19
Rhizoctonia solani	0.80	0.00	0.00	0.08
Myrothecium sp.	0.00	0.00	0.00	0.24
*Diplodia*sp.	0.00	0.00	0.08	0.00
Peronoslerosporasorghi	0.00	0.00	0.24	0.40
Phaeoisariopsis griseola	0.00	0.80	0.00	0.24

*: At the same sampling stage, % of colonized performing plants by a fungus is significantly different from % of colonized non-performing plants by the same fungus at the level of 5% according to LSD test.

endophytic fungi in sorghum plants.

Localization of endophytic fungi in different parts of the sorghum plant

Fungal isolation from plant leaf, stem and root material at maturity stage aimed to localize the part of the sorghum plant that would be useful for isolating endophytic fungi. The results of the present study indicated that eight fungal species (*Fusarium* spp., *F. culmorum*, *N. oryzae*, *Rhizopus* sp., *Melanospora zamiae*, *Alternaria* spp., *Cercospora* sp. and *Rhizoctonia solani)* were mainly

Table 3. Distribution of endophytic fungal species and their association with sorghum plant performance in two agro-ecological zones of Burkina Faso in 2009.

Fungal species	At 3-5 leaf stage				At plant maturity			
	Sudanian zone		Sahelian zone		Sudanian zone		Sahelian zone	
	P	NP	P	NP	P	NP	P	NP
Fusarium moniliforme	15.29*	3.52	12.50	7.50	9.80*	5.88	14.30	13.61
Fusarium pallidoroseum	2.35	1.17	2.50	0.00	0.19	0.00	0.13	0.00
Fusarium equiseti	1.17	0.00	0.00	0.00	0.00	0.00	0.83	0.55
Fusarium culmorum	0.00	0.00	0.00	0.00	0.39	0.00	0.41	0.13
Fusarium spp.	8.23	2.35	10.00*	0.00	3.13	3.52	5.00	5.27
Leptosphaeria sacchari	58.82	44.70	7.50	5.00	8.43	6.47	6.25	6.94
Phoma sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13
Macrophomina phaseolina	1.17	1.17	0.00	0.00	0.00	0.00	0.13	0.00
Cladosporium sphaerospermum	2.35	4.70	5.00	2.50	1.56	0.78	0.13	0.27
Colletotrichum sublineolum	1.17*	8.23	0.00	2.50	3.52	3.52	0.27	0.55
Colletotrichum gloerosporioides	1.17	3.52	0.00	0.00	0.00	0.19	0.00	0.13
Colletotrichum spp.	1.17	1.17	0.00	0.00	0.39	0.78	0.00	0.13
Exserohilum rostratum	4.70	4.70	2.50	5.00	0.39	0.19	1.66	1.25
Nigrospora oryzae	15.29	14.11	10.00*	0.00	5.88	5.09	6.66	6.52
Gloeocercospora sorghi	1.17	5.88	0.00	0.00	0.39	0.39	0.13	0.13
Rhizopus sp.	1.17	0.00	0.00	0.00	1.37	0.98	1.52	1.66
Curvularia lunata	5.88	9.41	0.00	2.50	1.37	0.57	0.13	0.13
Curvularia penniseti	0.00	0.00	0.00	0.00	0.19	0.00	0.00	0.00
Curvularia spp.	36.47	40.00	12.50	7.50	4.90	5.09	9.58	9.72
Acremonium strictum	0.00	0.00	0.00	0.00	1.37	0.58	0.55	0.13
Acremonium sp.	0.00	0.00	0.00	0.00	0.39	0.39	0.00	0.00
Penicillium sp.	20.00	10.58	20.00*	5.00	13.92	12.35	4.02	3.61
Trichothecium sp.	1.17	0.00	0.00	0.00	0.00	0.19	0.41	0.13
Epicoccum purpurascens	1.17	0.00	0.00	2.50	0.19	0.39	0.27	0.13
Bipolaris spicifera	0.00	0.00	0.00	0.00	0.00	0.00	0.27	0.13
Bipolaris sorghicola	1.17	0.00	0.00	0.00	0.19	0.00	0.13	0.27
Bipolaris spp.	1.17	0.00	0.00	0.00	0.39	0.00	0.27	0.69
Melanospora zamiae	1.17	0.00	0.00	0.00	0.19	0.00	0.83	0.41
Alternaria alternata	8.23	7.05	0.00	0.00	0.19	0.19	0.13	0.00
Alternaria longissima	4.70	7.05	0.00	0.00	0.19	0.00	0.27	0.27
Alternaria spp.	0.00	0.00	0.00	0.00	0.00	0.19	0.13	0.83
Ascochyta sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.41	0.00
Botryodiplodia theobromae	0.00	0.00	0.00	0.00	0.00	0.19	0.27	0.97
Cercospora sp.	4.70	1.17	0.00	0.00	0.39	0.98	0.13	0.00
Rhizoctonia solani	1.17	0.00	0.00	0.00	2.94	2.35	1.08	2.08
Myrothecium sp.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13
*Diplodia*sp.	0.00	0.00	0.00	0.00	0.00	0.58	0.00	0.00
Peronoslerosporasorghi	0.00	0.00	0.00	0.00	0.00	0.19	0.00	0.00
Phaeoisariopsis griseola	0.00	1.17	0.00	0.00	0.00	0.00	0.00	0.41

P: Performing plant; NP: non-performing plant. *: From the same zone and at the same sampling stage, % of colonized performing plants by a fungus is significantly different from % of colonized non-performing plants by the same fungus at the level of 5% according to LSD test.

encountered in sorghum roots, while four species (*L. sacchari*, *G. sorghi*, *Acremonium* sp. and *Bipolaris* spp.) were most commonly isolated from the sorghum leaf (Table 4). The following fungi were easily isolated simultaneously from two different parts of the plant: *F. equiseti*, *E. rostratum*, *Curvularia* spp. and *A. longissima* were mainly isolated from leaf and root; *C. sublineolum* and *Penicillium* sp. from leaf and stem and *F. moniliforme* and *Trichothecium* sp. from stem and root. The remaining fungi were invariably encountered in sorghum leaf, stem

Table 4. Distribution of endophytic fungi in different parts (leaf, stem and root) of sorghum plant at maturity stage.

Fungal species	Colonized plants by fungi (%)		
	Leaf	Stem	Root
Fusarium moniliforme	27.14b	38.57a	37.30a
Fusarium pallidoroseum	0.71a	0.00a	0.00a
Fusarium equiseti	2.50a	0.00b	1.15ab
Fusarium culmorum	0.00b	0.00b	2.30a
Fusarium spp.	12.50b	8.57b	18.84a
Leptosphaeria sacchari	33.21a	6.42c	23.07b
Phoma sp.	0.35a	0.00a	0.00a
Macrophomina phaseolina	0.35a	0.00a	0.00a
Cladosporium sphaerospermum	2.14a	2.50a	0.76a
Colletotrichumsublineolum	8.21a	5.00ab	1.92b
Colletotrichum gloeosporioides	0.71a	0.00a	0.00a
Colletotrichum spp.	1.42a	1.07a	0.00a
Exserohilum rostratum	3.57a	0.35b	5.00a
Nigrospora oryzae	20.71b	6.07c	29.23a
Gloeocercospora sorghi	2.14a	0.00b	0.00b
Rhizopus sp.	3.57b	1.78b	7.69a
Curvularia lunata	1.78a	0.07a	2.30a
Curvularia penniseti	0.00a	0.35a	0.00a
Curvularia spp.	25.35a	13.57b	31.15a
Acremonium strictum	2.50a	1.42a	1.53a
Acremonium sp.	1.42a	0.00b	0.00b
Penicillium sp.	25.00a	26.78a	16.92b
Trichothecium sp.	0.00b	0.35ab	1.53a
Epicoccum purpurascens	0.35a	0.35a	1.53a
Bipolaris spicifera	0.35a	0.00a	0.76a
Bipolaris sorghicola	0.35a	0.00a	1.15a
Bipolaris spp.	2.85a	0.00b	0.38b
Melanospora zamiae	0.35b	0.35b	3.07a
Alternaria alternate	0.35a	0.00a	0.78a
Alternaria longissima	0.35ab	0.00b	1.53a
Alternaria spp.	0.35b	0.35b	2.30a
Ascochyta sp.	0.00a	0,35a	0.00a
Botryodiplodia theobromae	0.00b	2.50a	1.15ab
Cercospora sp.	0.35b	0.35b	2.30a
Rhizoctonia solani	6.78b	2.50c	11.15a
Myrothecium sp.	0.00a	0.00a	0.38a
Diplodia sp.	0.71a	0.35a	0.00a
Peronoslerospora sorghi	0.35a	0.00a	0.00a
Phaeoisariopsis griseola	0.35a	0.00a	0.00a

Means within the same line followed by the same letter are not significantly different at 5% level, using the LSD test.

and root at low levels of infection.

DISCUSSION

With the objective of exploring potential endophytic fungi for control of fungal pathogens in sorghum, classical endophyte isolation and morphological identification methods were employed in order to identify all culturable fungi present in leaf, stem and root tissues of sorghum collected from local farmers fields in two agro-ecological zones in Burkina Faso. Endophytic fungi isolated from

these different tissue types were compared in order to detect tissue-specific differences in the communities. A total of 39 fungal species were identified representing 25 distinct genera with the most prevalent isolates being representatives of the *Fusarium*, *Leptosphaeria*, *Curvularia* and *Penicillium* genera. In the majority of cases, it was possible to identify fungal isolates to species level based on morphological characteristics and the use of several taxonomic keys. However, in some situations, it was not possible to identify isolates with certainty beyond genus level. This is particularly relevant for isolates belonging to the *Curvularia*, *Fusarium*, *Bipolaris*, *Colletotrichum* and *Alternaria* genera, which appeared quite frequently. Several unidentified fungal species belonging to *Trichothecium*, *Penicillium*, *Rhizopus*, *Cercospora* and *Diplodia* genera were also isolated.

These findings are largely in agreement with other sorghum-related studies in that several fungal species isolated were already known to be pathogens of sorghum (Zida et al., 2008). Sorghum grain mold most likely occurs due to a combination of *Curvularia lunata*, members of the *F. moniliforme* complex, *Alternaria* spp., *Bipolaria* spp., *Cladosporium* spp. or *Phoma* spp. *L. sacchari*, *C. sublineolum*, *F. moniliforme* and other fungi isolated during this study are known to occur as pathogens of sorghum (ICRISAT, 1980). Interestingly, these fungi were isolated from performing plants, appearing healthy and showing no obvious symptoms of disease. This could be tentatively interpreted as a pathogen suppression effect as a result of the presence of other competing organisms within the plants, or could more specifically be the result of *in situ* pathogen suppression by a fungal endophyte, as has already been described. In previous studies, *Fusarium* spp. has been found in plants of maize, sorghum and soybean without causing symptoms (Leslie et al., 1990) and also *L. sacchari* (as *Phoma sorghina*) has been reported as an endophyte in rice (Fisher and Petrini, 1992).

Future investigations will be needed in order to address these hypotheses. For example, *Fusarium verticillioides* has been described as a pathogen of sorghum; this fungus has also been shown to act endophytically and to reduce the severity of corn smut caused by *Ustilago maydis* on maize following co-inoculation of endophyte and fungal spore suspensions in greenhouse experiments (Lee et al., 2009). Despite the fact that these experiments took place under greenhouse conditions, one cannot rule out the possibility of a pathogen suppression effect by *F. verticilliodes* in the field. *F. moniliforme* is known to exist as an endophyte and a facultative pathogen transmitting both vertically as laterally (Bacon et al., 2001). It is also significant that *F. moniliniforme* is known to produce fumonisin mycotoxins in sorghum in addition to being a well-known pathogen causing head mold (Shetty and Bhat, 1997).

L. sacchari, isolated during this study, is a ubiquitous and common fungus in the tropics and subtropics, causing diseases of cereals and other *Gramineae* and forage crops (White and Morganjones, 1983). *L. sacchari* is also known to cause leaf spots of minor importance in a variety of hosts including sorghum and maize and leads to seedling loss in sorghum through pre- and post-emergence death (Zida et al., 2008). Furthermore, it has recently been found as a pathogen on wheat leaves in Argentina (Perello and Moreno, 2005). The isolation of *L. sacchari* from leaves, stems and roots in this study confirms that this fungus may exist as a pathogen of sorghum in Burkina Faso. However, since most of the plants collected during this study were apparently healthy, with no visible symptoms of disease, it is possible that *L. sacchari* was not in fact acting as a pathogen in these plants. A potential correlation between *L. sacchari* and *Curvularia* is particularly interesting owing to the recent observation that *Curvularia* species were among the endophytes with the greatest ability to significantly reduce the Black Pod Rot caused by *Phytophthora palmivora* in cocoa tree pods in Brazil (Hanada et al., 2010). One could speculate that the correlation between *Curvularia* spp. and *L. sacchari* was representative of an association between these two fungi whereby both are found simultaneously in plants, but the presence of *Curvularia* prevents development of disease by *L. sacchari*. It cannot be excluded that the correlations and effects observed in this study might be in part influenced by the presence of non-culturable fungi within the sorghum tissues. The endophyte isolation method employed (Petrini, 1986) relies on the growth of fungi which can be readily cultured on laboratory media (PDA), and therefore it does not provide any information on those fungi which might not be amenable to laboratory culture conditions. Future studies could also assess the presence of these fungi by employing DNA sequencing technologies. Nevertheless, the methods employed here have yielded a considerable number of potentially beneficial endophytes and interesting observations.

In conclusion, several studies have indicated a positive effect of fungal endophytes on pathogen suppression (Arnold et al., 2003; Hanada et al., 2010; Shittu et al., 2009). Other studies have reported cases in which endophytes have no effect on fungal infection. For example, *Neotyphodium coenophialum* presence was shown to have no influence on the severity of stem rust caused by *Puccinia graminis* in tall fescue seedlings. Studies indicated that endophytes may only be beneficial to plants under certain environmental conditions (Wali et al., 2006; Welty et al., 1991). Clearly, this is a complex area, and to our knowledge, the work presented here is the first thorough report concerning endophyte isolation from sorghum plants, representing a starting point for investigation into endophytic potential within sorghum. Investigation into molecular identification and pathogenicity tests of the isolated endophytic fungi, the effects of specific fungi on sorghum health as well as screening of

isolates with the potential to increase the stress tolerance will be the topic in future studies.

Conflict of Interests

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

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Improvement of soybean growth and productivity by inoculation with two yeast species in new reclaimed sandy soil amended with humic acid

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The use of biofertilizers and organic matter can eventually reduce the need for inorganic synthetic fertilizers which are potentially more detrimental to the environment. The objective of this work was to study the impact of soil inoculation with *Rhodotorula mucilaginosa* MB151 and *Saccharomyces cerevisiae* 66 in a soil inoculated with *Bradyrhizobium japonicum* 110 and amended with different concentrations of humic acid (HA) or fertilized with full dose of N (nitrogen), P (phosphorus) and K (potassium) as full NPK control on soybean growth and productivity. Field inoculation experiments were carried out during two successive seasons in a sandyloamy soil. The total microbial count, the physiological and the yield parameters of soybean were determined. The two yeast strains produce indole acetic acid and gibberellins. All the growth parameters of soybean were significantly enhanced due to application of yeasts, especially *S. cerevisiae*. The treatment T11 (*S. cerevisiae* + 3% HA) gave the significantly highest increase in N% and consequently the crude protein percent (6.37, 6.43; 39.81, 40.19) of soybean seeds at both seasons respectively. The soybean seeds oil percent increased as the HA% increased in the different treatments during the first season in comparison with control T1 (full NPK). The treatments T12 (*S. cerevisiae* + 4% humic acid) and T13 (S. cerevisiae + 5% humic acid) gave increase in seeds oil % equal 1.2 times the control T1. T11 (*S. cerevisiae* + 3% HA) gave significant increase in seed yield and straw yield (3.816 and 3.838; 5.377 and 5.380 Mg.ha^{1}) during the two seasons, respectively. It could be concluded that application of yeasts in soil amendment with HA, through the numerous direct or indirect mechanisms of action, allow significant enhancement in soybean growth and productivity.

Key words: Organic matter, *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae, Bradyrhizobium japonicum*, soybean.

INTRODUCTION

Excessive application of chemical fertilizers has led to health and environmental hazards. Therefore, sustainable

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ecological agriculture requires agricultural practices that are healthy to the environment and maintain the long-term balance of the soil ecosystem. In this context, use of microbial inoculants (biofertilizers) in agriculture represents an environmentally safely alternative to further applications of mineral fertilizers (Khan et al., 2007). The documented benefits of plant inoculation with beneficial microorganisms include reduced pathogen infection, improved fertilizer use efficiency, improved resistance such as drought, mineral deficiency and salinity (Kim et al., 2011; Amprayn et al., 2012). In addition, they produce phytohormones, siderophore and vitamin B12 that act as plant growth regulator (Pan et al., 2002).

Most of the research has focused on the use of particular bacterial species, commonly referred to as plant-growth promoting rhizobacteria (PGPR) (Vessey, 2003), or mycorrhizal fungi (Johansson et al., 2004); the role of other microbial species, including yeasts, has received less attention (Nassar et al., 2005).

Yeasts are unicellular fungi that proliferate primarily through asexual means and grow rapidly on simple carbohydrates (Botha, 2011). Because of their nutritional preference, yeast populations are generally an order of magnitude higher in the rhizosphere as opposed to the bulk soil (Botha, 2011). A diverse range of yeasts exhibit plant growth promoting characteristics, including pathogen inhibition (El-Tarabily and Sivasithamparam, 2006); phytohormone production and phosphate solubilization (Amprayn et al., 2012); nitrogen and sulphur oxidation (Al-Falih and Wainwright, 1995); siderophore production (Sansone et al., 2005), stimulation of mycorrhizal-root colonization (Alonso et al., 2008) and production of vitamin B12. Yeasts in the root zone may influence plant growth indirectly by encou-raging the growth of other plant growth promoting rhizo-microorganisms, through vitamin B12 production (Medina et al., 2004).

The application of composted organic matter to soil produces beneficial effects on the chemical, biochemical and physical quality of soil; increased soil microbial population and activity and its plant nutrition capacity (Arancon et al., 2004; Spaccini and Piccolo, 2009). Hence, a particular advantage of compost amendment to soil is the increase in colloidal humified organic matter that affects the quantitative and qualitative long term status of soil organic matter (Adani et al., 2007; Spaccini and Piccolo, 2009).

Moreover soil organic matter (SOM) is a basic component of the agroecosystem and acts as an essential link among the various chemical, physical and biological soil properties. It helps to prevent erosion and desertification and is a driving variable in environmental changes since it acts both as a source and as reservoir for carbon (Campitelli et al., 2006)

Soybean (*Glycine max* (L.) Merr.) is the most important oil seed crop in world with a seed protein content of 40-42% for human consumption and oil content of 20-22%. It is used as fodder for animal and is important in improved crop rotation systems (Carsky et al., 1997). When in symbiotic association with *Bradyrhizobium japonicum*, soybean plants can fix up to 200 kg N ha^{-1} yr^{-1} (Javaid and Mahmood, 2010).

Since, most of the research has focused on the use of PGPR and the role of other microbial species such as yeasts has received less attention, it is supposed that a good understanding of the role of soil yeasts in the rhizosphere hold a key to future sustainable agricultural practices.

Therefore, the objective of this work was to study the impact of soil inoculation with *Rhodotorula mucilaginosa* and *Saccharomyces cerevisiae* in a newly reclaimed soil inoculated with *B. japonicum* 110 and amended with different concentration of humic acid (HA) as organic matter on the growth parameters and productivity of soybean plants.

MATERIALS AND METHODS

Microbial strains and culture conditions

Yeast strains of *S. cerevisiae* 66 and *R. mucilaginous* MB151 were kindly provided by Microbiology Department, Soils, Water and Environment Research Institute (ARC), Giza, Egypt. The strains were grown on glucose peptone and yeast extract agar (GPY) medium (Difco, 1985). Whereas, *B. japonicum* 110 was kindly provided by the Biofertilizers Production Unit, Soil, Water and Environment Research Institute, Agricultural Research Center (ARC), Giza, Egypt. *B. japonicum* 110 was grown on yeast extract mannitol agar (YEM) medium (Vincent, 1970).

Plant growth-promoting characteristics of the two yeast strains

The ability of the two tested yeast strains to produce plant growth promoting hormones such as IAA was studied according to Glickmann and Dessoux (1995) while total gibberellins was studied according to the method described by Udagwa and Kinoshita (1961).

S. cerevisiae and *R. mucilaginosa* inocula preparation

The two yeasts *S. cerevisiae* and *R. mucilaginosa* were inoculated in 250 ml Erlenmeyer flasks containing 50 ml of liquid glucose peptone and yeast extract (GPY) medium. Then, they were incubated at 30°C for 48 h on a rotary shaker at 150 rpm.

Humic acid (HA) preparation

Mature compost with physical and chemical composition shown in Table 1 was used for extraction of humic acid substances. The extraction and the purification of humic acid (HA) were determined according to the methods described by Sanchez-Monedero et al. (2002) and Kononova (1966), respectively.

Elemental analysis [carbon (C), hydrogen (H), nitrogen (N), sulphur (S) and oxygen (O_2)] of the purified HA was performed by microanalyser (Table 2) as described by Goh and Stevenson (1971). The total acidity and carboxyl groups of HA were determined according to the method described by Dragunova (1958) and Schnitzer and Gupta (1965), respectively. However, phenolic groups were determined as described by Kononova (1966).

Table 1. Physical and chemical analysis of the used compost.

Macronutrient (%)			Organic	Organic	C/N	EC	pH	Parasite
N	P	K	carbon (%)	mater (%)	Ratio	(dS/m)		
1.35	0.52	0.55	25	43.1	18.5/1	3.21	7.6	Not detected

N: Nitrogen, P: phosphorous, K: potassium, C/N: carbon: nitrogen ratio EC: electrical conductivity.

Table 2. Characteristic of humic acid (HA) extracted from compost.

C%	N%	H%	S%	O_2%	Total acidity (mmole/100 g)	Carboxyl groups (mmole/100 g)	Phenolic groups (mmole/100 g)
50.0	4.1	5.0	1.0	39.9	425	195	230

C: carbon, N: nitrogen, H: hydrogen, S: sulfpher, O_2: oxygen.

Field trials

Two field experiments were carried out at Ismailia Research and Experimental Station, Ismailia Governorate, Egypt ((30° 35` 28.35`` N 32° 15` 6.56`` E), during the 2011 and 2012 summer seasons on a sandy loamy soil. This soil had the following physical and chemical characteristics: sand 70%; clay 29.3%, pH 7.73; electrical conductivity (EC)1.15 dSm^{-1}; organic carbon 0.143%; total N 624 ppm; available P 8.6 ppm; available K 348 ppm and $CaCO_3$ 1.5%. The experiments were conducted in a complete randomized plot design; where the plot size was 3 m in length x 3.5 m in width in 3 replicates. Each plot consisted of 6 lines with 3 m in length and 30 cm in width. Organic fertilizer, humic acid was randomly assigned to main plots with soil irrigation as 48 L ha^1 of humic acid with different concentration 1, 2, 3, 4 and 5%. In the sub-plot design, the two yeast species (*S. cerevisiae* and *R.mucilaginosa*) were distributed as biofertilizer. Their liquid cultures (10^8CFU) were added with soil irrigation at a rate of 24 L ha^1 in three equal doses after 15, 30 and 45 days of sowing. The soybean seeds [*Glycine max* (L.) Merrill] cv. Crawford was kindly provided by the Field Crops Research Institute, ARC, Giza, Egypt. The seeds were sterilized as described by Vincent, (1970) and then coated with *B. japonicum* 110 suspension (~10^8 cells.ml^1) using Arabic gum (40%) as an adhesive agent for 2 h before planting. The treated seeds were sown in hills (three seeds /hill, then after seed germination, the seedlings thinned to two seedlings/hill) on one side of the line at a distance of 20 cm apart.

Twelve treatments were included in the experiment and were arranged in a complete randomized plot design. The following treatments were used: Full NPK as control (T1); *R. mucilaginosa* without HA (T2); R. *mucilaginosa* + 1% HA (T3); *R. mucilaginosa*. + 2% HA (T4); *R. mucilaginosa* + 3% HA (T5); *R. mucilaginosa*. + 4% HA (T6); *R. mucilaginosa* + 5% HA (T7); *S. cerevisiae* without HA (T8);*S. cerevisiae* + 1% HA (T9); *S. cerevisiae* + 2% HA (T10); *S. cerevisiae* + 3% HA (T11); *S. cerevisiae* + 4% HA (T12); *S. cerevisiae*+ 5% HA (T13).

At soil preparation all plots received the recommended dose of phosphorus (15.5% P_2O_5) 360 kg ha^1 as calcium super phosphate and potassium (48% K_2O) 120 kg ha^1 as potassium sulphate, once after the first irrigation. Nitrogen (33.5% N) of 107.5 kg ha^1 as ammonium sulphate (36 nitrogen unit ha^1) was added during planting to activate nodulation. The plants were grown for 120 days, under field conditions. Water was supplied regularly as needed using sprinkler irrigation system.

Assays

Nodulation was estimated at 45 and 75 days after planting by counting the number of nodules (Nod no) in plant roots chosen randomly from each plots. Nodules were dried (60°C for three days) and the nodules dry weight (Nod DW) was measured. Nitrogenase activity was determined in an indirect way by acetylene reduction assay (ARA) according to Somasegaran and Hobben (1994). ARA was determined by GC using Hewlett Packard chromatography model HP (6890 GC) fitted with dual flam detector and 150 × 0.4 cm diameter stainless steel column fitted with propack - N × R 100-120 mesh. Nod No, Nod DW and ARA are the average of five plants from each treatment from each plot at 45 and 75 days.

Total nitrogen (N), phosphorous (P) and potassium (K) percentages (%) were determined in shoot dry matter and seeds of soybean at 45, 75 days and harvest according to Jackson (1958). The crude protein and oil percentage in seeds were also determined (AOCS, 1982). At harvest, shoot dry weight (Sh.DW) and pods number (Pods no.) were measured. The seeds and straw yield (Mg.ha^1) were also determined. All the tested parameters were determined during the two seasons.

Estimation of total microbial count in rhizosphere of soybean plants

The population dynamics of total microbial counts, including yeast were determined in the rhizosphere of soybean plants at 45 and 75 days by the plate count method according to Reinhold et al. (1985).

Statistical analysis

The data were analyzed statistically by applying Duncan's multiple range at P value 0.05 (Duncan, 1955), using a software Package "Costat", a product of Cohort software INC., Berkley, California.

RESULTS

Plant growth-promoting characteristics of the two yeast strains

Potentialities of the two yeast strains (*S. cerevisiae* and *R. mucilaginosa*) to produce phytohormones IAA and gibberellins were tested. Figure 1 shows that the two yeast strains have the ability to produce IAA and gibberellins. It was obvious that *S. cerevisiae* strain produced higher gibberellins (461 µg.ml^1) than *R.*

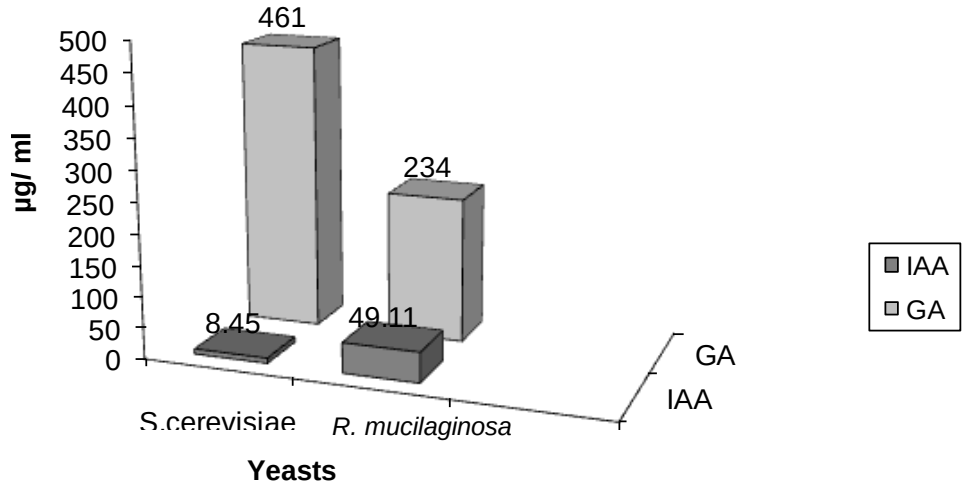

Figure 1. Growth hormones produced by the two yeast strains. IAA: indole acetic acid, GA: gibberellic acid.

Table 3. Effect of inoculation with the two yeast strains on total microbial count (CFU $\times 10^6$), yeast count (CFU $\times 10^4$) in soybean rhizospheric soil amended with humic acid.

Treatment	Season 2011				Season 2012			
	T. count (CFU×10^6) /g rhizosphere soil		T. yeast (CFU×10^4) /g rhizosphere soil		T. count (CFU × 10^6) /g rhizosphere soil		T. yeast (CFU×10^4) /g rhizospher soil	
	45 days	75 days	45 days	75 days	45 days	75 days	45 days	75 days
Control full NPK (T1)	15	32	3	7	22	51	5	10
Rhodotorulamucilaginosa (T2)	33	37	18	25	32	40	16	29
R. + Humic acid (1%) (T3)	40	38	23	31	31	51	25	37
R. + Humic acid (2%) (T4)	44	47	29	36	49	66	33	41
R. + Humic acid (3%) (T5)	61	88	42	58	95	99	47	55
R. + Humic acid (4%)(T6)	55	78	40	49	73	84	46	54
R. + Humic acid (5%) (T7)	49	54	35	43	52	68	38	47
Saccharomyces cerevisiae (T8)	40	46	22	30	49	66	24	33
S. + Humic acid (1%) (T9)	48	51	28	37	67	83	31	40
S. + Humic acid (2%) (T10)	59	65	33	45	79	98	40	48
S. + Humic acid (3%) (T11)	88	101	50	62	106	130	55	66
S. + Humic acid (4%) (T12)	79	90	44	56	96	124	49	59
S. + Humic acid (5%) (13)	66	82	39	51	88	102	45	53

T. count: total microbial count, T. yeast: total yeast count, CFU: colony forming unit, g: gram, NPK: nitrogen, phosphorous and potassium, *R.*: *Rhodotorula, S.*: *Saccharomyces*.

mucilaginosa (234 µg.ml^1) whereas *R. mucilaginosa* produced more IAA (49.11 µg.ml^1) than *S.* cerevisiae (8.45 µg.ml^1).

Total microbial count in the rhizosphere of soybean plants in soil inoculated with the tested yeast strains and amended with humic acid

All the treatments showed increase in the dynamics of total microbial populations (CFU × 10^6.g^1 rhizosphere) and total yeast count (CFU × 10^4.g^1 rhizosphere) in comparison with the treatment T1 (full NPK) in soybean rhizospheric roots during the two seasons at 45 and 75 days (Table 3). However, in treatment T5 and T11 the increases of total microbial populations and total yeast count were higher than in all the other treatments in soybean rhizospheric roots, during the two seasons at 45 and 75 days. The total microbial and yeast count were increased by the inoculation with *S. cerevisiae* more than

Table 4. Nodules number, nodules dry weight (g) and acetylenes reduction assay (ARA) (µmole C2H4.g1 dry nodule) in soybean roots inoculated with the two yeast strains and amended with humic acid.

Treatment	Season 2011						Season 2012					
	Nod. no. plant^{1}		Nod DW (g.plant^{1})		ARA (µmol.g^{1}nodules)		Nod. no.plant^{1}		Nod DW (g.plant^{1})		ARA (µmol.g^{1}nodules)	
	45 d	75 d	45 d	75 d	45 d	75 d	45 d	75 d	45 d	75 d	45 d	75 d
Control full NPK (T1)	10.00	25.0	0.15	0.31	0.05	2.31	13.00	35.0	0.22	0.75	0.11	3.61
Rhodotorulamucilaginosa (T2)	20.60*	61.8*	0.36*	0.42*	0.31	6.55*	19.80*	63.0*	0.40*	0.51	0.41	22.53*
R. + Humic acid (1%) (T3)	22.90*	68.7*	0.48*	0.49*	0.61	10.10*	22.90*	68.0*	0.39*	0.60	0.65	24.51*
R. + Humic acid (2%) (T4)	24.00*	89.7*	0.40*	0.60*	1.31*	13.55*	22.80*	90.1*	0.40*	0.70	1.51*	28.00*
R. + Humic acid (3%) (T5)	33.3*	122.0*	0.57*	0.70*	2.31*	25.10*	32.40*	181.0*	0.56*	0.82	3.55*	42.20*
R. + Humic acid (4%)(T6)	29.9*	99.0*	0.53*	0.61*	2.22*	22.00*	28.90*	101.3*	0.49*	0.71	3.12*	33.35*
R. + Humic acid (5%) (T7)	28.40*	85.2*	0.43*	0.52*	1.33*	13.76*	26.13*	87.0*	0.41*	0.54	1.71*	27.52*
Saccharomyces cerevisiae (T8)	24.80*	74.4*	0.39*	0.66*	0.41	5.70*	24.13*	83.0*	0.43*	0.51	1.09	24.50*
S. + Humic acid (1%) (T9)	26.60*	78.0*	0.44*	0.70*	0.53	11.50*	27.30*	88.3*	0.49*	0.55	1.10	26.57*
S. + Humic acid (2%) (T10)	32.03*	96.9*	0.50*	0.74*	3.20*	28.30*	28.2*	110.0*	0.53*	0.81	4.11*	32.53*
S. + Humic acid (3%) (T11)	29.10*	135.0*	0.56*	0.76*	5.11*	44.18*	36.00*	155.0*	0.58*	0.87	6.15*	49.65*
S. + Humic acid (4%) (T12)	37.50*	112.5*	0.67*	0.85*	4.61*	41.30*	40.60*	117.1*	0.68*	0.83	4.80*	47.51*
S. + Humic acid (5%) (13)	30.70*	92.1*	0.55*	0.73*	3.78*	35.90*	30.13*	103.3*	0.56*	0.70	4.20*	46.4*
LSD at 0.05	1.77	1.85	0.09	0.06	1.02	1.21	3.04	3.59	0.11	0.14	1.04	1.68

Nod DW: nodule dry weigh, ARA: acetylene reduction assay, g: gram, d: day, NPK: nitrogen, phosphorous and potassium, R.: *Rhodotorula*, S: *Saccharomyces*, LSD at 0.05: least significant difference at P value 0.05. *: Significant result.

the inoculation with *R. mucilaginosa.* Moreover, the enhancement in the total microbial populations and yeast count increased in the second season.

Root-nodulation related characters of soybean plants

Nod no., Nod Dw. per plant and ARA were significantly higher in almost all the treatments in which plants were inoculated with the *B. japonicum* combined with each of the two yeast species *R. mucilaginosa* or *S. cerevisiae* and humic acid as compared to the treatment T1 (full NPK) (Table 4). Furthermore, the data in Table 4 showed that the amendment of soil with different concentrations of HA improved soybean nodulation as well as the related characters. During the two seasons, the treatments T5, T6, T11 and T12 gave the significantly highest values of Nod no, Nod DW and ARA. The nitrogenase activity values increased significantly with the treatment T5 inoculated with *R. mucilaginosa* + 3% HA + *B. japonicum* (25.10; 42.2 µmol ethylene h^{-1} .g^{1} Nod DW) at 75 days during the two seasons, respectively. However, the increase in nitrogenase activity values in treatment T11 inoculated with *S. cerevisiae* + 3% HA + *B. japonicum* during the two seasons was higher than that in the treatment T5 at 45 and 75 days, respectively. In the second season, the results come in the same trend as the first one (Table 4), even at 45 or 75 days of growth. The treatments T5 and T11 proved that they are still the superior ones that gave the significant highest values of nitrogenase activity.

Shoot inorganic mineral contents of soybean dry shoots

N, P and K percentages in shoot dry matter were increased in inoculated plants with *R. mucilaginosa* + *B. japonicum* + HA and *S. cerevisiae* + *B. japonicum* + HA in both growth

Table 5. Effect of inoculation with the two yeast strains and amendment with humic acid on N, P and K percentage in soybean shoots during the two successive seasons.

Treatment	Season 2011						Season 2012					
	N (%)		P (%)		K (%)		N (%)		P (%)		K (%)	
	45 d	75 d	45 d	75 d	45 d	75 d	45 d	75 d	45 d	75 d	45 d	75 d
Control full NPK (T1)	1.33	2.31	0.37	1.73	1.82	0.77	1.51	2.20	0.36	1.70	1.90	1.00
Rhodotorulamucilaginosa(T2)	0.90	1.45	0.29	0.49	1.30	0.75	0.95	1.65	0.30	0.60	1.51	0.82
R. + Humic acid (1%) (T3)	0.96	1.65	0.31	0.53	1.51	0.83	1.00	1.76	0.33	0.63	1.56	0.85
R. + Humic acid (2%) (T4)	1.00	1.81	0.34	0.85	1.79	0.80	1.21	1.93	0.34	0.94	1.83	0.87
R. + Humic acid (3%) (T5)	1.40	2.51*	0.40	1.77	1.93*	1.00*	1.52	2.61*	0.42*	1.91*	1.59	1.05
R. + Humic acid (4%)(T6)	1.45	2.31	0.36	1.52	1.91	0.92*	1.33	2.33*	0.35	1.72	2.00*	0.92
R. + Humic acid (5%) (T7)	1.30	1.95	0.34	1.31	1.81	0.88	1.37	2.00	0.35	1.56	1.95	0.90
Saccharomyces cerevisiae (T8)	0.94	1.54	0.30	0.55	1.40	0.77	1.10	1.75	0.39	0.60	1.75	0.80
S. + Humic acid (1%) (T9)	0.98	1.60	0.33	0.61	1.53	0.80	1.21	1.82	0.40	0.66	1.87	0.83
S. + Humic acid (2%) (T10)	1.10	1.85	0.32	0.87	1.81	0.86	1.33	1.96	0.40	1.00	1.96	0.90
S. + Humic acid (3%) (T11)	1.40	2.56*	0.43*	1.82	2.00*	1.10*	1.62	2.67*	0.50*	2.00*	2.11*	1.21*
S. + Humic acid (4%) (T12)	1.37	2.41	0.36	1.56	1.94*	0.95*	1.48	2.56*	0.45*	1.93*	1.96	1.00
S. + Humic acid (5%) (13)	1.31	1.99	0.35	1.41	1.90	0.90*	1.43	2.44*	0.42*	1.72	1.99	0.95
LSD at 0.05	0.168	0.141	0.059	0.102	0.099	0.116	0.127	0.122	0.052	0.17	0.095	0.185

N: nitrogen, P: phosphorous, K: potassium, d: day, NPK: nitrogen, phosphorous and potassium, R: *Rhodotorula*, S: *Saccharomyces*, LSD at 0.05: least significant difference at P value 0.05, *: Significant result.

periods 45 or 75 days during the two seasons, especially the treatments T5 and T11 that received 3% HA (Table 5).

Data in Table 5 showed that the significantly highest value of N% in soybean shoot dry matter were in treatment T5 (2.51 and 2.61, during the first and second season, respectively) at 75 days. In addition, treatment T11 gave the significantly highest value of N% (2.56 and 2.67, at 75 days), during the first and second season, respectively.

P % increased significantly in treatment T5 during the second season at 45 and 75, but the increase was insignificant in the first season as compared to control T1 (full NPK). However, T11 showed significant P % increase in both seasons at 45 and 75 days as compared to the control T1. Furthermore, T11 gave significant K% in both seasons at 45 and 75 days compared to the control T1.

Comparing the data obtained in Table 6, it was found that treatments T5, T6, T11 and T12 significantly increased N% and consequently the crude protein of soybean seeds in both seasons. However, the highest of them (6.37, 6.43; 39.81, 40.19) were obtained in treatment T11 at both seasons, respectively. In addition, results in Table 6 shows that the seed oil % increased as the HA % increased in the different treatments during the first season in comparison with control T1 (full NPK). On the other hand, the seed oil % decreased in all treatments as compared to control T1 in the second season.

Table 7 shows the effect of the different treatments on the NPK content of soybean straw. The data proved that T5 and T11 gave significant increase in NPK % in soybean straw during the first season as compared to the treatment T1 (control full NPK). However, in the second season, T5 and T11 gave the significant increase in straw N% only. On the other hand, T11 showed the highest value of N% (1.55) and P% (0.53) in second season.

As shown in Table 8, the yield components of soybean plants inoculated with some yeast strains in soil amended with humic acid proved that, the treatment T11 in both seasons is considered the best treatment. It showed the significant highest plant Sh.DW (44.6 and 44.5 g plant^{1}) and the significant highest number of pods per plant (32.3 and 32.5). In addition T11 showed significant increase in seed yield (3.816 and 3.838 Mg.ha^{1}) as well as it gave significant increase in straw yield (5.377 and 5.380 Mg.ha^{1}) during the two seasons, respectively.

DISCUSSION

For a sustainable agriculture system, it is necessary to utilize renewable inputs which can maximize the ecological benefits and minimize the environmental hazards. The present study have assessed the influence of two yeast strains (*R. mucilaginosa* and *S. cerevisiae*) in a soil amended with *B. japonicum* 110 and different concentrations of humic acid (HA) on growth and productivity of soybean plants under two field experiments.

The increase of total microbial count and total yeast

Table 6. Crude protein, oil and total nitrogen (%) in seeds of soybean plants inoculated with some yeast strains in soil amended with humic acid.

Treatment	Season 2011			Season 2012		
	Protein (%)	Oil (%)	Nitrogen (%)	Protein (%)	Oil (%)	Nitrogen (%)
Control full NPK (T1)	34.37	20.00	5.50	35.00	30.80	5.60
Rhodotorulamucilaginosa(T2)	28.44	18.80	4.50	28.75	18.10	4.60
R. + Humic acid (1%) (T3)	30.00	19.60	4.80	31.69	19.20	5.07
R. + Humic acid (2%) (T4)	32.50	21.00*	5.20	34.56	21.30	5.53
R. + Humic acid (3%) (T5)	38.75*	20.80*	6.20*	38.31*	21.60	6.13*
R. + Humic acid (4%)(T6)	37.50*	21.70*	6.00*	37.69*	20.90	6.03*
R. + Humic acid (5%) (T7)	34.19	22.20*	5.47	35.19	21.90	5.63
Saccharomyces cerevisiae (T8)	28.56	18.63	4.57	29.19	18.70	4.67
S. + Humic acid (1%) (T9)	31.25	19.30	5.00	32.69	19.17	5.23
S. + Humic acid (2%) (T10)	33.31	22.03*	5.33	32.93	22.43	5.27
S. + Humic acid (3%) (T11)	39.81*	21.83*	6.37*	40.19*	22.53	6.43*
S. + Humic acid (4%) (T12)	37.69*	23.30*	6.03*	37.69*	23.40	6.03*
S. + Humic acid (5%) (13)	36.88*	23.43*	5.90*	36.44*	23.10	5.83
LSD at 0.05	0.332	0.51	0.18	1.21	1.08	0.24

NPK: nitrogen, phosphorous and potassium, R: *Rhodotorula*, S: *Saccharomyces*, LSD at 0.05: least significant difference at P value 0.05, *: Significant result.

Table 7. Effect of inoculation with the two yeast strains and amended with humic acid on N, P and K percentage in soybean straw.

Treatment	Season 2011			Season 2012		
	N (%)	P (%)	K (%)	N (%)	P (%)	K (%)
Control full NPK (T1)	1.20	0.04	0.07	1.34	0.44	0.09
Rhodotorulamucilaginosa (T2)	0.92	0.23*	0.07	0.91	0.23	0.08
R. + Humic acid (1%) (T3)	0.99	0.25*	0.08	0.94	0.26	0.08
R. + Humic acid (2%) (T4)	1.05	0.31*	0.08	1.05	0.32	0.08
R. + Humic acid (3%) (T5)	1.47*	0.44*	0.09*	1.49*	0.44	0.09
R. + Humic acid (4%)(T6)	1.32*	0.41*	0.09*	1.37	0.42	0.09
R. + Humic acid (5%) (T7)	1.18	0.38*	0.08	1.23	0.41	0.08
Saccharomyces cerevisiae (T8)	0.90	0.22*	0.05	0.92	0.21	0.08
S. + Humic acid (1%) (T9)	0.98	0.25*	0.07	0.99	0.27	0.08
S. + Humic acid (2%) (T10)	1.12	0.28*	0.07	1.13	0.33	0.08
S. + Humic acid (3%) (T11)	1.51*	0.49*	0.09*	1.55*	0.53*	0.09
S. + Humic acid (4%) (T12)	1.41*	0.43*	0.08	1.46*	0.45	0.09
S. + Humic acid (5%) (13)	1.28*	0.39*	0.08	1.28	0.44	0.09
LSD at 0.05	0.054	0.018	0.013	0.045	0.023	0.009

N: nitrogen, P: phosphorous, K: potassium, d: day, NPK: nitrogen, phosphorous and potassium, R: *Rhodotorula*, S: *Saccharomyces*, LSD at 0.05: Least Significant Difference at P value 0.05, *: Significant result.

count in the rhizosphere of soybean plants proved that inoculation with both yeast strains + *B. japonicum* + organic matter (humic acid) increased the microbial populations (Fierer et al., 2007; Botha, 2011). The increase of total microbial count and yeast populations in soil amended with organic matter was due to the act of simple organic carbon compounds found in humic acid associated with root exudates of soybean plants that are readily assimilated by yeasts and other microorganisms (Cloete et al., 2009; Botha, 2011).

Our study illustrated that the different treatments used led to enhancement of the plant growth, because yeasts are capable of directly enhancing the plant growth by the production of plant growth regulators (El-Tarabily and

Table 8. Yield components of soybean plants inoculated with the two yeast strains in soil amended with humic acid.

Treatment	Season 2011				Season 2012			
	Sh.DW g.plant^{1}	Pods no. Plant^{1}	Seeds yield (Mg.ha^{1})	Straw yield (Mg.ha^{1})	Sh.DW g.plant^{1}	Pods no. Plant^{1}	Seeds yield (Mg.ha^{1})	Straw yield (Mg.ha^{1})
Control full NPK (T1)	41.5	21.8	3.580	5.16	43.5	25.3	3.601	5.208
Rhodotorulamucilaginosa (T2)	30.7	19.7	2.281	3.508	32	19.4	2.164	3.544
R. + Humic acid (1%) (T3)	33.2	22.3	2.448	3.869	32.7	22.8	2.440	3.952
R. + Humic acid (2%) (T4)	35.7	23.7*	2.756	4.261	34.7	24.6	2.873	4.239
R. + Humic acid (3%) (T5)	41.2	29.9*	3.420	5.016	38.4	30.8*	2.703	4.947
R. + Humic acid (4%)(T6)	39.1	27.9*	3.265	4.748	36.6	28.6*	2.451	4.751
R. + Humic acid (5%) (T7)	37.3	25.8*	2.947	4.674	34.9	27.6*	3.163	4.599
Saccharomyces cerevisiae (T8)	33	19.7	2.252	3.698	35.8	20.3	2.230	3.815
S. + Humic acid (1%) (T9)	37.6	23.2*	2.778	3.751	36.9	23.9	2.67	4.280
S. + Humic acid (2%) (T10)	39.4	25.4*	3.016	4.465	38.7	25.9	3.018	4.526
S. + Humic acid (3%) (T11)	44.6*	32.3*	3.816*	5.377*	44.5	32.5*	3.838*	5.380*
S. + Humic acid (4%) (T12)	43*	30.3*	3.590	5.168	40.9	31.8*	3.654*	5.004
S. + Humic acid (5%) (13)	40.5	27.7*	3.375	4.834	39.3	28.7*	3.373	4.783
LSD at 0.05	0.943	0.853	0.031	0.048	1.295	1.26	0.035	0.005

Sh.DW: shoot dry weight, Pods no.: pods number, g: gram, Mg: mega gram, ha: hectare, NPK: nitrogen, phosphorous and potassium, R: *Rhodotorula*, S: *Saccharomyces*, LSD at 0.05: least significant difference at P value 0.05, *: Significant result.

Sivasithamparam, 2006; Cloete et al., 2009). After growth for 75 days, soybean plants inoculated with *S. cerevisiae* + 3% HA + *B. japonicum* gave higher nodule number, nodule dry weight and nitrogenase activity. Moreover, many authors (Abd El-monem et al., 2008) studied a wide diversity of soil yeasts for their potential as bio-fertilizers. Organic fertilizers consisting of combinations of yeast strains as well as organic and inorganic components are already commercially available, which declares that some of the products are capable of re-establishing the sustainability of ecosystems, as well as enhancing the productivity of farmland for various crops (Pang et al., 2003; Botha, 2011). Our data proves that *S. cerevisiae* and *R. mucilaginosa* have the ability to produce IAA and gibberellins. Plant performance can also be increased as a result of the production of plant growth regulators compounds includes indole-3-acetic acid, indole-3-pyruvic acid, gibberellins and polyamines by yeasts (Botha, 2011).

Soil yeasts representing the genera *Candida*, *Saccharomyces, Geotrichum, Rhodotorula* and *Williopsis* have the potential to contribute to the nitrogen and sulphur cycles within soil (Al-Falih, 2006; Botha, 2011). In addition, these yeasts may be able to solubilize insoluble phosphates thus making these nutrients more readily available to plants (Botha, 2011).

Furthermore, contents of N, P and K were also higher in plants inoculated with both yeast types + *B. japonicum* in soil amended with humic acid as organic matter after growth for 45 and 75 days. The increasing N, P and K levels affected positively the plant growth, in addition to the increase of total yeast count in the soybean rhizosphere. This can be explained on the basis that yeasts are capable of indirectly enhancing the plant growth (El-Tarabily and Sivasithamparam, 2006; Cloete et al., 2009). Singh et al. (1991) found that inoculation of legumes with *S. cerevisiae* increases nodulation as well as *Arbuscular mycorrhiza* (AM) fungal colonization therefore a variety of yeasts are known to occur in the rhizosphere (Botha, 2011), and the interaction between *Mycorrhizal* fungi and soil yeasts is expected.

Alonso et al. (2008) found that yeast genera *Cryptococcus* and *Rhodotorula* were able to solubilize low soluble phosphorus sources and accumulate polyphosphates, affected root growth of rice seedlings and it was suggested that a tripartite interaction exists between the plants, AM fungi and microorganisms. Another research group concluded that both *Ascomycetous* and *Basidiomycetous* yeasts may exert a positive effect on *Glomus mosseae* colonization of cowpea as a result of vitamin B12 production, which stimulates AM development (Boby et al., 2008).

Application of humic acid + *B. japonicum* + yeasts resulted in the increase of soybean yield and other yield traits. This increases could be mainly attributed to the directly or indirectly enhancement in the rhizosphere by yeasts (El-Tarabily and Sivasithamparam, 2006; Cloete et al., 2009).

The results showed increase in seeds oil and protein contents, especially in the first season. The increase of

crude protein % mainly due to the increase of N percentage which indicate that both bio- organic matter can provide plants with essential nutrients elements required for oil and protein formation (Schmidt et al., 2000; Mekki and Ahmed, 2005). Furthermore, yeast is also a natural source of cytokinins that stimulates cell proliferation and differentiation, controlling shoot and root morphogenesis and chloroplast maturation which lead to vegetative growth stimulation (Ezz El-Din and Hendawy, 2010).

The reduction in N, P and K in soybean straw may be due to the increase of translocation rate of their element during flowering and seed formation stages. This is due to the fact that N, P and K are used for numerous plant growth processes (Miller, 2000).

In conclusion, plant growth promoting yeasts (PGPY) in addition to soil amendment with HA can be a true success story in sustainable agriculture. In fact, through their numerous direct or indirect mechanisms of action, PGPY and HA may allow significant reduction in the use of chemical fertilizers. These beneficial events producing plant growth promotion and increases in crops yield, can take place simultaneously or sequentially. There is important synergism observed on plant growth when the inoculants used contain a mixture of organisms. In order to have future beneficial inoculants for field grown crops, one approach should consider performing inoculation assays containing a mixture of soil organisms and amended soil with HA. This association could contain a mixture of PGPY stimulating plant growth at different growth stages, and showing one or more of the known PGPY mechanisms of action. It could also stimulate beneficial symbiotic organisms like AM fungi, rhizobia and *Mycorrhizae* helper bacteria (Son et al., 2001; Antoun and Prėvost, 2005).

Conflict of Interests

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

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Effect of bacteria biomass detachment on the ammonium oxidation yield

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Nitrification yield can be affected by fixed biomass or biofilm density. Infact, biofilm detachment may influence the nitrification. The present experiment investigated the effect of detachment biomass cells on nitrifying bacteria expressed via nitrification rate. We monitored nitrification rates before and after biomass detachment from gravel sampled in a small-scale model of wastewater treatment using macrophytes in vertical and horizontal filtersystems. The ammonia-oxidizing bacteria (AOB) number decreased after cell detachment whereas the number of nitrite-oxidizing bacteria (NOB) was lower and saving a constant value of 3.0 MPN/100 ml. Despite this detachment, the yield of ammonium oxidation in the vertical filter remains constant but the reaction required more time. After washing, the NO_3^--N concentration at the bottom of horizontal filter with fine gravel is more important (1.24 mg/l) than that observed at the medium (1.1 mg/l) and the top (0.8 mg/l) of basin; whereas, at the horizontal filter with coarse gravel, the nitrification performance is more important at the medium of basin with NO_3^--N concentration value of 1.14 mg/l than those obtained at the top (0.7 mg/l) and the bottom (0.98 mg/l).

Key words: Autotrophic bacteria, detachment, nitrification, turbidity.

INTRODUCTION

Nitrogen is present in the environment in a wide variety of chemical forms including organic nitrogen, ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), nitrous oxide (N_2O), nitric oxide (NO) or inorganic nitrogen gas (N_2). The ammonia, nitrite and nitrate form are toxic to living (WHO, 2006). Exposure to high levels of nitrates or nitrites has been associated with increased incidence of cancer in adults and brain tumors, leukemia and nasopharyngeal (nose and throat) tumors in children (Sanchez-Echaniz et al., 2001; Pogoda and Preston, 2001; USEPA, 2006).

In biological wastewater treatment processes, the nitrification is achieved by two types of bacteria, that is, ammonia-oxidizing bacteria (AOB) responsible for nitrite formation, and nitrite-oxidizing bacteria (NOB) for

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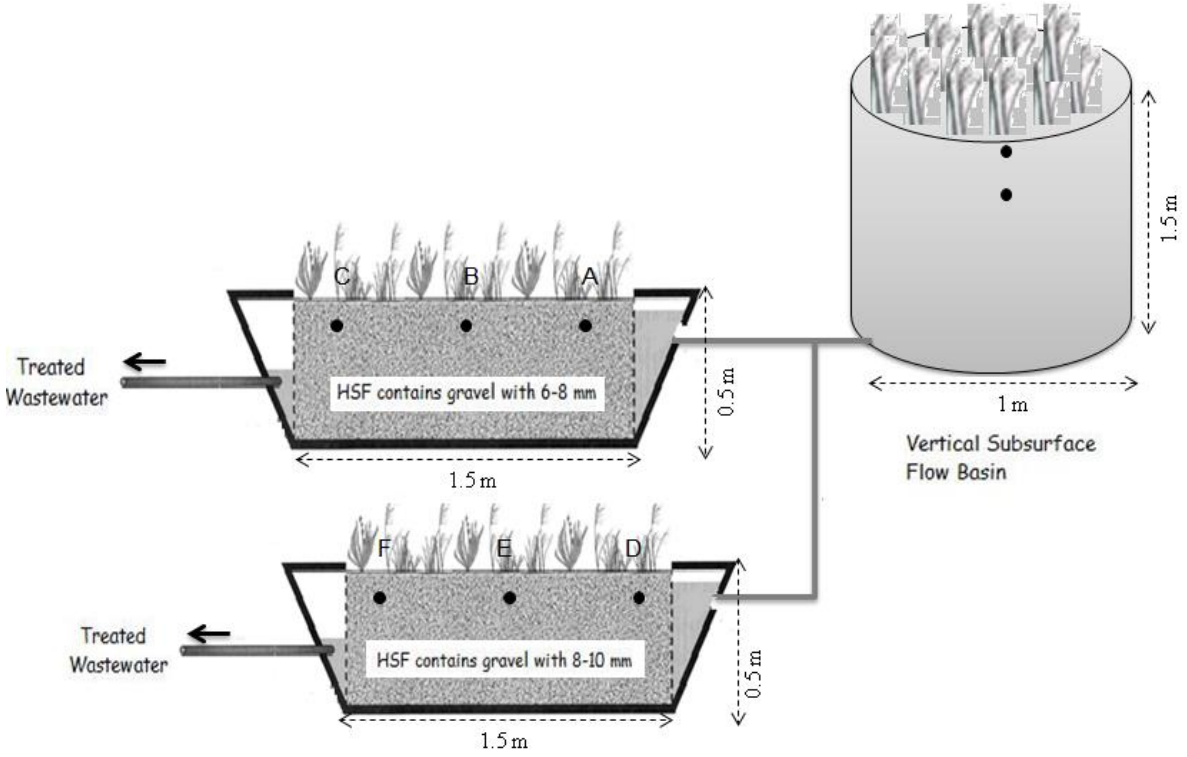

Figure 1. Schematic representation of the constructed wetlands system (small-scale model) placed under greenhouses. HSF: horizontal subsurface flow. The samples were taken from the top (A), middle (B) and the bottom (C) of the HSF with fine gravel. In addition others coarse gravel samples were taken at the top of the HSF (D), middle (E) and the bottom (F) gravel sampling point.

conversion of nitrite to nitrate (Ruiz et al., 2003; Tay et al., 2002). At temperatures higher than 25°C, the growth rate of ammonia-oxidizing microbes is higher than nitrite-oxidizing (Hellinga et al., 1998). Microbial biomass detachment can directly reduce the fixed biofilm (Stoodley et al., 1999; Tijhuis et al., 1996). Approximately 60 to 80% of nitrogen in domestic and municipal wastewater is in the form of ammonium (Gerardi, 2010). Strong ammonium concentrations can contribute to biofilm detachment from the filter of constructed wetlands. The biofilm detachment appears to be the major cause of no constructed wetlands efficiency in terms of nitrates reduction. Nevertheless, the biofilm is detached from the filterand washed out. Wash-out of solids proportional to the flow rate is foreseen only at higher flow rates. It is assumed that detached parts of the biofilm are retained within the pores and metabolized until washed out by a peak flow (Langergraber, 2008).

The biofilm detachment may reduce autotrophic bacterial populations despite the heterotrophic layer could have protecting nitrifiers from detachment (Michaud et al., 2006). The decrease of nitrifying bacteria number can induce a relatively low potential nitrification. Continuous detachment from a constant-thickness biofilm results invariability in nitrification rates. Detachment frequency can influence the competition between heterotrophic and autotrophic bacteria within the biofilm (Morgenroth and Wilderer, 2000; Rittmann et al., 2002).

These experiments investigated the effect of detachment of nitrifying bacteria from gravel on nitrification rate.

MATERIALS AND METHODS

Pilot-scale wastewater treatment

A pilot-scale model of wastewater treatment using macrophytes was installed in greenhouses in Gembloux, Belgium. Macrophytes were planted in vertical flow beds with a support medium composed of peagravel and non-limestone gravel from 6 to 8 mm in diameter (Figure 1). Two horizontal flow beds contained two different sizes of gravel. The first flow basin contained coarse gravel of 8-10 mm in diameter and the second flow basin included fine gravel of 6-8 mm. Macrophytes were planted in these beds. Sixty liters of bovine liquid manure diluted with clear water to reach 150 mg BOD_5/l was added daily to each system.

Sampling (gravel-biofilm)

The gravel was sampled to follow the biofilm development. Two samples were taken from the vertical filter at 5 and 20 cm depth. Six other samples were also considered at different positions from the horizontal flow basins at 5 cm depth (Figure 1).

Table 1. Effect of washing on the numbers of nitrifying and denitrifying bacteria

Nitrifying and denitrifying bacteria	Number of bacteria (MPN/100 ml)	
	First washing	Fourth washing
Ammonia-oxidizing bacteria (AOB)	23.0 ± 0.0	3.0 ± 0.0
Nitrite-oxidizing bacteria (NOB)	3.3 ± 0.4	3.0 ± 0.0
Denitrifying bacteria	161.5 ± 96.9	5.5 ± 2.7

Ammonium analysis

Ammonium was assayed using the indophenol blue-ISO 7150/1 (Merck-Spectroquant) method. Merck reagents Spectoquant ammonium was used. The optical density (OD) was determined at 692 nm by a spectrophotometer (Spectronic® 20 Genesys^{M}). The OD_{692} value was converted to NH_4^+mg/l, using the Excel/Fiexcel/Calcdos.

Nitrate analysis

For two milliliters of the sample were two milliliters of sodium salicylate solution (5 g/l) added. After mixing, the solution was evaporated at 60°C for 2 h and cooled in desiccators. Two ml of H_2SO_4 concentrated was added. After ten minutes, 15 ml of distilled H_2O and 15 ml of NaOH/sodium potassium tartrate (40/6%, vol/vol) were added. The mixture was brought to a final volume of 50 ml and OD was measured at 420 nm using a spectrophotometer UNICAM.

Turbidity measurements

Turbidity is the measure of relative clarity of a liquid. It is an optical characteristic of water and is an expression of the amount of light that is scattered by material in the water when a light is shined through the water sample. The turbidity of the solutions was measured by ISO 2100P turbidimeter HACH® and expressed in nephelometric turbidity units (NTU).

Nitrifying and denitrifying bacteria enumeration

The autotrophic nitrifying bacteria (AOB and NOB) and the heterotrophic denitrifying populations were enumerated using the most probable number (MPN) method (Lorch et al., 1995). Preparation and composition of the AOB, NOB and denitrifying medium were as described by Alef (1995). An incubation period of four weeks was used. After incubation, ammonia-oxidizing bacteria were counted with the MPN-Griess method, while NOB was counted with both the MPN-diphylamine (Both et al., 1990). Denitrifying bacteria presence was expressed by gas that can be observed after three weeks of incubation at room temperature in an anaerobic jar containing nitrogen gas. MPN values were calculated according to the statistical tables of De Man (1983).

Experimental protocol of biofilm detachment

Bacterial enumeration after successive washing

In this experiment, a single piece of gravel was removed from the top (F) of the horizontal filter and placed in a sterile tube. Three milliliters of sterile distilled water were added followed by agitation at 2 rpm during 4 s. The bacterial suspension in the tube was enumerated for nitrifying and denitrifying bacteria. The gravel was washed three more times following the same way and the number of bacteria was determined after each washing. This experience was released in triplicate.

Nitrification after washing

The kinetics of nitrification was followed after washing. Gravel was sampled from vertical and horizontal filter. For this, for a weight of 100 g of gravel was added a volume of 200 ml of sterile distilled water. After agitation at 2 rpm during 10 s the suspension was separated from the gravel and this fraction is considered as the first wash fraction. Four successive washes were applied and each obtained fraction was collected separately. The turbidity of the different fraction was determined. After the fourth wash, a volume of 200 ml of solution A[$(NH_4)SO_4$ 20 mg/l with pH 7,6 and 500 mg/l of $CaCO_3$ as carbon source] was added to the sample gravel included in bottles and the bottles were incubated at 25°C during increasing times (5 min, 4, 8, 24 and 48 h). Then, a volume of 10 ml was sampled and analyzed for the various nitrogen forms.

The kinetics of nitrification was followed before washing (as control samples). Each gravel sample (100 g), undergoing a successive washing, was placed into 500 ml bottle. After addition of 200 ml of solution A, bottles were incubated at 25°C. Then, a volume of 10 ml was taken at different time (5 min and 4, 8, 24 and 48 h) and analyzed for the various nitrogen forms.

Statistical procedures

Pearson's correlation coefficient (*r*) was used to show correlation between the analyzed parameters data using Statistical Package for the Social Sciences (SPSS) software (SPSS for Windows, SPSS Inc., Chicago, Il, USA).

RESULTS AND DISCUSSION

Detachment of nitrifying bacteria on gravel

The nitrifying and denitrifying bacteria decreased with subsequent washings (Table 1). The population of ammonia-oxidizing bacteria (AOB) in suspension was higher than 23 MPN/100 ml. After the fourth wash, this number decreased to attain 3.3 MPN/100 ml. In the same way, the number of denitrifying bacteria was affected by washing, as these populations decreased from 161.5 to 5.5 MPN/100 ml before and after fourth washing, respectively. The nitrite-oxidizing bacteria (NOB) populations present in suspension of sample (single piece of

Table 2. Biomass cell suspension expressed by the turbidity values obtained after the first and fourth sample washing at vertical filter (at 5 and 20 cm of depth), horizontal filter characterized by fine gravel (in the top, medium and bottom of filter) and horizontal filter characterized by coarse gravel (in the top, medium and bottom of filter).

Washing	Vertical filter (cm)		HF with fine gravel			HF with coarse gravel		
	5	20	Top	Medium	Bottom	Top	Medium	Bottom
First washing	57 ± 2^{d}	97 ± 2^{h}	63.5 ± 1.3^{e}	76.4 ± 2^{f}	88.5 ± 1.5^{g}	35.3 ± 2^{c}	29 ± 1.3^{b}	23 ± 2.2^{a}
Fourth washing	4.4 ± 2^{b}	7 ± 1^{bc}	6.9 ± 1.9^{bc}	0.7 ± 1.6^{a}	8.3 ± 1.4^{c}	9.5 ± 2^{c}	3.2 ± 0.16^{ab}	3.8 ± 0.3^{ab}

HF: Horizontal filter; (a, b, c, etc.): In each line for each sample, mean values followed by a different symbol are significantly different according to Student–Newman–Keuls test at $P < 0.05$.

gravel) were lower, saving a constant value of 3.3 MPN/100 ml. For this reason after washing, the number of NOB populations remained almost constant (3.0 MPN/100 ml). This may be due to the low number of NOB that failed to form a thick biofilm on the gravel. Thus, after washing, the detachment will be too minor.

In this study, the autotrophic bacteria (AOB) are detached and their concentration decreased. Similar study by Derlon (2008) showed that detachment causes a decrease in the number of autotrophic bacteria. The heterotrophic bacteria with fast growth in the outer layers of biofilm (substrate concentration and detachment rate are high) may cover nitrifying bacteria with slow growth in internals layers of the biofilm (Nogueiro et al., 2002). Thus, the heterotrophic bacteria affect positively the nitrifying bacteria by protecting them against detachment, when the oxygen levels were sufficient for their maintenance under the biofilm matrix (Furumai and Rittmann, 1994). In this study, the insufficient oxygen level in the horizontal filter (where the sample was taken) prevents the heterotrophic bacteria from protecting the autotrophic bacteria against the detachment.

Biomass density in suspension

Results showed that the turbidity was inversely proportional to the number of gravel washings (Table 2). In the vertical filter, the turbidity after the first washing was much higher (97 NTU at 20 cm) than the turbidity after thes ubsequent washings (7NTU after 4^{th} wash). The statistical analysis indicated significant differences according to the Student–Newman–Keuls test at $P<0.05$ obtained between turbidity after the first and fourth washing of the gravel sample taken at 20 cm of depth from vertical filter (Table 2). In the vertical filter, we found that the turbidity of the first washing of gravel taken from 20 cm was higher (97 NTU) than that for gravel sampled at 5c m of depth (57 NTU). In addition, the statistical analysis indicated that turbidity marks significant differences according to Student–Newman–Keuls test at $P<0.05$ after the first washing of the gravel sample taken at 5 and 20 cm of depth from vertical filter (Table 2).

Generally, the turbidity was greater at the bottom of the horizontal beds (88.5 NTU during the first washing than that obtained at the top and medium of horizontal filter (63.5, 76.4 NTU, respectively). The bottom of horizontal filter consisted of more fine gravel and it may be that the microbial loading is greater in the bottom of filter. The statistical study indicated significant differences according to the Student–Newman–Keuls test at $P<0.05$ obtained between turbidity at the bottom of horizontal filter characterized by fine gravel and the turbidity obtained at the top and medium of horizontal filter (Table 2). Also, it is probable that the microbial biomass at the bottom is less fixed than that at top and medium of horizontal filter. At the top of horizontal filter characterized by coarse gravel the turbidity value is higher than the turbidity value obtained at the medium and the bottom of this basin with values of 35.3, 29 and 23 NTU, respectively (Table 2). The fine gravel loaded per unit mass of microorganisms indicated less fixed microbial biomass than those obtained with coarse gravel. The microorganisms loading are more important in the filter with fine gravel than the filter with coarse gravel. The turbidity values after the first wash of fine gravel saved a value ranging between 60 and 100 NTU. The statistical study indicated significant differences according to the Student–Newman–Keuls test at $P<0.05$ obtained between turbidity of sample characterized by fine and coarse gravel from horizontal filter after the first washing (Table 2).

The nature of the carrier media used requires development of a very thin, evenly distributed and smooth biofilm to enable transport of substrate and oxygen to the biofilm surface. The turbulence sloughs off excess biomass and maintains adequate thickness of biofilm. Biofilm thickness less than 100 µm for full substrate penetration is usually preferred (Odegaard et al., 1994). Nevertheless, extremely high turbulence detaches biomass from the carrier and therefore is not recommended.

Ammonium oxidation in vertical and horizontal filters

The gravel from 5 cm incubated for 8 h at 25°C did not show a reduction of NH_4^+-N amount expressed by constant value of ammonium saved at the level of samples from before and after washing 13.84 ± 3.6 and

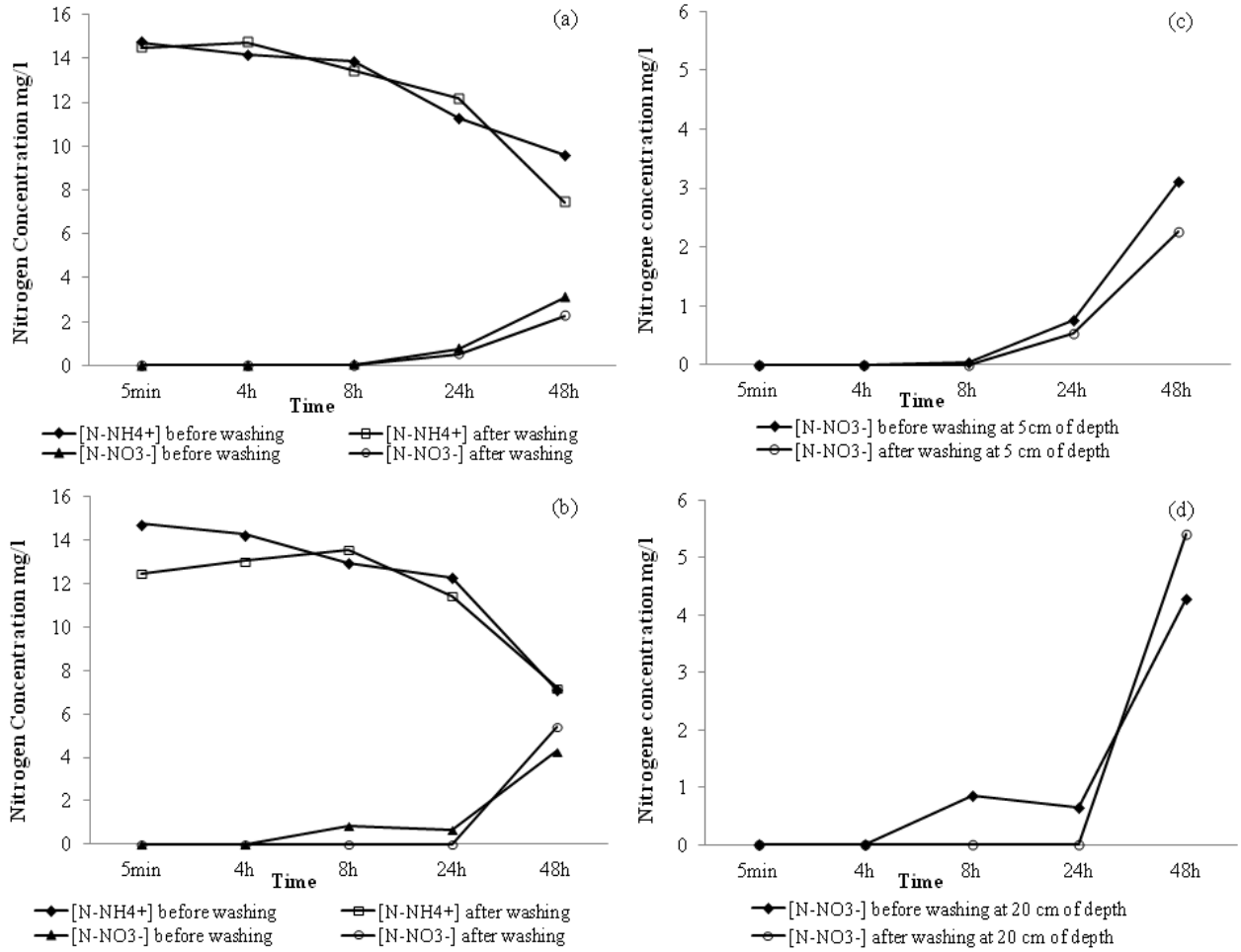

Figure 2. Evolution of nitrate oxidation on gravel sampled from vertical filter at 5 cm (a) and 20 cm of depth (b), NO_3^--N concentration at 5 (c) and 20 (d) cm of depth after first and fourth washing.

13.43 ± 2.5mg/l, respectively (Figure 2a). After incubation for 24 h, an increase of NO_3^--N concentration was observed, in samples before and after washing 0.8 and 0.5 mg/l, respectively. For samples taken from 20cm of depth and before washing the ammonium oxidation started after 8 h of incubation (Figure 2b), therefore, after washing, ammonium oxidation started after 24 h of incubation.

This result shows that washing sample gravel delayed ammonium oxidation. Washing or detachment seems to affect the nitrification performance by delaying nitrification. Using a bench-scale aerated biofilter, Ohashi et al. (1995) established that is no nitrification due to the biofilm detachment by daily backwash. However, their subsequent trials with lower substrate loading and backwash rates allowed stable nitrification.

However, after washing gravel sampled from vertical filter and following incubation during 48 h ammonium oxidation is more important at 20 cm (5.41 mg/l) than that obtained at 5 cm of depth (2.26 mg/l) (Figure 2c and d). The obtained result supposes that at 20 cm of depth microorganisms loading is more important than that at 5 cm of depth. Also, a significant, positively correlation between NO_3^--N concentration at 5 and 20 cm of depth before (r = 0.97) and after (r = 0.973) washing was obtained. In addition, a highly significant positively correlation between NO_3^--N concentration at 20 cm of depth before and after washing was obtained, with r = 0.977.

Nitrification performance of horizontal filter characterized by fine and coarse gravel after washing was grouped in Figure 3. NO_3^--N concentration at the bottom of horizontal filter with fine gravel is more important (1.24 mg/l at 48 h of incubation) than that observed at the medium and the top of basin saving values of 1.1 and 0.8 mg/l, respectively after 48 h of incubation (Figure 3a).

We supposed that nitrifying microorganisms are more important at the bottom of filter even after washing. Whereas, after washing the horizontal filter with coarse gravel, the nitrification performance is more important at the medium of basin with NO_3^--N concentration value of 1.14 mg/l than those obtained at the top and the bottom

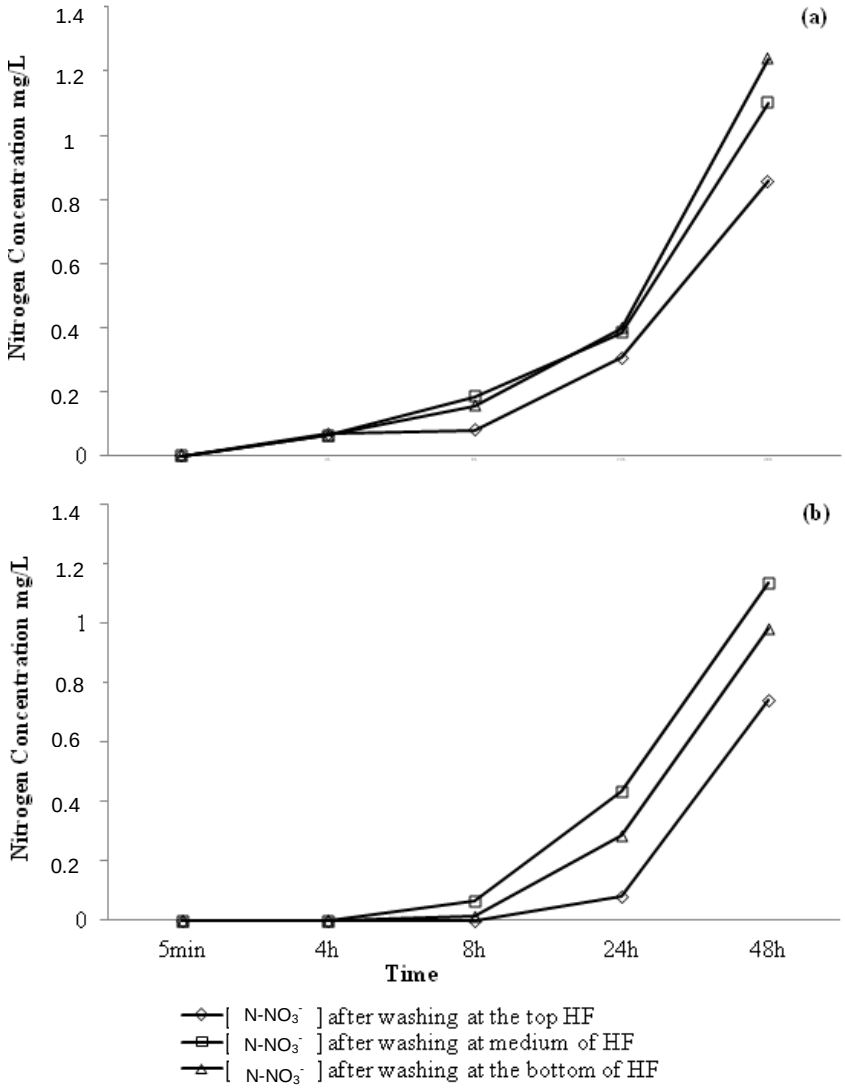

Figure 3. Evolution of nitrate oxidation at the top, medium and bottom of horizontal filter characterized by fine (a) and coarse (b) gravel after washing. HF: Horizontal filter.

of basin with values of 0.7 and 0.98 mg/l, respectively.

A statistical significance was showed at $P<0.05$ for NO_3^--N concentration before and after washing at horizontal filter. A positively correlation between NO_3^--N concentration at the top, medium and the bottom of horizontal filter characterized by fine gravel after and before washing (respectively, r=0.933, 0.93 and 0.966) was observed. After washing, a significant positively correlation ($P<0.01$) between NO_3^--N concentration at the top and the medium (r = 0.975), between the top and the bottom (r =0.966) and between the medium and bottom (r=0.999) of horizontal filter characterized by fine gravel were obtained. However, no significant correlation was noted at the top, medium and the bottom of horizontal filter characterized by coarse gravel after and before washing.

In this study, the detachment from a constant thickness biofilm resulted in nitrification reduction. However, a stable nitrification was obtained by an extension of the treatment period. Other studies showed that the washing induce reduction of the average of nitrification rates and reduced number of autotrophic bacteria (Elenter et al., 2007).

When dynamic of biofilm detachment is imposed on the system, a drop in the efficiency of nitrification is observed. In addition, some studies indicated that the effectiveness of nitrification drop 98 to 25% when the events of detachment is imposed, meaning that autotrophic bacteria are within biofilm (Derlon, 2008). In this investigation and unlike other studies, a stable and effective nitrification was obtained after washing. This difference may be due to the growth of nitrifying bacteria

after washing or presence of substrate even in lower loading, thereby allowing a stable nitrification (Ohashi et al., 1995).

Conclusion

The decrease in AOB and denitrifying bacteria populations was affected by the number of washings, while the number of NOB present in suspension was lower and had a constant value of 3.0 MPN/100 ml. Washing seems to affect the nitrification performance by delaying nitrification.

Despite this detachment for gravel sampled from vertical filter, ammonium oxidation yield has been obtained by an extension of the treatment period. After washing, the ammonium oxidation was more important at 20 cm (5.41 mg/l) than at 5 cm of depth (2.26 mg/l). This supposes that at 20 cm of depth microorganisms loading was more important than at 5 cm depth.

In horizontal filter with fine gravel, the nitrification performance was more important at the bottom of filter even after washing. Whereas, at the horizontal filter (coarse gravel), the nitrification performance was more important at the medium of the basin.

Since the yield of nitrification is unaffected by detachment, enhancing detachment by acting in some physico-chemical parameters may lead to clogging prevention in fixed-biofilm wastewater treatment processes.

Conflict of Interest

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

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