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Full Length Research Paper

Optimization of biomass production of *Acetobacter pasteurianus* KU710511 as a potential starter for fruit vinegar production

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The objective of the present work was first the isolation of novel acetic acid bacteria strains from natural Moroccan habitats, and then, the evaluation of their ability to produce microbial starters for vinegar production on a large scale. The strains were isolated from figs, dates, cactus, and traditional fruit vinegars. Four strains, selected from a total of 63 isolates, were confirmed as belonging to *Acetobacter* species according to biochemical and molecular studies based on 16s rRNA sequence analysis. Acetous fermentation tests, performed on date and apple fermented juices using selected *Acetobacter* strains, showed a high capacity of acidification. The most efficient strain KU710511, isolated from Morrocan cactus (*Opuntia ficus-indica*), was identified as *Acetobacter* strain closely related to *A. pasteurianus* and yielded 42.5 g/L acidity in apple juice. Cell growth optimization was carried out for KU710511 using response surface methodology (RSM). The linear, quadratic, and interaction effects of four factors-ethanol, acetic acid, glucose concentrations and pH-were studied by the application of a central composite design. Thirty experiments were designed to predict the maximum concentration of cell biomass. The optimal calculated values of ethanol, acetic acid, glucose and pH allowing the prediction of the maximum biomass production (2.21 g/L) were 28.18, 10.12, 15.15 and 5.33 g/L, respectively. Subsequently, further batch fermentations were carried out in a 6-L lab-bioreactor at optimal conditions. The results were in line with the predicted values. It can be concluded that the studied strain is well suited to be used as a parental strain to prepare a starter for fruit vinegar production.

Key words: Isolation, vinegar, starter, *Acetobacter*, acetic fermentation, response surface methodology.

INTRODUCTION

Acetic acid bacteria (AAB) is a group of microorganisms known to have unique fermentation ability, so-called "oxidative fermentation," a process where ethanol is converted to acetic acid (Saichana et al., 2014). They are

ubiquitous organisms that are well adapted to sugar and ethanol rich environments (Bartowsky and Henschke, 2008). Vinegar, from the French vin aigre, meaning “sour wine,” can be made from almost any fermentable carbohydrate source, including apples, dates, grapes, pears, coconut, honey, etc. (Johnston and Gaas, 2006).

Morocco is one of the main fruit and vegetable producing countries on the southern shore of the Mediterranean Sea. Thousands of tons are commercialized annually in the national and international market. According to a citrus packing stations association, 30 to 40% of estimated losses of fruits and vegetables are generated annually on the production sites and from the processing plants (L’economiste, 2010). These substandard fruits are, in most cases, improper to be commercialized on the local market. Thus, transforming them via biotechnological processes to obtain exotic products with the local knowledge is, therefore, essential (Benkerroum, 2013; Ndoye et al., 2007a).

Vinegar is a product of high nutritional and cultural value (Johnston and Gaas, 2006). It is obtained from double stage fermentation, alcoholic and acetic, performed, respectively, by yeasts and acetic acid bacteria. The use of traditional processes, where both fermentation steps are performed spontaneously, generates improper vinegar because of the possibility to contain, among other substances, mycotoxins. The presence of these compounds in food is of great concern for human health due to their properties to induce severe toxicity effects at low dose levels (Fernández-Cruz et al., 2010).

In order to produce biological type vinegar that meets the chemical and microbiological standards, it was necessary to select endogenous strains from Moroccan natural habitats, which are able to perform efficient acetic acid fermentation. In fact, until recently, the production of industrial vinegar in Morocco has required industrial imported starter cultures. Obtaining valid starters from local screened bacteria will help to avoid the need to import them. AAB are obligate aerobes and their growth is highly dependent upon the availability of a carbon source, nitrogen source, molecular oxygen, and growth factors (Hidalgo et al., 2010; Liu et al., 2008). Many physicochemical factors are observed as influencing the growth and productivity of AAB. pH of the fermenting must, temperature, and the concentration of ethanol are, beside dissolved oxygen, the main factors that influence the growth of AAB (Drysdale and Fleet, 1988). Furthermore, glucose acts as a principal carbon source for most strains of AAB (Guillamón and Mas, 2009). In addition, it was demonstrated previously that acetic acid would have a stimulatory effect on cell growth of AAB at low concentration (De Ory et al., 2004).

Response surface methodology (RSM) has been extensively used for optimization of medium composition and conditions of fermentation (Cui et al., 2006). It has been reported that this method is more effective compared to conventional techniques, which are extremely laborious and time-consuming. Furthermore, conventional methods do not guarantee the determination of optimal conditions and are unable to detect interactions between two or more factors (Cui et al., 2006; Liu et al., 2008). In this study, RSM was selected in order to search for the crucial influencing growth factors and to exhibit their synergistic interactions for biomass production of selected Acetobacter strains. Thus, the objective of this work was, firstly, the isolation of novel acetic acid bacteria from a variety of Moroccan foods (fruits and vinegars) and the identification of endogenous isolates, which can be used for the production of bacterial starters. Secondly, an examination of their performance and the optimization of cell growth depending on the growth factors were performed using RSM.

MATERIALS AND METHODS

Samples and microorganisms

Samples used for isolation of AAB were recovered from different regions of Morocco and grouped into two groups: (1) Fruits including apple (*Malus domestica;* variety *Golden delicious*, from Midelt), dates (*Phoenix dactylifera*; variety *black Bousthami*, from Zagora), figs (*Ficus carica* L. from Ouarzazate) and cactus (*Opuntia ficus-indica*; *Moussa*, from Sidi Ifni); (2) Vinegars: Namely apple vinegar originating from Midelt and cactus vinegar from Ait Baamrane, both manufactured in a traditional manner. Immediately after collection, the samples were stored at a low temperature (4°C) to protect them from deterioration.

Isolation procedure

Fruit samples were cut into small slices and transferred in a GYEA enrichment medium that consisted of glucose 2% (w/v), yeast extract 1% (w/v), ethanol 2% (v/v) and acetic acid 1% (v/v). Samples were incubated under agitation (120 rpm) at room temperature (25-30°C) for one week. Then, 0.2 ml of liquid samples were diluted and inoculated in modified YGM/Mg2+ solid medium composed of yeast extract 5 g/L, glucose 20 g/L, mannitol 20 g/L, $MgSO_4$ 0.5 g/L, agar 15 g/L, pure ethanol 2% (v/v) and acetic acid 0.5 % (v/v) (Lisdiyanti et al., 2000). Ethanol and acetic acid were added to the medium aseptically after sterilization. Glucose and mannitol were sterilized separately. Cycloheximide and nisin were used in culture media to inhibit the growth of fungi and lactic acid bacteria, respectively (Kadere et al., 2008). The screening procedure was completed in Carr, Frateur and GYC solid media on plates at 30°C (Sievers and Swings, 2005). The isolated and purified strains were stored on GYC agar at 4ºC for a few days and

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in Microbank vials at -20ºC for long-term storage.

Identification of selected strains

Biochemical and morphological identification tests were performed to confirm that the selected isolates belong to Acetobacter genius. Conventional biochemical tests, including gram staining, catalase, oxidase, and oxidation of ethanol, were performed following the guidelines of Bergey's Manual of Systematic Bacteriology (Sievers and Swings, 2005). Other biochemical tests such as growth in the presence of high glucose concentrations, ammonium utilization and different carbohydrate assimilation were performed on presumed Acetobacter strains.

In addition, presumed Acetobacter strains were submitted to 16s rRNA sequence analysis by amplification of genomic DNA with universal primers 16SP0 5'-GAAGAGTTTGATCCTGGCTCAG-3' for the coding segment and 16SP6 5'-CTACGGCTACCTTGTTACGA-3' for the non-coding segment (Mounir et al., 2016). DNA was extracted from fresh cells grown on solid YGM/Mg2+ medium using the Promega extraction kit (Promega, USA). Then, the PCR reaction was monitored in a 200 µl Eppendorf tube containing 25 µl of Ready Mix (Promega, USA). The conditions of the PCR reaction, carried out in a thermocyclor (Eppendorf, France) were as follows: The first denaturation cycle of the DNA at 95°C for 5 min, 25 denaturation cycles at 95°C for 30 s, the primers annealing at 55°C for 30 s, the primers elongation at 72°C for 2 min, and a final elongation cycle at 72°C for 10 min. Thereafter, the PCR products were separated and visualized using electrophoresis at 100V for 20 min on 1% agarose gel in a phosphate TAE buffer 50x containing 1 µg/ml of ethidium bromide. The PCR reaction products were purified using the kit PCR Preps Wizard (Promega, USA) and quantified on agarose gel.

Finally, the purified PCR product was sequenced according to the Sanger method using a Big Dye Kit and a 3730 DNA analyzer (Applied Biosystems) (Ndoye et al., 2006), and the CodonCode Aligner program was used to pile up the products of the sequencing reaction. The sequences were then compared to those deposited in GenBank, using the BLAST algorithm (NCBI).

Analytical methods

Acetic acid was determined by titration using 0.5 M sodium hydroxide and phenolphthalein as indicator. This rapid method was used for immediate monitoring of acid production during fermentation. Biomass was estimated by optical density (O.D) measurement at 540 nm. Calibration curve was established between O.D and bacterial dried weight (g/L) determined using gravimetrical method (Chen et al., 2011; Ndoye et al., 2007c). Samples were passed through cellulose nitrate membrane (0.45 µm pore size) using a vacuum system. The membrane was then dried (105°C) until a constant weight.

High performance liquid chromatography (HPLC) was used to determine glucose, ethanol, acetate, and gluconate in cultures. Culture samples were collected and centrifuged at 13,000 rpm for 10 min and the supernatants were filtered through a 0.2 µm cellulose acetate membrane (Sartorius Minisart). The HPLC analyses were performed using an Agilent 1110 series HPLC equipped with a Supelcogel C610H column preceded by a Supelguard H precolumn (using a column heater at 40°C) and a differential refractive index detector (RID, detection cell maintained at 35°C). An isocratic mobile phase consisting of 0.1% H_3PO_4 (in MilliQ water) was used at a flow rate of 0.5 mL/min. Analysis was completed within 35 min and operated at a maximum pressure of 60 bars.

The bioconversion capacity of selected bacteria was determined by calculating the following parameters:

1. Stoichiometric yield:

$$Ys = \frac{n_f(AA) - n_i(AA)}{n_i(Eth) - n_f(Eth)} \text{ x } 100$$

n_i(AA) and n_i(Eth): initial acetic acid and ethanol moles, respectively. n_f(AA) and n_f(Eth): Final acetic acid and ethanol moles, respectively.

2. Theoretical acetic acid concentration (C_t): Considering the reaction

$C_2H_5OH + O_2 \rightarrow CH_3COOH + H_2O$

The theoretical acetic acid concentration C_t expressing the mass (g) of acetic acid formed in 100 ml was calculated as follows:

$$C_t = \frac{C_i\ (Eth) - C_f\ (Eth)}{M\ (Eth)}\ x\ M\ (AA) + C_0$$

C_i (Eth): initial ethanol concentration. C_f (Eth): final ethanol concentration. M (Eth): ethanol molar mass. M (AA): acetic acid molar mass. C_0: starting acetic acid concentration in the medium.

3. Bioconversion efficiency (E):

$E = (C_r / C_t) \times 100$

C_r: Real acetic acid concentration determined by titration. C_t: theoretical acetic acid concentration.

4. Productivity (P): quantity of acetic acid produced per liter and per hour (g/L/h).

Evaluation of bioconversion ability of the selected bacteria on fruit musts

In this part of the study, the ability of selected strains to achieve efficient acetic fermentation on alcoholic fermented fruit juices was evaluated in order to select the most efficient strains with regard to acetic acid and biomass production. Alcoholic musts used to perform acetic fermentation were obtained from date and apple juices. These fruits were chosen on the basis of their availability on the local market and on their significant valorization potential.

Fruit juice extraction

Date juice was prepared from black Bousthami date variety (south-east of Morocco). This variety was chosen because of its availability and its low cost. The soft consistency and sugar content of this variety give it very interesting properties to be transformed; it is widely used in the most popular, traditional preparations of soft dates in the south of Morocco (Harrak et al., 2012). The preparation of date juice was performed according to the soaking method recommended by Nancib et al. (2001), except that the temperature used for the extraction was 65°C for 2 h. The mixture was then filtered through a filter cloth to obtain clear juice. Apple juice was extracted using a Robot-Coupe centrifuge (J 80 Ultra, France) by pressing mature, small size apples. Date and apple juices were used immediately for fermentation.

Alcoholic fermentation

Alcoholic fermentation was achieved using a commercial baker's *Saccharomyces cerevisiae* "Rafiaa" strain. The yeast was obtained

Table 1. Coded levels (between brackets) and corresponding real levels of the independent variables involved in the design.

Variable		Levels				
		(-2)	(-1)	(0)	(1)	(2)
X1	Ethanol (g/L)	10	20	30	40	50
X2	Acetic acid (g/L)	6	8	10	12	14
X3	Glucose (g/L)	10	12.5	15	17.5	20
X4	pH	4	4.5	5	5.5	6

from LESAFFRE Company (Morocco) in the form of active dried granules. pH of date juice, which exceeded the optimum value of the yeast, was adjusted to 3.5 by adding 0.5 N citric acid (Colin and Conroy, 1998). The yeast was activated by mixing the appropriate amount (0.6 g/L of inoculated juice) with 500 ml of warm juice (35 to 40°C). After a rest period of 15 min, active yeast was used to inoculate a 30 L plastic drum of both apple and date juices.

Acetous fermentation

Prior to main fermentation, strains were screened using an enrichment medium composed of glucose 2% (w/v), yeast extract 1% (w/v), peptone of casein 0.5% (w/v), mannitol 2% (w/v), ethanol 2% (v/v) and acetic acid 1% (v/v). 500 ml flasks containing preculture media were incubated at 30°C on a shaker (120 rpm) for 2 days. The alcohol concentration of fruit musts was adjusted to 4% (v/v) which is the optimum for cell growth according to results reported by Kommanee et al. (2012) and Romero-Cortes et al. (2012). Afterwards, selected strains were evaluated for their ability to perform acetous fermentation of alcoholic fruit musts.

Optimization of biomass production of selected bacteria

Experimental design

In this part of the study, we investigated the effect of glucose, pH, acetic acid, and ethanol on the biomass production of selected bacteria using response surface methodology (RSM). The fermentation cultures were carried out in Erlenmeyer flasks (250 mL) containing 100 mL GYEA culture medium supplemented by 2.5 g/L fructose. Glucose, ethanol, acetic acid, and pH were then fixed based on the experimental design. Table 1 summarizes the independent variables involved in the design in their real and coded levels. Minimum and maximum levels of influencing variables studied were: 10 to 50 (g/L) for ethanol, 6 to 14 (g/L) for acetic acid, 10 to 20 (g/L) for glucose and 4 to 6 for pH. The lower and upper limits were determined with reference to previous experiments (data not shown). Accordingly, these conditions generated an experimental design (Table 5) with 30 runs determined by: 2^k=16 factorial points, 2k=8 axial points, and N_0=6 central points designed as replications.

By designating cellular biomass as "Y", the quadratic polynomial equation 1 which describes the variation of the response "Y" is as follows:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

Where β_0, β_i, β_{ii}, β_{ij} are constant regression coefficients of the model and X_i , X_j (i=1-4; j=1-4; i ≠ j) represent the independent variables. The coefficient of determination R^2 was used to express the quality of fit of the polynomial model. The experiments were carried out in duplicates and the mean values were calculated.

The *Acetobacter* strain selected for its best performance in terms of acetic acid production on alcoholic juices (apple and date) was chosen to maximize growth depending on the composition of the fermentation medium.

Statistical analysis

Central composite design was used to predict bacterial biomass production (Table 5). Data gained from the experiments were subjected to a second order multiple regression analysis to obtain parameters estimated for the mathematical model. Statistical analysis [regression and analysis of variance (ANOVA)] was carried out using Minitab software (v. 17 .1.0, UK, 2003). The contour plot and the 3D response surface analysis were made by keeping two independent variables at constant level and changing the other two independent variables, and then calculating the response "Y".

Batch fermentation in a bioreactor

The selected strain for which the optimization of biomass production was carried out was tested in 6-L scaled bioreactor (INFORS, France). The influencing parameters studied were taken in their statistically predicted optimal values to prepare the cultivation medium. The reactor was aerated using a continuous flow of filtered sterile air at a rate of 1 VVM. Microbial cells were first precultured in 500 ml baffled flask at 30°C for 36 h and then the liquid broth was inoculated into a fermenter. Bacterial growth was monitored by measuring dry matter (g/L) along with the produced acetic acid and residual ethanol.

RESULTS AND DISCUSSION

Identification of isolated bacteria

The present study aimed at isolating AAB strains from local Moroccan products destined for bacterial starter culture production to use in industrial vinegar production. The incubation of the inoculated culture media made of apple, cactus and date juice extracts at 30°C for 7 days resulted in an increase in the growth of presumptive acetic acid bacteria in the fermentation media. This was confirmed by the increase in the turbidity of the culture media and the development of the characteristic odor of acetic acid. Except for isolates obtained from figs, strains obtained from apple and date fruits (AF and DF), cactus vinegar (CV), and apple vinegar (AV) were able to convert Carr medium color from blue to yellow and could develop colonies surrounded by bright rings in Frateur and GYC media. These macroscopic observations show that the four groups of isolates were capable of converting ethanol present in the Carr medium in acetic acid, which resulted in a color change (Mounir et al., 2015; Sharafi et al., 2010).

According to biochemical tests (Table 2), isolated bacteria were gram-negative, catalase-positive, and oxidase-negative. Morphologically they appeared on GYC agar as smooth colonies in single or paired cocci and sometimes rod-shaped. A motility test made on a mannitol

Table 2. Biochemical and morphological identification of selected bacteria.

Test	Isolates [a]												
	***A. Aceti* (LMG1625)**	**FF1 G1 [b]**	**FF2 G2**	**AF1 G3**	**AF2 G4**	**CF1 G5**	**DF1 G6**	**DF2 G7**	**CV1 G8**	**CV2 G9**	**AV1 G10**	**AV2 G11**	**AV3 G12**
Morphology	Rods	Irregular	Irregular	Ovoid	Spherical	Ovoid	Ovoid	Ovoid	Ovoid	Spherical	Ovoid	Ovoid	Spherical
Arrangement	Pair	Single	Single	Pair/ single	Pair/ single	Pair/ single	Pair/ single	Pair/ single	Pair/ single	Pair/ single	Pair/ single	Pair/ single	Pair/ single
Motility	+	-	-	+	+	+	+	+	+	+	+	+	+
Gram staining	-	-	+	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-
Production of acetate from ethanol	+	-	-	+	+	+	+	+	+	+	+	+	+
Over-oxidation capacity	+	-	-	+	+	+	+	+	+	+	+	+	+
Cellulose production	-	-	-	-	-	-	-	-	-	-	-	**+**	-

[a]FF, fig fruit; AF, apple fruit; CF, cactus fruit; DF, date fruit; CV, cactus vinegar; AV, apple vinegar; [b]G1 - G12, groups of bacteria respectively formed of a number of 6, 4, 3, 7, 4, 5, 4, 8, 5, 6, 7 and 4 isolates.

nitrate motility medium showed that all selected bacteria were found to be motile. In addition to these results, molecular identification was performed according to phylogenetic analysis based on the sequencing of the gene coding for 16S ribosomal RNA. According to NCBI blast algorithm, the representative selected strains, which were isolated from apple fruit (AF), cactus vinegar (CV), and cactus fruit (CF), respectively, were closely related to *A. pasteurianus* [97-99% homology (query cover)], whereas the selected isolate from date fruit (DF) was related to *A. pomorum* with 98% homology.

Interestingly, the cultures of a selected strain, isolated from traditional apple vinegar (AV2 group), exhibited the formation of a non-soluble substance, which accumulated in the form of filaments. After identification with Lugol's solution (1/10) and Congo red, this substance was recognized as cellulose. The 16s rDNA sequence analysis revealed that this bacterium was assigned to *Komagataeibacter xylinus* (98% homology). Further studies aimed at the optimization of the production of this substance could be considered. Therefore, this phenomenon represents a disadvantage for the use of this bacterium for industrial production of vinegar; it is, however, more suited to be involved in industrial production of cellulose (Fu et al., 2014; Jozala et al., 2015; Qureshi et al., 2013). The GenBank accession numbers for the 16s rRNA sequences of the two selected bacteria, CV01 and AF01, isolated from cactus and apple fruits are KU710511 and KU710512, respectively. These bacteria were selected for further studies.

Evaluation of bioconversion ability of the selected bacteria

This part of the study aimed to evaluate the ability of the selected *Acetobacter* strains to perform acetous fermentation on apple and date fruit musts (Table 3). The bioconversion capacity of tested strains was estimated by calculating fermentation performance indicators (stoichiometric yield, theoretical acetate production, bioconversion efficiency and productivity) as described in the material and methods section. The first stage alcoholic fermentation was achieved using a commercial *S. cerevisiae* strain and allowed to obtain a final ethanol concentration after 10 days of 8.24 and 9.12% (v/v), respectively, for apple and date alcoholic musts. This result corroborated the depletion of reducing and total sugars and, consequently, with °Bx variation which represents the percent of soluble dry matter by weight (grams per 100 milliliter of water) (Table 3). The alcoholic fermenting media were adjusted for their starting ethanol and acetic acid concentrations of 4 and 1% (v/v), respectively before inoculation with active cells taken in their exponential growth phase

Table 3. Chemical analysis of apple and date juices and musts used for the bioconversion assay.

Parameter		Fruit juice		Fruit musts	
		Apple Golden delicious	Date Black bousthammi	Apple Golden delicious	Date Black bousthammi
pH		3.86±0.06	5.85±0.02	ND	ND
°Bx	% (w/w)	13.30±0.23	15.76±0.11	4.06±0.07	5.12±0.10
Acidity	% (w/w)	0.64±0.13	0.36±0.09	0.79±0.11	0.81±0.17
Ethanol	% (v/v)	ND	ND	8.24±0.08	9.12±0.33
Ash content	% (w/w)	0.22±0.05	1.45±0.12	0.18±0.1	1.33±0.23
Reducing sugars	% (w/v)	8.2±0.97	5.8±0.65	Traces	Traces
Total sugars	% (w/v)	12.2±1.45	17.65±1.39	3.16±0.16	5.41±0.21

(10^6 to 10^7 CFU/mL).

Figure 1 shows that the four tested bacteria were able to produce acetic acid from ethanol in both date and apple fruit fermented juices. The overall acetate production process finished in 5 days in both cases. However, the kinetics of oxidation and the final acetic acid concentration differed from one strain to another. A maximum amount of acetic acid productions (42.5 and 36.5 g/L) were obtained for the CV01 *Acetobacter pasteurianus* strain, respectively, for apple and date acetous fermentation processes. This was confirmed by the calculation of performance indicators. Indeed, the results summarized in Table 4 show that the CV01 strain exhibited the highest productivity (P) level in the two experiments compared to the others (0.27 and 0.22 g/L/h for apple and date fermentation media, respectively). On the other hand, the stoichiometric yield, which represents the moles of acetic acid produced per mole of ethanol consumed in the liquid medium (Ndoye et al., 2007b), ranged from 76.96 to 97.81% for the overall strains. Except for the AV1 strain, the final produced amount of acetic acid and then the productivity (P) level were higher in the apple fermentation process compared to the date process. This allowed us to claim that the fermented apple juice was more suited for acetic fermentation compared with the fermented date juice. Considering these results, the CV01 and AF01 strains were selected for further studies.

Optimization of biomass production of CV01 *Acetobacter* strain

An RSM experiment was performed to evaluate the effect of four independent variables (ethanol, acetic acid, glucose, and pH) on biomass production (g/L) of the selected CV01 *Acetobacter* strain isolated from cactus vinegar. According to the generated experimental design, 30 experiments were implemented separately in 500 ml baffled flasks incubated on a shaker (120 rpm) at 30°C. Flasks were inoculated by fresh cells grown on a plate (24 to 48 h) and the bacterial biomass was estimated after 72 h using the gravimetrical method. The coded, actual, and predicted values of the independent variables and their responses are shown in Table 5. The bacterial biomass ranged from 1.563 (run number 18) to 2.251 (run number 26). The analysis of variance (*F*-test) results are given in Table 6. These results could be explored for evaluating the overall quality of the model. The given value of the coefficient of determination R^2 (0.968) implies that 96.80% of the sample variation of biomass concentration was attributed to the independent variables, and only 3.20% of the total variation of biomass cannot be explained by the model. This suggests that the accuracy and general predictive ability of the polynomial model was acceptable since the R^2 value was higher than 0.9 (Li et al., 2005). In addition, the observed values of Fisher (*F*-value) and the corresponding probability (*P*-value) of the model (respectively, 32.38 and <0.001) show that the model is highly significant.

The effects of each factor and their interactions on bacterial biomass were estimated through the regression analysis shown in Table 7. The corresponding *P* values of regression coefficients are used as a tool to verify the significance of each coefficient, which in turn may indicate patterns of interaction between the coefficients (Cui et al., 2006). Statistically, the smaller the *P*-values, the greater the significance of the corresponding coefficient (Liu et al., 2003). Results reported in Table 7 show that the regression coefficients of all the quadratic terms and two of the linear coefficients (β_1 and β_4) were significant at the 1% level; furthermore, two of the cross-products (β_1 x β_2 and β_1 x β_4) were also found to be significant at the 1% level. Taking into account only terms found to be significant, the fitted second order polynomial equation for the prediction of the biomass production (g/L) of CV01 *Acetobacter* strain is shown below:

$$\text{Biomass (g/L)} = 2.1647 - 0.0620X_1 + 0.0581X_4 - 0.1215X_1^2 - 0.0758X_2^2 - 0.0550X_3^2 - 0.0404X_4^2 - 0.0335X_1X_2 + 0.0307X_1X_4 \quad (2)$$

Ethanol (X_1) and acetic acid (X_2) had a negative effect on

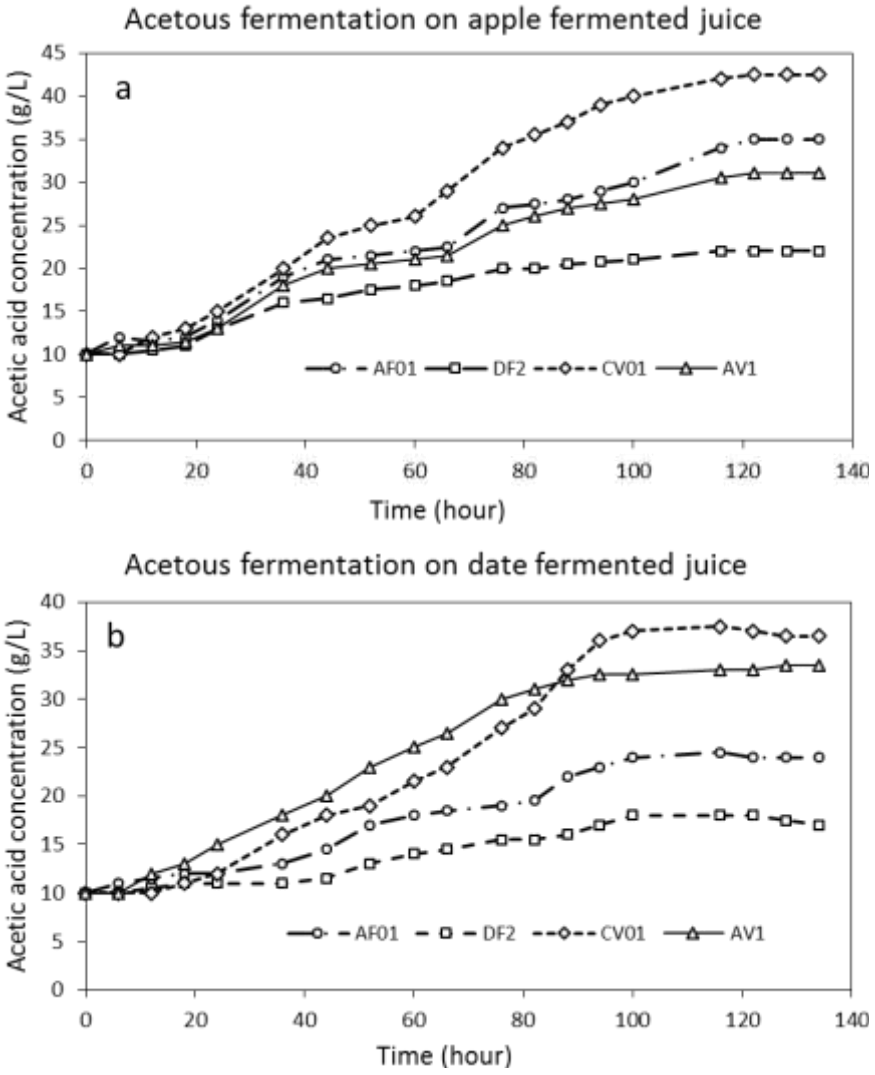


Figure 1. Acetous fermentation on apple (a) and date (b) fermented juices of four selected *Acetobacter* strains AF01, DF2, CV01 and AV1 isolated respectively from apple fruit, date fruit, cactus vinegar and apple vinegar. Fermentation was carried out in 500 ml flasks at 30°C.

Table 4. Calculation of performance indicators for selected strains subjected to acetous fermentation on fruit fermented juices.

Strain	AA_f[a] (% m/v)		Eth_f[b] (% v/v)		C_t[c] (% w/v)		Ys[d](%)		E[e] (%)		P[f] (g/L.h)	
	Apple	Date	Apple	Date	Apple	Date	Apple	Date	Apple	Date	Apple	Date
AF01	3.50	2.40	1.27	2.37	3.70	2.61	92.62	86.87	94.62	91.90	0.21	0.12
DF2	2.20	1.70	2.43	3.08	2.55	1.91	77.31	76.96	86.20	89.02	0.10	0.06
CV01	4.25	3.65	0.42	1.25	4.54	3.72	91.82	97.47	93.62	98.15	0.27	0.22
AV1	3.10	3.35	1.65	1.57	3.32	3.40	90.38	97.81	93.28	98.46	0.18	0.20

[a]Final acetic acid concentration; [b]final ethanol concentration (obtained at the end of acetous fermentation from fruit musts with starting ethanol concentration of 4% (v/v)); [c] theoretical acetic acid concentration; [d] stoichiometric yield; [e] bioconversion efficiency; [f] productivity.

cellular biomass, whereas glucose (X_3) and pH (X_4) had a positive effect. This finding agrees with several previous studies (Garrido-Vidal et al., 2003; González-Sáiz et al., 2009; Macías et al., 1997). In particular, Chen et al. (2011) reported the same effect of glucose and pH on biomass production of *Acetobacter* sp. CCTCC M209061

Table 5. Central composite design matrix and the responses of the dependent variable biomass.

Runs	Coded independent variable levels				Actual values of independent variables				Biomass (g/L)	
	X1	*X2*	*X3*	*X4*	Ethanol (g/L)	Acetic acid (g/L)	Glucose (g/L)	pH	Experimental	Predicted
1	-1	-1	-1	-1	40	8	12.5	4.5	1.887	1.902
2	1	-1	-1	-1	20	8	12.5	4.5	1.793	1.762
3	-1	1	-1	-1	20	12	12.5	4.5	1.912	1.915
4	1	1	-1	-1	40	12	12.5	4.5	1.633	1.641
5	-1	-1	1	-1	20	8	17.5	4.5	1.919	1.885
6	1	-1	1	-1	40	8	17.5	4.5	1.755	1.788
7	-1	1	1	-1	20	12	17.5	4.5	1.956	1.924
8	1	1	1	-1	40	12	17.5	4.5	1.702	1.694
9	-1	-1	-1	1	20	8	12.5	5.5	1.947	1.930
10	1	-1	-1	1	40	8	12.5	5.5	1.891	1.913
11	-1	1	-1	1	20	12	12.5	5.5	2.041	1.999
12	1	1	-1	1	40	12	12.5	5.5	1.839	1.847
13	-1	-1	1	1	20	8	17.5	5.5	1.929	1.911
14	1	-1	1	1	40	8	17.5	5.5	1.965	1.938
15	-1	1	1	1	20	12	17.5	5.5	2.000	2.006
16	1	1	1	1	40	12	17.5	5.5	1.923	1.898
17	-2	0	0	0	10	10	15	5	1.762	1.803
18	2	0	0	0	50	10	15	5	1.563	1.555
19	0	-2	0	0	30	6	15	5	1.865	1.875
20	0	2	0	0	30	14	15	5	1.825	1.848
21	0	0	-2	0	30	10	10	5	1.930	1.928
22	0	0	2	0	30	10	20	5	1.927	1.962
23	0	0	0	-2	30	10	15	4	1.883	1.887
24	0	0	0	2	30	10	15	6	2.091	2.119
25	0	0	0	0	30	10	15	5	2.163	2.165
26	0	0	0	0	30	10	15	5	2.251	2.165
27	0	0	0	0	30	10	15	5	2.126	2.165
28	0	0	0	0	30	10	15	5	2.145	2.165
29	0	0	0	0	30	10	15	5	2.182	2.165
30	0	0	0	0	30	10	15	5	2.124	2.165

Table 6. Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of CV01 biomass production.

Source	df	Sum of squares	Mean square	*F*-value	*P*-value
Model	14	0.745	0.053	32.38	<0.001**
Linear	4	0.176	0.044	26.77	<0.001**
Quadratic	4	0.530	0.132	80.66	<0.001**
Crossproduct	6	0.038	0.006	3.92	0.015*
Residual total error	15	0.025	0.002	-	-

**Significant at 1% level; * significant at 5% level; R^2 = 0.9680; Adj. R^2 = 0.9381.

grown on synthetic medium. The fitted model given in equation 1 indicates that ethanol concentration (X_1) had a significant linear effect (P<0.001) on cellular biomass of the studied strain as it has the higher coefficient followed by pH (X_4). Only the linear coefficient of pH (X_4) and the interaction term (X_1X_4) had a positive value, which indicates a direct effect on biomass production. In contrast, the linear coefficient of ethanol (X_1) along with the quadratic terms (X_1^2, X_2^2, X_3^2 and X_4^2) and the interaction term (X_1X_2) had a negative effect that

Table 7. Regression analysis of a polynomial model for optimization of biomass production of CV01 strain.

Term	Estimated coefficients	*t*- Statistic	*P*-value
Intercept (β_0)	2.1647	130.74	<0.001**
β_1	-0.0620	-7.49	<0.001**
β_2	-0.0066	-0.80	0.436
β_3	0.0085	1.02	0.323
β_4	0.0581	7.02	<0.001**
β_1^2	-0.1215	-15.68	<0.001**
β_2^2	-0.0758	-9.78	<0.001**
β_3^2	-0.0550	-7.10	<0.001**
β_4^2	-0.0404	-5.22	<0.001**
$\beta_1 \times \beta_2$	-0.0335	-3.30	0.005**
$\beta_1 \times \beta_3$	0.0108	1.07	0.303
$\beta_1 \times \beta_4$	0.0307	3.03	0.008**
$\beta_2 \times \beta_3$	0.0066	0.65	0.527
$\beta_2 \times \beta_4$	0.0139	1.37	0.191
$\beta_3 \times \beta_4$	-0.0004	-0.04	0.966

** Significant at 1% level.

decreases cellular biomass.

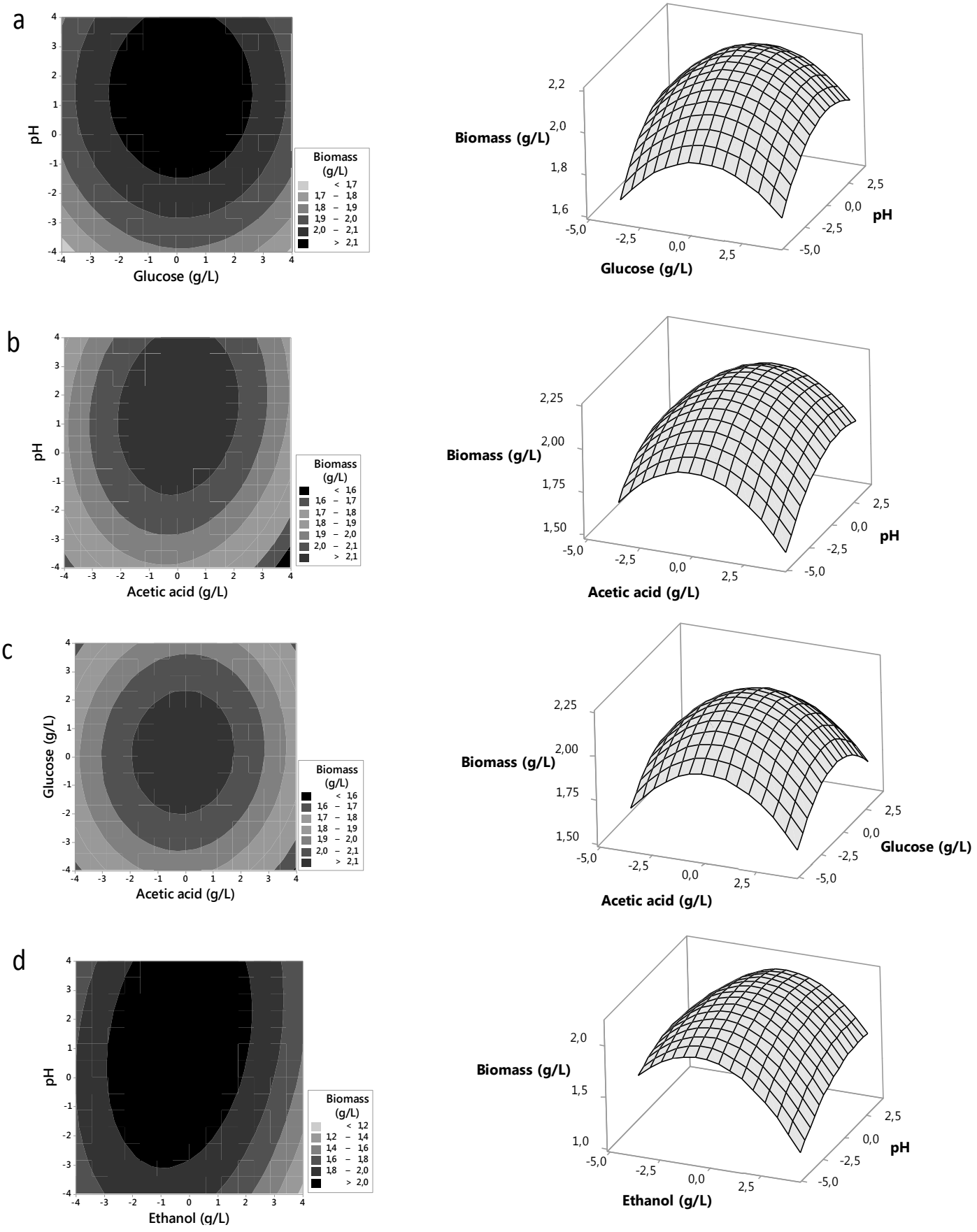
In order to better understand the relationship between the cellular biomass and the independent variables (X_1, X_2, X_3 and X_4), 3D response surface plots were formed based on the second order polynomial model. The shape of the corresponding contour plots indicates whether the mutual interactions between the independent variables are significant or not (Cui et al., 2006). Figure 2 (a-f) illustrates the fitted response surfaces and corresponding contour plots that provide a geometrical representation of changes in the predicted concentration cellular biomass, in response to modifications to two experimental parameters and maintaining the two others constant. Globally, variables exhibited a significant interaction. In fact, it is known that an elliptical contour plot indicates a significant interaction between variables (Liu et al., 2008). All six contour plots show similar relationships with respect to the effect of each variable. The three-dimensional plots and their respective contour plots facilitate the location of optimum experimental conditions (Liu et al., 2008). It is noteworthy that the produced biomass concentration of CV01 *Acetobacter* strain was sensitive when pH and ethanol concentration were subjected to a small alteration. The optimal values of variables required to obtain the maximum value of biomass concentration were gained by moving along the major and the minor axis of the contour plots. The predicted optimal values for the variables gained using the response optimizer command of the software were as follows: X_1= 28.18 g/L, X_2=10.12 g/L, X_3=15.15 g/L and X_4=5.33. The studied variables taken at these levels allow a production of a fit cellular biomass of approximately 2.21 g /L.

Batch fermentation in a 6-L bioreactor

A batch fermentation was performed for the CV01 strain in a 6 L Lab-fermenter (INFORS, France) in order to test the accuracy of the regression fitted model. The fermentation medium was prepared based on the predicted optimal composition. Thus, the medium was composed of 28.18 g/L ethanol, 10.12 g/L acetic acid, and 15.15 g/L glucose along with mannitol 20 g/L and fructose 2.5 g/L, and the pH was fixed at 5.33. Figure 3 shows the batch profile of CV01 *Acetobacter* strain in a lab-bioreactor and the variation of produced biomass, acetic acid and residual ethanol versus time. As can be seen in Figure 3, after a short adaptation time, the ethanol concentration started to decrease with a corresponding increase in acetic acid, and they reached the final concentration of 4.09 and 42.27 g/L for residual ethanol and acetic acid, respectively. On the other hand, bacterial biomass concentration increased slightly at the beginning of fermentation, then exponentially after 15 h. Bacterial biomass concentration reached a maximum level of 2.294 g/L after 80 h of fermentation. Consequently, the experimental value of produced biomass is shown to be slightly higher than the fit value (2.2 g/L). The raison might be related to the improvement of the volumetric oxygen transfer coefficient (K_La) in the bioreactor (better aeration and stirring systems) (De Ory et al., 2004).

Conclusions

The conversion of ethanol to acetic acid for the


a
pH
Glucose (g/L)
Biomass (g/L)
< 1,7
1,7 – 1,8
1,8 – 1,9
1,9 – 2,0
2,0 – 2,1
> 2,1
Biomass (g/L)
Glucose (g/L)
pH
b
pH
Acetic acid (g/L)
Biomass (g/L)
< 1,6
1,6 – 1,7
1,7 – 1,8
1,8 – 1,9
1,9 – 2,0
2,0 – 2,1
> 2,1
Biomass (g/L)
Acetic acid (g/L)
pH
c
Glucose (g/L)
Acetic acid (g/L)
Biomass (g/L)
< 1,6
1,6 – 1,7
1,7 – 1,8
1,8 – 1,9
1,9 – 2,0
2,0 – 2,1
> 2,1
Biomass (g/L)
Acetic acid (g/L)
Glucose (g/L)
d
pH
Ethanol (g/L)
Biomass (g/L)
< 1,2
1,2 – 1,4
1,4 – 1,6
1,6 – 1,8
1,8 – 2,0
> 2,0
Biomass (g/L)
Ethanol (g/L)
pH

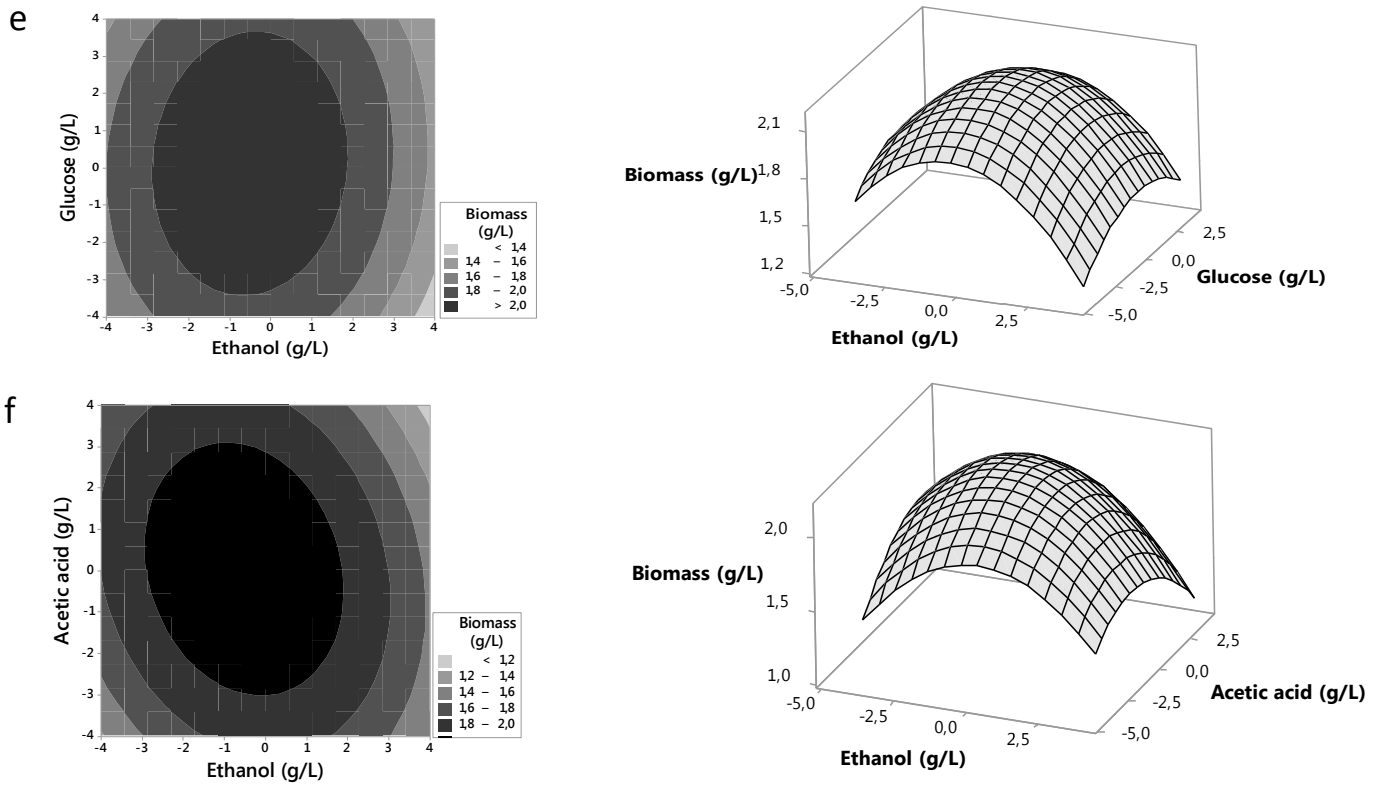


Figure 2. Surface plot and corresponding contour plot of the combined effects, respectively, of (a) glucose and pH; (b) acetic acid and pH; (c) acetic acid and glucose; (d) ethanol and pH; (e) ethanol and glucose, and (f) ethanol and acetic acid on the cellular biomass production of CV01 *Acetobacter* strain.

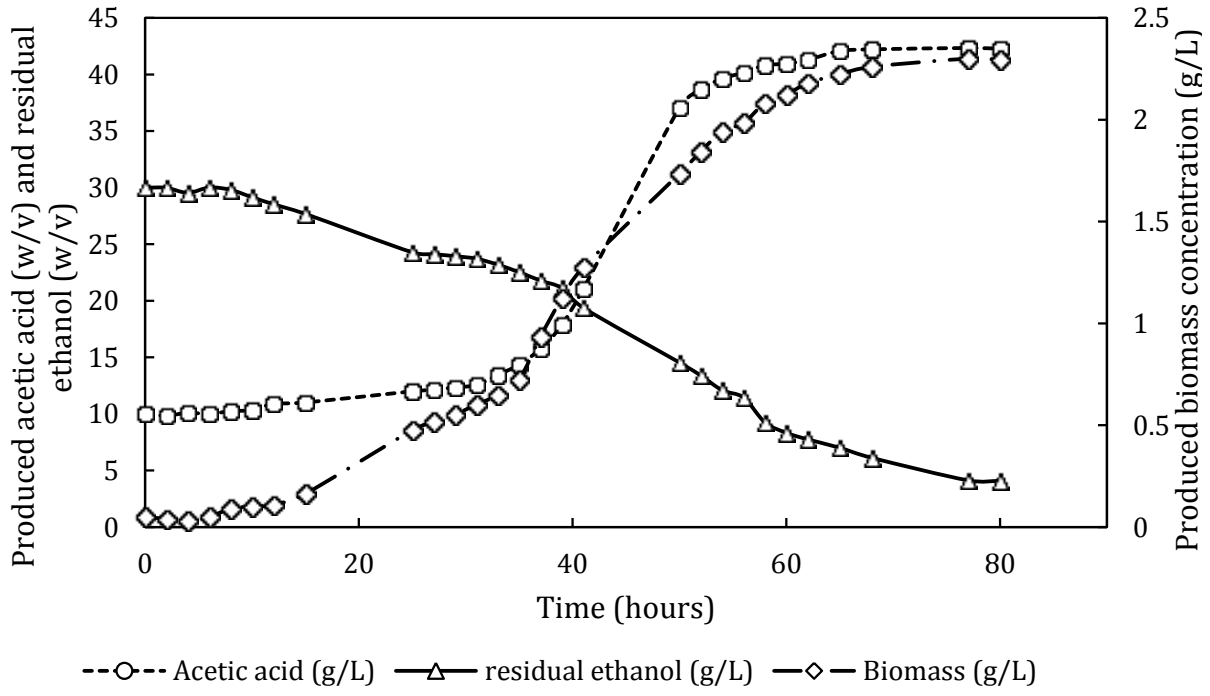


Figure 3. Batch fermentation profile of *Acetobacter* CV01 strain in a 6-L bioreactor at 30°C (a), and at thermal stress condition (b). Cultures were carried out using the optimized culture medium composition. The presented results are the means of two independent replicates.

production of vinegar is the most well-known application of AAB strains. In this paper, novel bacteria considered to be *Acetobacter* species according to a phylogenetic study based on 16s rDNA gene sequence analysis were isolated. These strains were subjected to an oxidation ability test performed on date and apple juices. Maximum acetic acid productions (42.5 and 36.5 g/L) were obtained for the CV01 *A. pasteurianus* strain isolated from cactus vinegar, respectively, for apple and date acetous fermentation processes. Response surface methodology (RSM) was applied to maximize the production of cell biomass of the CV01 *Acetobacter* strain for an industrial starter production objective. It was concluded that the predicted optimal values for the studied variables (ethanol, acetic acid, glucose and pH), allowing the maximum biomass production of 2.2 g/L, were, respectively, as follows: X_1= 28.18 g/L, X_2=10.12 g/L, X_3=15.15 g/ and X_4=5.33. Finally, a batch fermentation was carried out in a 6-L lab-bioreactor and the results were in line with the predicted values. It was concluded that the CV01 strain was well suited to be used as parental strain to prepare a starter for vinegar fruit production. Consequently, the conservation of this strain through freeze-dried powder lyophilisation is to be considered.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of pond depth and lining plastic color on growth and nitrogen fixing capacity of the cyanobacteria, *Anabaena* sp. E3

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Cyanobacteria are a cheap source of nitrogen and quite suitable for farmers of developing countries. Although, they live in a diverse range of environments, different environmental variables influence their nitrogen fixing ability. Thus, this study evaluated the effect of pond depth and lining plastic colors on nitrogen fixing capacity of *Anabaena* species strain E3. Factorial combinations of four pond lining plastic colors and two depths were laid out in a complete randomized design with three replications. The ANOVA results revealed that the 20 cm depth pond had a higher mean growth rate (0.063 OD day^{-1}), dissolved oxygen (17.63 mg L^{-1}), dry biomass (0.58 g L^{-1}) and total nitrogen (27.77 mg L^{-1}) than the 40 cm depth. The highest mean growth rate (0.089 OD day^{-1}), dissolved oxygen (19.07 mg L^{-1}), dry biomass (0.66 g L^{-1}) and total nitrogen (41.60 mg L^{-1}) were registered in the treatment with a transparent lining plastic color. Moreover, it was noted that, the test strain under the treatment combinations of 20 cm depth and transparent plastic lining expressed the highest mean optical density (1.227), heterocyst frequency (2.92%) and pH (10.28). Therefore, for mass-production of E3-strain-based biofertilizer, the strain should be grown in ponds of 20 cm depth with transparent plastic.

Key words: Cyanobacteria, dry biomass, growth rate, shallow pond, total nitrogen.

INTRODUCTION

Cyanobacteria (formerly classified as “blue-green algae”) are among the largest, most diverse and widely distributed group of prokaryotes. They are highly adaptable, and some species exhibit wide ecological tolerance and gliding mobility. They can be found in almost any environment, including extreme ones (e.g. benthos, cold and hot deserts, and Antarctic dry valleys) (Cohen and Gurevitz, 2006; Stal, 2007). Although, all existing cyanobacteria have the ability to perform oxygenic photosynthesis, they use H_2O as an electron donor, and some are able to grow as anaerobic photo-autotrophs using H_2S as an alternative electron donor

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(Cohen et al., 1986). This represents a unique additional capability of anoxygenic photosynthesis in these organisms. Some species produce toxins that affect animals and humans. People may be exposed to cyanobacterial toxins by drinking or bathing in contaminated water. The most frequent health effects are caused by drinking water containing the toxins (cyanobacteria), or by ingestion during recreational water contact (Ian, 1996). Cyanobacteria are equipped with nitrogenase enzymes, thus they can fix atmospheric nitrogen (N_2) into a biologically accessible form and thereby play a key role in the nitrogen cycle of the biosphere (Bothe et al., 2010; Zehr, 2011). Consequently, they are used as biofertilizer to maintain and improve soil fertility (Ahmed, 2001).

One way to improve soil fertility is the use of inorganic fertilizers. Adugna and Hiruy (1988) reported that the majority of Ethiopian soils gave a large yield response to applied nitrogen. Nevertheless, the use of this input among smallholder farmers is currently very low in the country. High fertilizer costs, marketing problems and poor infrastructure are some of the major reasons for low use of fertilizers (Schneider and Anderson, 2010). Moreover, synthetic N fertilizers have lower agronomic use efficiency due to losses of applied N through volatilization, leaching and denitrification (Havlin et al., 2010). Excess use of chemical fertilizers may result in multi-nutrient deficiencies and nutrient imbalance in soil. Furthermore, it also generates several environmental problems including acidification of water (Choudhury and Kennedy, 2005). The Ethiopian Ministry of Agriculture has identified 19 soil types throughout the country. The big proportion of the country's landmass is covered by lithosols, nitosols, cambisols and regosols in order of their importance (MoA, 2000). The country's soils, similar to other agricultural soils of the tropics, are generally low in nitrogen and phosphorus (Desta, 1982; Pulschen, 1987).

Therefore, the use of alternative options for soil fertility replenishment is indispensable to maintain soil fertility and productivity. Cyanobacteria offer an economically attractive and ecologically sound alternative to chemical fertilizers for realizing the ultimate goal of increased productivity (Mishra and Pabbi, 2004; Rajasulochana and Krishnamoorthy, 2014; Shweta and Kritika, 2015).

Phylogenetic analysis on endogenous Cyanobacteria *Anabaena* sp. E strains was performed in Colorado State University, USA, and *Anabaena torulosa* A525, *Anabaena oscillarioides* BECID22, *Anabaena sphaerica* RPAN 38 and *A. sphaerica* UTEX B 1616 were found to be close phylogenetic neighbors of *Anabaena* sp. E3 with greater than 97% similarity as summarized in Table 1. The phylogenetic tree for *Anabaena* sp. E3 and other similar endogenous cyanobacterial strains is also presented in Figure 1.

Although, the cyanobacteria live in a diverse range of environments, a number of environmental variables influence their photosynthetic and nitrogen fixing ability (Behl, 2013). Prior to this, a research was conducted (in 2012 at the same geographical location) to evaluate the effect of cyanobacterial based biofertilizer on maize yield, indicating that strains grown in ponds with two colors produced different cyanobacterial biomasses. This observation ignited an idea that pond plastic color may also influence the growth of *Anabaena* sp. E3. In general, studies on an organism's atmospheric nitrogen fixation capacity have to be done in laboratory settings prior to mass-production and formulation as biofertilizer. Therefore, the objectives of this study were (1) to evaluate the effect of pond depth and lining plastic color on nitrogen fixing capacity of cyanobacteria *Anabaena* sp. E3 and (2) to develop recommendations for pond depth and lining plastic color that optimize growth and development of cyanobacteria *Anabaena* sp. E3.

Table 1. Estimate of evolutionary divergence between *Anabaena* sp. E3 and reference cyanobacterial species registered in GenBank.

Strains	Similarity with E3 (%)
Anabaena torulosa A525	97.05
Anabaena oscillarioides BECID22	97.36
Anabaena sphaerica RPAN 38	97.34
Anabaena sphaerica UTEX B 1616	97.34

MATERIALS AND METHODS

Description of the study area

The laboratory phase of the experiment was carried out in the soil microbiology laboratory at Hawassa University, College of Agriculture and the glasshouse phase at Hawassa University main campus, Hawassa, Ethiopia, from March 2014 to October 2014.

Source of cyanobacterial strain

Heterocytous cyanobacteria, *Anabaena* sp. E3 was used. The strain was previously isolated from a pigeon pea field in Ziway, Ethiopia by Colorado State University, USA and was obtained from soil microbiology laboratory of Hawassa University College of Agriculture. In an earlier study, in comparison with strains E2, E5 and E9, the test strain in this study was proven to be the strain with the highest nitrogen fixing capacity under different environmental conditions (Girma et al., unpublished data).

Experimental details

Strain activation and inoculum preparation

Allen-Arnon medium was prepared (Allen and Arnon, 1955) using tap water. The only source of N in the Allen-Arnon medium is the ammonium metavanadate (NH_4+VO_3). For strain activation, *Anabaena* sp. E3 was transferred from a preserved sample to Allen-Arnon media in a 1:10 ratio and incubated for two weeks as

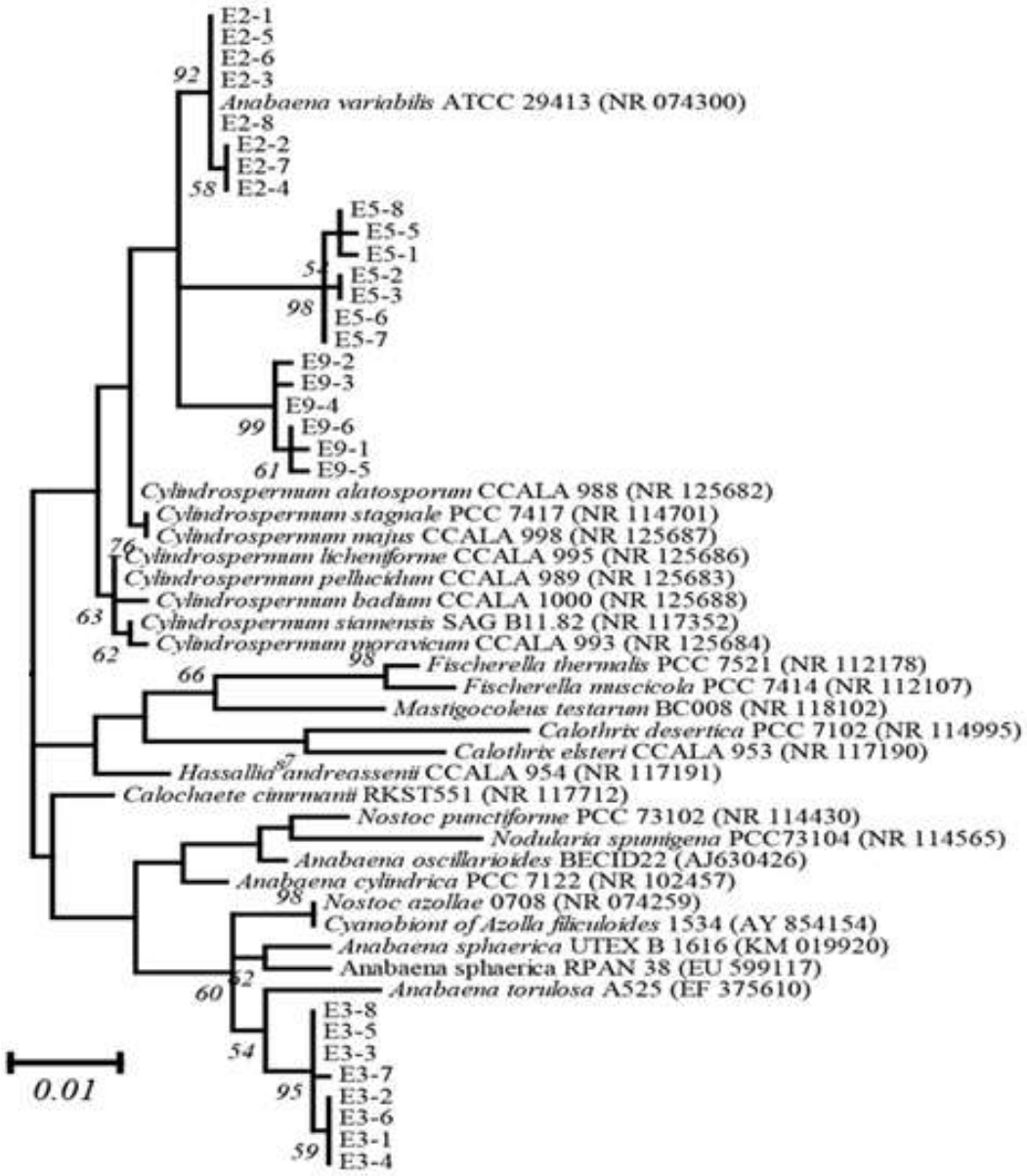

Figure 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the relationships among the different initial Ethiopian cyanobacterial strains and reference cyanobacterial species registered in GenBank. Accession numbers are given in parentheses. Trees were constructed by the maximum likelihood method using MEGA version 5 (Tamura et al., 2011). Bootstrap values over 50% are shown at each node. Bars, % estimated substitution expected number of changes per site.

shown in Figure 2. Then, the activated culture was transferred to fresh media made of double distilled water and grown for two weeks.

For inoculant culture preparation, the strain was inoculated in growing media in a 1:50 culture dilution. The culture was maintained under uniformly controlled conditions of continuous light of 2500 lux (Briand et al., 2004) during the day time (12 h) using cool fluorescent tubes at 27-30°C (Heino et al., 2009). Air was supplied to the growing culture in the box by means of a compressor for six hours day time only. The cultures were allowed to grow for two weeks before the formulation of secondary metabolites (Amal et al., 2010). The inoculant was described microscopically at the beginning and weekly throughout this phase of the experiment and hence, normal and healthy appearance of the inoculum culture was verified.

Pond design

A variety of cultivation systems for microalgae was developed. The only one used on a large scale and a commercial basis is the shallow, open raceway. These ponds are usually not more than 30 cm deep, and the water with nutrients and microalgae is circulated with a paddle wheel (Janssen, 2002). A factorial combination of two

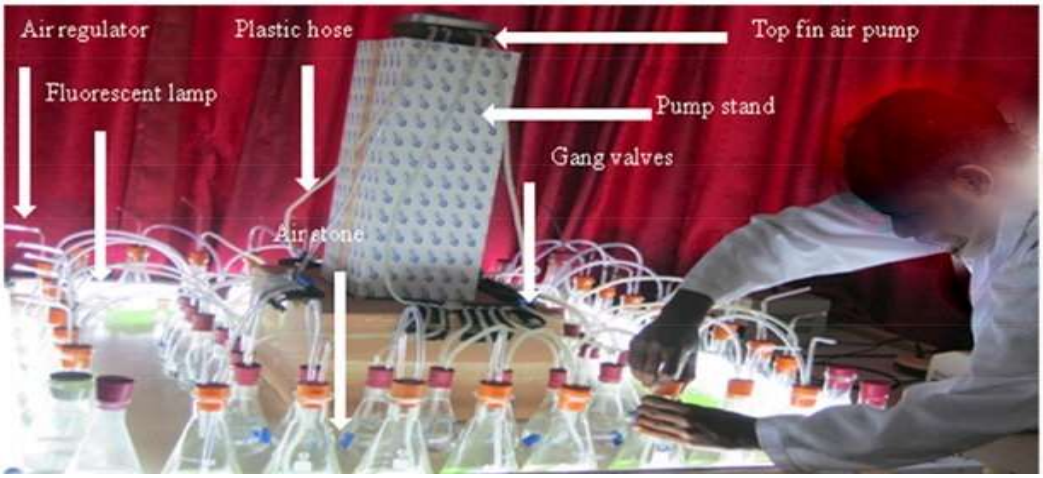

Figure 2. Cyanobacteria *Anabaena* species strain E3 activation for inoculum preparation.

Figure 3. Aeration by modified rotating paddle wheel.

depths (40 and 20 cm) and four pond lining colors (transparent, black, blue and red), altogether eight treatments were used. To control the effect of extraneous variables and assume any significant difference in the outcome variables is fairly due to the pond depth and lining plastic color, the treatments were laid out in a complete randomized design with three replications. In total, 24 experimental units were constructed. Plastic sheets purchased from the local market were used for pond lining after the ponds were constructed from wood in the glasshouse. The size of the two sets of twelve ponds were 50 (width) x 100 (length) x 20 cm (depth) and 50 (width) x 100 (length) x 40 cm (depth). Air was supplied to the growing culture in the ponds by means of a modified paddle wheel (rotating wheel) as shown in Figure 3. The wing length of the paddle wheel in ponds with a 20 cm depth was 10 cm whereas in 40 cm depth ponds it was 20 cm. As a result, the entire culture was set in motion to optimize gas exchange with the atmosphere in a similar manner in all the ponds.

Strain E3 was cultured in the Allen-Arnon media as described above, and this was used as the inoculant culture. The ratio of inoculant to media was 1:15. Four pond lining plastic colors (transparent, black, blue and red) and two depths (20 and 40 cm) with three replicates were assigned to the experimental units. The air temperature range in the glasshouse was 27-38°C, and the light incidence range was 5745.6 to 7711.2 lux with 12 h light/dark cycle for two weeks.

Data collection and analysis

Cyanobacterial growth attributes

Optical density (OD): Optical density was determined at 655 nm at the end of the experiment using JENWAY model 6300 spectrophotometer (Briand et al., 2004).

Growth rate (GR): The growth of cyanobacterial culture was calculated with the optical density result from the following equation (Tang et al., 1997; Gokason et al., 2007): $GR= (X_2-X_1) /(T_2-T_1)$,

Table 2. Interaction effects of pond depth and lining plastic color on optical density of E3 at the end of the two weeks growth period.

Treatment	Optical Density (655nm)				
Pond depth	**Trans**	**Blue**	**Red**	**Black**	**Depth mean**
20 cm	1.227[a]	0.955[c]	0.815[d]	0.614[e]	0.90
40 cm	1.083[b]	0.927[c]	0.804[d]	0.595[e]	0.85
Lining plastic color mean	1.15	0.94	0.81	0.60	
LSD (5%) = 0.08					
CV (%) = 4.74					

Means across all rows and columns followed by the same letter are not statistically different at $p < 0.05$.

where X_1 is the OD at the beginning of a time interval T_1, and X_2 is the optical density at the end of time interval T_2.

Heterocyte frequency (HF): Heterocyte frequency was determined by counting the number of heterocytes (nitrogen fixing cells) in a given filament under microscopic observation and expressed as % frequency (Singh et al., 2011).

Dry biomass (DB): The dry biomass of cyanobacteria was measured by taking 100 ml of culture from each pond at the end of the growth period and filtering it through Whatman No. 42 filter paper, then oven drying at 75°C for 24hrs. Finally, the measured g $100mL^{-1}$ value was converted to g L^{-1}.

Dissolved oxygen (DO): Dissolved oxygen in the culture was measured by Thermo Scientific ORION 5 STAR at the end of the growth period.

pH: The pH of cultures was measured every day during midday time throughout the growth period of the culture by Thermo Scientific ORION 5 STAR.

Total nitrogen (TN): After two weeks of culturing, total nitrogen of each culture was determined using Kjeldahl procedure as described by Nelson and Sommers (1980) and APHA (1999).

Statistical analysis

The triplicate sets of data for various parameters were subjected to analysis of variance (ANOVA) in accordance with the experimental design (completely randomized design) using Statistical Analysis System (SAS, 2003) to quantify and evaluate the source of variation. The means were compared using the least significant difference (LSD) test at the 5% significance level. Simple correlation analysis was conducted between cyanobacterial strain nitrogen fixing capacity and growth attributes.

RESULTS AND DISCUSSION

Chemical properties of the water source

The mean pH of tap water was 7.04, which was within the acceptable range for cyanobacteria growth (Roger and Reynaud, 1979). In addition to this, the amount of total nitrogen in the tap water was found to be 0.30 mg L^{-1}.

Growth performance of strain E3

Optical density

The main effects of pond depth ($p<0.01$), lining plastic color ($p<0.001$) and their interactions ($p<0.05$) were significant on final culture optical density at the end of the experiment. The maximum mean optical density (1.227) was recorded in the 20 cm deep transparent plastic lined ponds whereas minimum OD (0.595) was recorded in 40 cm deep black plastic lined ponds (Table 2). The depth effect was only significant in the ponds with transparent plastic lining; for the blue, red and black plastics, depth did not significantly affect OD.

In cyanobacterial culture, light is the source of energy that drives photosynthesis for assimilation of inorganic carbon which results in increased synthesis of carbohydrates, and thereby increased growth and optical density (Briand et al., 2004). Therefore, the difference in optical density with respect to the interaction effect of the two factors could be attributed to the highest illumination due to shallow depth (20 cm) and the higher light intensity reflected back to the culture as a result of transparency of the lining plastic color. This was consistent with other findings like that of Bernhard et al. (1966). A study conducted by Fernandez et al. (1998) revealed that a transparent glass tank reflected more light back to the culture and thus recorded highest optical density values. Similarly, Park et al. (2011) noticed that an increase in light intensity resulted in higher photosynthetic activity and higher optical density. Since all photoautotrophic organisms, that is cyanobacteria, photosynthetic eubacteria, algae and higher plants, are able to convert light energy into chemical energy by means of photosynthesis, undoubtedly light becomes the most limiting factor in a number of ways. But the light requirement varies from organism to organism. A laboratory scale study by Behl (2013) indicated that higher light intensity resulted in higher optical density, growth rate and dry biomass for cyanobacteria *Synechococcus* BG0011. Different light intensities such as 3000, 4000, 5000 and 6000 lux light were directly

Table 3. Growth parameters of strain E3 at the different pond depth and lining plastic colors. Growth rate (GR), dissolved oxygen (DO), dry biomass (DB) and total nitrogen (TN).

Treatment	GR (OD day^{-1})	DO (mg L^{-1})	DB (g L^{-1})	TN (mg L^{-1})
Pond lining plastic color				
Transparent	0.089^{a}	19.07^{a}	0.66^{a}	41.60^{a}
Blue	0.058^{b}	17.67^{b}	0.53^{b}	25.56^{b}
Red	0.049^{c}	16.90^{b}	0.46^{c}	20.85^{c}
Black	0.039^{d}	15.19^{c}	0.37^{d}	17.22^{d}
LSD (5%)	0.003	0.84	0.02	1.32
Pond depth				
20 cm	0.063^{a}	17.63^{a}	0.584^{a}	27.77^{a}
40 cm	0.054^{b}	16.79^{b}	0.428^{b}	24.85^{b}
LSD (5%)	0.0021	0.59	0.014	0.93
CV (%)	4.15	3.99	3.30	4.09

Means across all columns followed by the same letter are not statistically different at $p < 0.05$.

proportional to light intensity with cyanobacterium *Arthrospira maxima* growth rate, optical density and dry biomass (Pandey et al., 2011). Furthermore, cyanobacterial culture maintained under higher illumination produced chlorophyll concentrations of 25 mg L^{-1} which was slightly higher than the low-light counterparts that produced 20 mg L^{-1} thus, resulting in increased optical density (Wen et al., 2005). The rapid rate of increase in culture absorbance at A_{655} obtained in ponds with highest illumination was largely due to an increase in the volume of cells, their granular content and division rate.

Growth rate

The difference in growth rate of the E3 strain was observed to be highly significant ($p < 0.001$) among the pond liners with different plastic colors. The highest (0.089 OD day^{-1}) and the lowest (0.039 OD day^{-1}) growth rates were observed in ponds lined with transparent and black plastic, respectively (Table 3).

The difference in growth rate could be related to the difference in light intensity saturation that was being reflected by the culture (Bernhard et al., 1966). A study conducted by Alan and David (1990) revealed that cyanobacterial culture grown under high light intensity up to the saturation point induces a higher growth rate than the low light intensity. Similarly, a study conducted by Oliveira et al. (2014) revealed a progressive change in growth response curve and biomass for cyanobacteria (*Anabaena* PCC7120 and *Anabaena variabilis*) related to an increase in light intensity. The same research team indicated that biomass yield of *Nostoc* species F105 was found to be a function of light intensity.

Growth rate of the culture strain was found to be significantly ($p < 0.001$) influenced by the pond depth. However, the interaction of depth and lining color was not significant. Higher growth rate value (0.063 OD day^{-1}) was obtained in ponds with 20 cm depth, whereas a lower value (0.054 OD day^{-1}) was obtained in ponds with 40 cm depth (Table 3).

The lower growth rate of *Anabaena* sp. E3 in 40 cm depth ponds could be due to the decrease in photon flux density of the incoming light. Sunlight heated the surface and was absorbed by the culture; in a relative sense, in 40 cm deep ponds, the photon flux density and light intensity probably decreased with increasing depth as compared to the 20 cm deep ponds. Thus, the lower growth rate was recorded in 40 cm deep ponds. This phenomenon was visualized by Janssen (2002) for ponds with 30 cm depth; the photon flux density was 1000 µmol m^{-2}s^{-1} but decreased with increasing depth. A potential toxin producing cyanobacterial strain of *Cylindrospermopsis raciborskii* (coiled and straight) showed faster growth rate when exposed to higher light intensity (Oliveira et al., 2011). Similarly, the growth rate of cyanobacterial strain PCC 7936 was greatly influenced by higher light intensity as observed by Otero and Vincenzini (2003).

Heterocyte frequency

The main effects of pond lining plastic color, depth and their interaction were highly significant ($p < 0.001$) on the mean heterocyte frequency of the strain grown under different treatment combinations. The maximum (2.92%) and minimum (1.17%) mean heterocyst frequencies were observed in shallow (20 cm) transparent and deep (40 cm) black plastic lined ponds, respectively (Table 4).

The maximum heterocyte frequency in 20 cm transparent plastic lined ponds could be in response to the increased light intensity which resulted in an increased

Table 4. Interaction effect of pond depth and lining plastic color on heterocyte frequency of E3.

Treatment	Heterocyte frequency (%)				
Pond depth	**Trans**	**Blue**	**Red**	**Black**	**Depth mean**
20 cm	2.92[a]	2.21[b]	1.92[c]	1.64[d]	2.17
40 cm	2.15[b]	1.72[d]	1.64[d]	1.17[e]	1.67
Lining plastic color mean	2.535	1.965	1.78	1.405	
LSD (5%) = 0.116					
CV (%) = 3.672					

Means across all rows and columns followed by the same letter are not statistically different at $p < 0.05$.

population of heterocyte along the filament. Similarly, Pisciotta et al. (2010) noted the presence of double heterocytes resulting from an increase in the symmetry of cell division due to incubation of cyanobacteria in a pond having relatively higher illumination. A research report by Adams and Carr (1981) indicated that incubation of *Anabaena cylindrica* at high light intensity resulted in an increase in the frequency of pairs of adjacent double heterocytes (DHC).

In addition, heterocyte formation increased with increased cyanobacteria growth in response to optimum intensity of light (Sven et al., 2010). In the same way, heterocyte frequency of cyanobacteria strain PCC 7936 increased significantly with higher light intensity (Otero and Vincenzini, 2003).

Dissolved oxygen

The difference in mean dissolved oxygen level measured during the growth period of strain E3 was found to be highly significant ($p < 0.001$) among the ponds lined with different plastic colors. The highest (19.07 mg L^{-1}) and the lowest (15.19 mg L^{-1}) dissolved oxygen concentration were observed in ponds lined with transparent and black plastic color, respectively (Table 3).

Pond lining plastic color could reflect a relatively higher light intensity and could produce a higher photosynthetic rate of oxygen evolution. Correspondingly, Baosheng and Kunshan (2002) reported that an increase in cyanobacterial photosynthesis could result in increased dissolved oxygen concentration in the suspension. Pond water day time DO levels could increase to more than two fold saturation with intense photosynthetic activity (Garcia et al., 2000).

The amount of mean dissolved oxygen under the two depths was found to be significantly ($p < 0.01$) influenced by pond depth. However, the interaction of depth and lining color was not significant. Higher (17.63 mg L^{-1}) and lower (16.79 mg L^{-1}) dissolved oxygen of the culture strain was recorded in ponds with 20 and 40 cm depth, respectively (Table 3).

The higher dissolved oxygen level of *Anabaena* sp. E3 in ponds with 20 cm depth could be due to the higher illumination in shallow depth that comparatively enhanced photosynthetic rate and hence oxygen evolution. Similarly, Huisman et al. (2004) noted that increased light intensity resulted in higher photosynthetic activity and consequently, this resulted in enhanced oxygen production. Furthermore, Kranz et al. (2010) suggested that the effect of optimum light could increase optical density and growth rate of a cyanobacterial culture by promoting photosynthetic activity which in turn resulted in increased oxygen level.

pH of the culture

The difference in mean pH of the culture measured during the growth period was found to be significant ($p < 0.05$) among the different treatment combinations. The maximum mean pH (10.28) was recorded in 20 cm deep transparent plastic lined ponds whereas the minimum (8.88) was recorded in 40 cm deep black plastic lined ponds (Figure 4).

Prior to the inoculation of the test strain in the different treatment units, the pH of the cyanobacterial growing culture was 7.97. Nevertheless, pH increased in the whole treatment units during the growth period.

Higher illumination due to the shallow pond depth (20 cm) and transparency of the lining color resulted in higher photosynthetic activity which in turn caused an increased consumption of CO_2 and HCO_3^-. This phenomenon leads to the accumulation of OH^- ions in the medium (Park and Craggs, 2010). Photosynthetic rates can be considerable within millimeter-thin layers of cyanobacteria at the air–water interface because of high light intensities and efficient gas exchange with the atmosphere in shallow ponds (20 cm) which were lined with transparent plastic; accordingly, high CO_2 uptake resulted in a relatively higher pH value (Briand et al., 2004). Moreover, Gao et al. (2012) indicated that an increase in cyanobacterial biomass caused an elevation in pH (9-10.5) for weeks in the shallow and tidal-fresh regions of the Sassafras

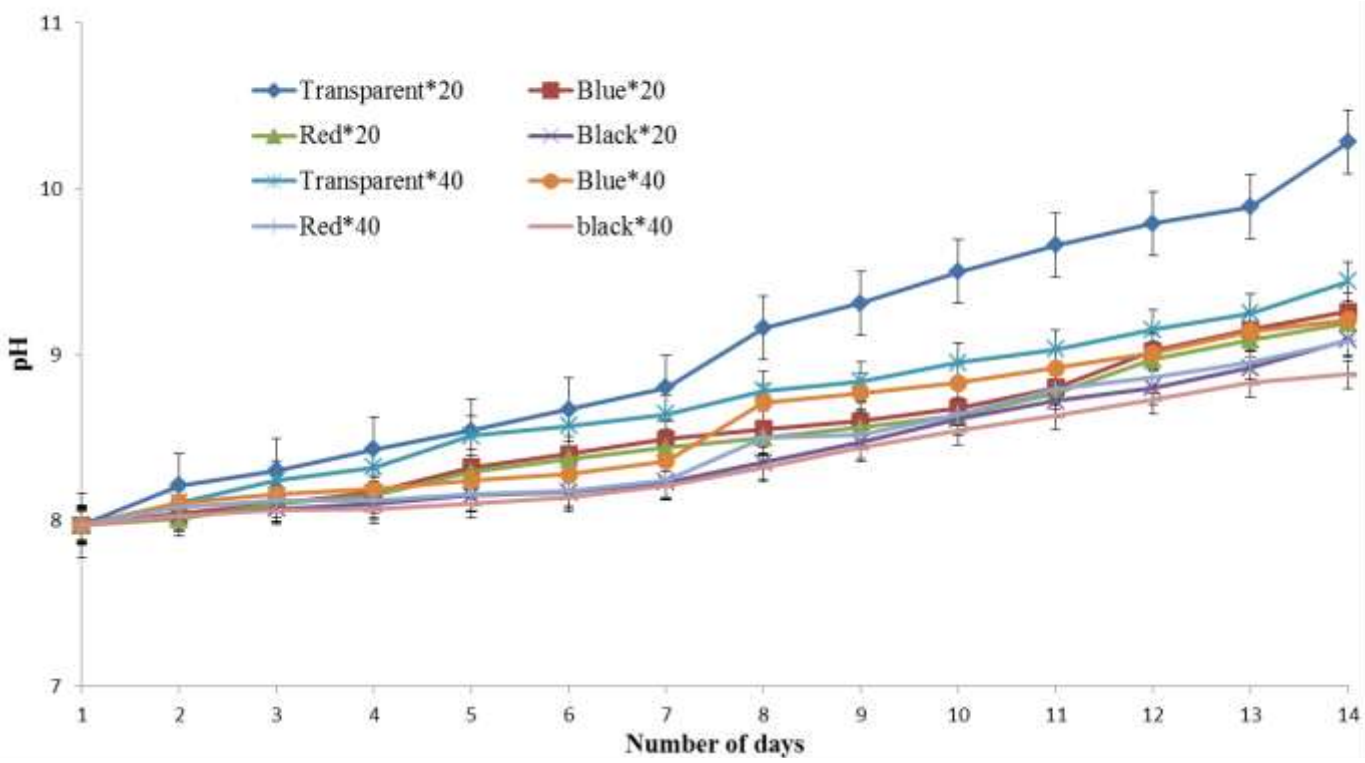

Figure 4. The pH of growing culture in different treatment units and it was measured during the midday time. Error bars indicate the 95% confidence intervals based on the Students T distribution. The numbers 20 and 40 after the respective lining plastic colors indicates depth of the ponds in cm.

River, a tributary of the Chesapeake Bay (USA).

Dry biomass

The difference in dry biomass was observed to be highly significant ($p < 0.001$) among the ponds lined with different plastic colors. The highest (0.66 g L^{-1}) and lowest (0.37 g L^{-1}) dry biomass were recorded in transparent and black plastic color lined ponds, respectively (Table 3).

This difference in dry biomass among the ponds lined with different plastic colors could be a reflection of the difference in light intensity reflected by the culture that had direct impact on photosynthetic rate and thus the dry biomass (Tredici, 1999). The influence of depth on dry biomass was highly significant ($p < 0.001$). However, the interaction of depth and lining color was not significant. Higher (0.58 g L^{-1}) and lower (0.43 g L^{-1}) dry biomass of cyanobacteria *Anabaena* sp. E3 was recorded in ponds with 20 and 40 cm depth, respectively (Table 3).

The difference in dry biomass under the two depths could be due to variation in the efficiency of light utilization, which can be expressed as the biomass yield of light energy in grams of dry weight of cyanobacteria *Anabaena* sp. E3 per amount of light energy absorbed. The poor volumetric dry biomass productivity of the culture strain in 40 cm deep ponds could be related to the relatively long light path which decreased photosynthetic efficiency. Thus, lower biomass was observed (Janssen, 2002). In addition, the findings of this research strongly agreed with Sven et al. (2010) that optimum light intensity had a pronounced effect on the growth and dry biomass accumulation of cyanobacterial culture. In addition, the report of Sven et al. (2010) again found that the biomass of cyanobacterial culture did not increase even by two fold when grown at a low light intensity of 2000 lux. However, higher biomass of 1.2366 g L^{-1} was acquired when grown under light intensity of 6000 lux.

Total nitrogen content

The difference in total nitrogen was observed to be highly significant ($p < 0.001$) among the ponds lined with different plastic colors. The highest (41.60 mg L^{-1}) and lowest (17.22 mg L^{-1}) total nitrogen concentrations were recorded in ponds lined with transparent and black plastics, respectively (Table 3).

The significant difference in total nitrogen content of *Anabaena* sp. E3 in ponds with different lining color could be attributed to relatively higher nitrogenase enzyme activity of the cells resulting from the difference in light

intensity (Levitan et al., 2010). Similarly, the effect of optimum light intensity can stimulate photosynthesis and nitrogen fixation of cyanobacteria (Kranz et al., 2010). The same research study reported that gross photosynthesis increased with light intensity, which in turn, increased total nitrogen, carbon content and growth rate of cyanobacterium, *Trichodesmium* IMS101. Moreover, high light intensity increases cellular iron uptake which could eventually be responsible for higher nitrogen fixation (Milligan and Harrison, 2001).

Total nitrogen content of the culture was found to be significantly ($p < 0.001$) influenced by pond depth. However, the interaction of pond depth and lining color was not significant. Higher nitrogen concentrations were recorded in ponds with 20 cm (27.77 mg L^{-1}) depth as compared to the 40 cm depth (24.85 mgL^{-1}) (Table 3).

The significant difference in total nitrogen content under the two depths could be attributed to variation in photon flux density which further created variation in illumination and intensity of light (Janssen, 2002). Similarly, it was observed that an increase in light intensity up to the saturation point could accelerate photosynthesis and thus enhance both growth and N_2 fixation of cyanobacteria (Alan and David, 1990). The total nitrogen content was positively and significantly ($p \leq 0.001$) correlated with optical density ($r = 0.92$), growth rate ($r = 0.98$), heterocytes frequency ($r = 0.93$), dissolved oxygen ($r = 0.84$) and dry biomass ($r = 0.85$). These correlations indicate that TN increased due the increase in these parameters.

Conclusion

Biological nitrogen fixation through microbial processes ensures great quantitative impact on the nitrogen cycle and has tremendous potential for the contribution of fixed nitrogen in the soil. Cyanobacterial based biofertilizer mass production conditions can greatly influence distribution and concentrations of photosynthetic and UV-screening pigments which, in turn, can critically alter light attenuation, photosynthetic rates and nitrogenase activity. Therefore, from the study findings, it could thus be concluded that the test strain in this study should be grown in ponds of 20 cm depth lined with transparent plastic for large-scale production. However, the issue related to cyanotoxins cannot be overlooked, thus further research aiming at exploring the safety of this strain should be taken into consideration for large-scale production and dissemination of biofertilizer derived from this strain.

Conflict of interest

The authors declare that there is no conflict of interest with regards to this study.

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Review

Plant growth promoting rhizobacteria: Beneficial effects for healthy and sustainable agriculture

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It is unanimously admitted that the chemical fertilizers and pesticides used in modern agriculture create a real environmental and public health problems. One of the promising solutions to substitute these agrochemicals products is the use of bio-resources, including plant growth promoting rhizobacteria (PGPR). The PGPR focused more and more scientific attention in recent decades. These rhizospheric bacteria colonize actively the root system of plants and improve their growth and yield. The PGPR use different mechanisms of action to promote plant growth. These mechanisms were grouped into three clusters according to the PGPR effects on plant physiology. These groups are as follow: (i) biofertilization including biological fixation of atmospheric nitrogen, phosphate solubilization, siderophores production and exopolysaccharides production; (ii) phytostimulation including production of indole acetic acid, gibberellins, cytokinins and ethylene; and (ii) biocontrol including induction of systemic resistance, competition for iron, nutrient and space, production of antibiotics, lytic enzymes, hydrogen cyanide and volatile compounds. In view of the latest advances in PGPR biotechnology, this paper proposes to do the review on PGPR in rhizosphere and describes the different mechanisms used by PGPR to promote the plants growth and health. In prospect to a healthy and sustainable agriculture, respectful of environment, the PGPR approach revealed as one of the best alternatives.

Key words: Rhizosphere, plant growth promoting rhizobacteria (PGPR), root colonization, biofertilization, biocontrol, biostimulation, interaction plant-microorganisms, sustainable agriculture.

INTRODUCTION

The galloping growth of world population estimated around 7 billion people and may reach 8 billion by 2020 (Glick, 2012), generates several problems including food insecurity and famine. So it is urgent to double the agricultural production in order to reduce the risk of malnutrition and increased poverty (Soulé et al., 2008). In response to this, new seeds varieties of high-yield were introduced into agricultural production systems in several countries. The use of these new varieties is accompanied by a growing and excessive use of chemical fertilizers and pesticides. Although the use of these chemical products has many advantages such as the ease to

handle and convincing results, they generate the environmental and public health problems. Among these problems, (i) groundwater and crop products contamination by heavy metals from the use of these agricultural inputs, (ii) interruption of the natural ecological cycle of nutrients, (iii) destruction of the soil biological communities, and (iv) physical and chemical deterioration of agricultural soils, can be mentioned. Indeed, the prolonged use of mineral fertilizers without addition of organic matter leads to the poor soils in organic matter, more sensitive to wind and rain erosion (Alalaoui, 2007). Koo et al. (2009) asserted that heavy metals contamination of groundwater and crop products is one of the major causes of the cancer occurrence.

The growing necessity to protect our natural resources, invites to a more restrictive use of fertilizers, pesticides and herbicides from chemical origin. Thus, in order to reduce or change the agrochemical used products and institute sustainable agriculture, respectful of the environment, the use of bio-resources such as plant growth promoting rhizobacteria (PGPR) focuses more and more on the scientific attention.

Indeed, Hiltner (1904), a German researcher has firstly defined the rhizosphere as soil area surrounding the root, directly or indirectly influenced by root and which has a strong microbial activity. The rhizosphere contains different groups of microorganisms such as the fungi, algae, nematodes, actinomycetes, protozoa and bacteria. The group of bacteria is subdivided into three subgroups (neutral, negative or positive) according to their effects on the plant physiology. Thus, PGPR is a group of bacteria capable to actively colonize the plant root system and improve their growth and yield (Wu et al., 2005). The term PGPR was proposed for the first time by Kloepper et al. (1980) and was used specifically for the fluorescent *Pseudomonas* involved in the biological control of pathogens and enhancing plant growth. Later Kapulnik (1981) extended this term to the rhizobacteria capable to promote directly plant growth. Today, the term PGPR is used to refer to all rhizospheric bacteria capable to improve the plant growth by one or more mechanisms (Haghighi et al., 2011). This reviewed article presents the PGPR in rhizosphere and describes the different mechanisms used by PGPR to promote the plants growth and health.

RHIZOSPHERE

According to the foremost definition given by the German scientist, Hiltner L., rhizosphere refers to the soil area surrounding a plant's root, directly or indirectly influenced by the root and which has a strong microbial activity (Hiltner, 1904). The term of rhizosphere comes etymologically from *rhiza* (root) and *sphera* (surroundings). The rhizosphere is subdivided into three separate parts (Figure 1). The first part (Exorhizosphere) corresponds to soil adherent to the root and remains attached to it after vigorous shaking. The second part (Rhizoplane) corresponds to interface soil/root and finally the third part (Endorhizosphere) is the intercellular space between the root tissues inhabited by endophyte bacteria, which does not form symbiotic structures (Bowen and Rovira, 1999).

This particular environment is the seat of important and intense interactions between plant, soil and various associated microorganisms (Nihorimbere et al., 2011; Lemanceau et al., 2006). Several biochemical interactions between plants and soil microorganisms have been reported by Pinton et al. (2007). The biological and physico-chemical characteristics of the rhizosphere depend largely on the nature of the various compounds released by the plant root (exudates) in rhizosphere. The process to excrete the exudates is called rhizodeposition. The roots secrete at the apex a mucilage constituted of carbohydrate polymers that the primary function is to protect root against desiccation (Bais et al., 2006). The root exudates are transported through the cell membrane and excreted in the rhizosphere. The composition and concentration of exudates are strongly influenced by the following factors: plant species, stage of development and plant nutrition, soil type and environmental conditions (temperature, soil water potential and light intensity) (Kochian et al., 2005). The root exudates effects depend on their ability to disseminate as far as possible from rhizoplane (Gupta and Mukerji, 2002).

The rhizosphere is very rich in nutrients such as sugars, amino acids, organic acids, hormones (Badri et al., 2009), nucleotides, fatty acids, sterols, growth factors, enzymes, flavonoids and other small molecules from the plant root exudates. These compounds serve many functions and they pose a significant carbon cost for the plant. The microorganisms found in this medium, require energy for their metabolism. The root exudates will also condition the diversity and density of microorganisms in the rhizosphere. The root exudates can attract beneficial and pathogenic microorganisms as well.

RHIZOSPHERIC MICROFLORA

Rhizosphere is the zone of a few millimeters around the plants root system (Compant et al., 2010), contains a sizeable microbial population (about 10^8-10^9 CFU/g of soil) (Schoenborn et al., 2004). The rhizospheric microflora is naturally made of a complex assembly of prokaryotic and eukaryotic microorganisms (Cardon and

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Gage, 2006). It constitutes of bacteria, fungi, algae, nematodes, actinomycetes and protozoa. The microbial structure of rhizosphere varies according to the plant species, stages of development and soil type (Broeckling et al., 2008). Proteobacteria and Actinobacteria are the microorganisms most frequently found in the rhizosphere of several plant species (Singh et al., 2007). The density and structure of rhizospheric microbial communities (Kowalchuk et al., 2010; Latour et al., 2009) and their metabolic activity (Nannipieri et al., 2008) are significantly different from those of bare soil. Through the root exudates, the plant can limit and/or guide the rhizosphere colonization and create its microbial community. Thus, the microbial population (diversity and load) of rhizosphere depends partly to the quantity and quality of exudates (microorganisms-exudates affinity), but also to microbial interactions (Somers et al., 2004).

RHIZOBACTERIA

Among the microbial community of rhizosphere, bacteria (rhizobacteria) are the most known (95%) and the most abundant because of their high growth rate and ability to use different carbon and nitrogen sources (Glick, 2012). The rhizobacteria concentration in the rhizosphere can reach 10^{12} CFU/g of soil (Foster, 1988). However, in the soils of stressed ecosystem, the load of rhizobacteria may be less than 10^{4} CFU/g of soil (Timmusk et al., 2011).

These rhizobacteria can affect the plants physiology through different ways. Thus, the interactions between rhizobacteria and plant can be beneficial, harmful or neutral (Ordookhani and Zare, 2011). The presence of neutral rhizobacteria in the rhizosphere has probably no effect on plant health. In opposite, phytopathogenic rhizobacteria (*Desulfovibrio*, *Erwinia*, *Agrobacterium*, *Enterobacter* and *Chromobacter*, etc.) affect negatively the plant growth, whereas the beneficial rhizobacteria (*Azospirillum*, *Pseudomonas*, *Bacillus,* etc.) affect positively plant growth and yield through various mechanisms of action. The beneficial rhizobacteria are known under the name 'PGPR'.

PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

PGPR are a group of bacteria capable to actively colonize the plants root system and improve their growth and yield (Wu et al., 2005). They colonize all ecological niches of root to all stages of plant development, even in the presence of a competing microflora. PGPR represent about 2 to 5% of total rhizospheric bacteria (Antoun and Kloepper, 2001). The term PGPR was proposed by Kloepper et al. (1980) and has been used for a long time, especially for fluorescent *Pseudomonas* involved in the pathogens biological control and enhancing plant growth. Later, Kapulnik et al. (1981) extended this term to the rhizobacteria capable to directly promote plant growth. Today, the term of PGPR is used to refer to all bacteria living in the rhizosphere and improve plant growth through one or more mechanisms (Haghighi et al., 2011). A wide range of species belonging to the genus *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus* and *Serratia* was reported as PGPR (Saharan and Nehra, 2011).

The PGPR effects depend on ecological and soil factors, plant species, plant age, development phase and soil type (Werner, 2001). For example, a bacterium which promotes plant growth through nitrogen fixation or phosphorus solubilization (compounds often present at low dose in many soils), certainly not produce beneficial effects to the plant when the soil receives chemical fertilizers. Also, the mutant bacterium *Pseudomonas fluorescens* BSP53a, hyper producing indole acetic acid (IAA) and stimulating root development of blackcurrant (*Ribes nigrum* L.) inhibits root development of Cherry (*Prunus avium* L.) (Dubeikovsky et al., 1993).

MECHANISMS OF ACTION USED BY PGPR TO PROMOTE PLANT GROWTH AND HEALTH

Current knowledge of mechanisms used by PGPR although this is not yet completely elucidated, it is possible to classify them into three groups (Biofertilization, Phytostimulation and Biocontrol) according to the PGPR effects on plant physiology (Table 1).

Root colonization

Root colonization is an essential step in the biological control of pathogens and in the improvement of plants growth by PGPR. The fundamental elements for efficient colonization include the ability of microorganisms to survive after inoculation, to grow in spermosphere (region surrounding the seed) in response to exudates production by seed, to fix on surface of the first roots, and to colonize the entire root system (Nelson, 2004). Especially for endophilic microorganisms, the root colonization includes four steps: (i) attraction, (ii) root recognition, (iii) root adhesion and (iv) root invasion (Nihorimbere et al., 2011). These steps are influenced by biotic and abiotic factors.

Indeed, the seeds colonization is the first step in the root colonization process. The microorganisms that are established on the seeds during the germination can grow and colonize the roots along their length from where they emerge and grow in the soil. Seed colonization during soaking phase has a significant effect on plant growth. Through the markers utilization, Trivedi et al.

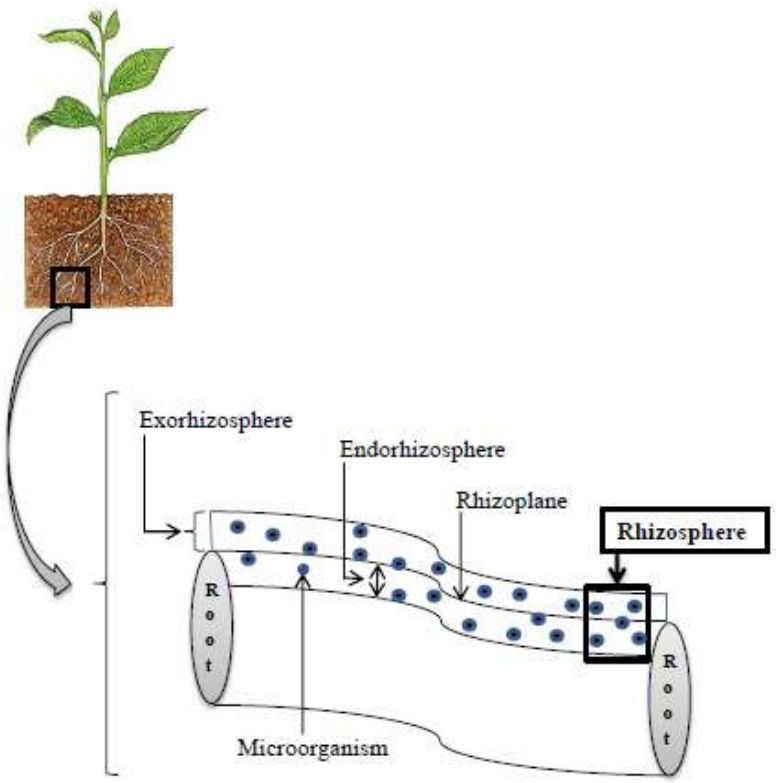

Figure 1. Schematic representation of rhizosphere.

Table 1. Mechanisms used by PGPR to promote plant health and growth;

Functions	Mechanisms	References
Biofertilization	Phosphate solubilization	Yazdani et al. (2009)
	Siderophores production	Vansuyt et al. (2007)
	Exopolysaccharides production	Sandhya et al. (2009)
	Biofixation of atmospheric nitrogen	Weyens et al. (2010)
Phytostimulation	Ethylene production	Glick et al. (2007)
	Cytokinins production	Kang et al. (2009)
	Gibberellins production	Kang et al. (2009)
	Indole Acetic Acid production	Ashrafuzzaman et al. (2009)
Control of pathogens	Antibiotics production	Ongena et al. (2005)
	Lytic enzymes production	Joshi et al. (2012)
	Hydrogen cyanide production	Lanteigne et al. (2012)
	Volatile compounds production	Trivedi et al. (2008)
	Induction of systemic resistance	Doornbos et al. (2012)
	Competition for Iron, nutrient and space	Innerebner et al. (2011)

(2005) showed that rhizobacteria that have promoted the tea growth (*Bacillus subtilis, Bacillus megaterium, and Pseudomonas corrugata*) are those that effectively colonized their root system. These bacteria have greatly colonized the maize rhizosphere (Trivedi and Pandey, 2008).

Biofertilization

The improvement of soil fertility is one of the strategies commonly used to increase agricultural production. PGPR participates in soil fertilization through the biofixation and biosolubilization process.

Biofixation of atmospheric nitrogen

Nitrogen is the main limiting nutrient for plant growth (Munees and Mulugeta, 2014; Chapin and Aerts, 2000). It is the fourth important element of plant dry mass. Nitrogen is an essential constituent of nucleotides, membrane lipids and amino acids (enzymatic and structural proteins) (Marschner, 1995). The most part of this element is in gaseous form (N_2) inaccessible to animals and plants (Pujic and Normand, 2009). The biological fixation of atmospheric nitrogen is an important microbial activity for the maintenance of life on the earth through photosynthesis performed by photosynthetic organisms. About 175 million tons of atmospheric nitrogen are reintroduced annually in life cycle through the biological fixation.

The biological nitrogen fixation is limited to prokaryotes that possess (unlike plant) an enzymatic complex (the dinitrogenase) which catalyses the reduction of atmospheric nitrogen into ammonia ($N_2 + 4H_2 \quad 2NH_3 + H_2$) (Weyens et al., 2010). Nitrogen-fixing bacteria include both free rhizospheric prokaryotic (e.g. *Achromobacter*, *Acetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Azomonas*, *Bacillus*, *Beijerinckia*, *Clostridium*, *Corynebacterium*, *Derxia*, *Enterobacter*, *Herbaspirillum*, *Klebsiella*, *Pseudomonas*, *Rhodospirillum*, *Rhodopseudomonas* and *Xanthobacter*) (Tilak et al., 2005) and symbiotic rhizospheric prokaryotes that fix nitrogen only in association with certain plants. This latter group comprises rhizobia (*Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium* and *Allorhizobium*) associated with leguminous plants and *Frankia* strains, filamentous sporulating bacteria associated with Actinorhizal plants (Gray and Smith, 2005).

Several studies showed that the co-inoculation of *Bradyrhizobium* and PGPR can positively influence the symbiotic nitrogen fixation through the increase of nodules number, nodule dry weight, seed yield, nutrients availability and improvement of nitrogenase activity (Son et al., 2006).

As previously announced, certain non-symbiotic bacteria are also capable to fix atmospheric nitrogen that will be transferred to plants (Bashan and Levanony, 1990). The discovery of nitrogen fixation by non-symbiotic bacteria was made by Beijerinck (1901). However, it is unanimously accepted that non-symbiotic bacteria fix less nitrogen than symbiotic bacteria (James and Olivares, 1997). Despite their low nitrogen fixation power, some PGPR are very effective. The inoculation of several cultures with diazotroph PGPR especially *Azotobacter* and *Azospirillum* has improved the yield of annual and perennial grasses (Tilak et al, 2001). The wheat inoculation with *Azotobacter* has increased their yield of 30% (Gholami et al., 2009; Kloepper and Beauchamp, 1992).

Under normal conditions, the fixing microorganisms benefit from the nitrogen without excretion of nitrogen compounds. But at their death and after decomposition, nitrogen is available to plants providing an average of 25 kg $Nha^{-1}year^{-1}$ at the continents. In most ecosystems and through this process, the fixing microorganisms participate to accumulation of nitrogen compounds over time (Vitousek et al., 2002). This process is then sufficient to maintain the stock of nitrogen compounds in the ecosystem and to restore the losses.

Phosphate solubilization

Phosphorus is a second mineral element after nitrogen that the deficiency crucially limits plant growth (Nisha et al., 2014). Phosphorus represents about 0.2% of plant dry weight and is an essential constituent of nucleic acids, phytin and phospholipids. Phosphorus plays a major role in photosynthesis, respiration, storage and transfer of energy and cell division and elongation (Sagervanshi et al., 2012). It is essential for seed formation which contains the highest phosphorus content of the plant.

The plant absorbs the phosphorus in mono and dibasic ($H_2PO_4^-$, HPO_4^{2-}) soluble forms (Keneni et al., 2010; Ramos Solano et al., 2008). Unfortunately, the great proportion of soil phosphorus (about 95-99%) is in the form of insoluble inorganic phosphates (apatite) or insoluble organic phosphates (inositol phosphate, phosphomonesters and phosphotriesters) unassimilated by the plant (Pérez-Montano et al., 2014; Khan et al., 2007). Phosphorus (highly reactive) is immobilized by precipitation with cations Ca^{2+} and Mg^{2+} in alkaline soils and with Fe^{3+} and Al^{3+} in acid soils (Hesham and El-Komy, 2005). Thus, when applied to an agricultural soil, the soluble inorganic phosphate, the great proportion of this phosphate is rapidly immobilized after application and becomes unavailable to the plant (Vikram and Hamzehzarghani, 2008).

Fortunately, some PGPR possess the ability to solubilize the soil insoluble phosphate in order to make available to the plant. These PGPR are referred by the acronym "Phosphate Solubilizing Bacteria, PSB". The PSB group contains the genus *Pseudomonas*, *Azospirillum*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Arthrobacter*, *Alcaligenes*, *Serratia*, *Enterobacter*, *Acinetobacter*, *Azotobacter*, *Flavobacterium* and *Erwinia* (Zaidi et al., 2009). *Pseudomonas* and *Azospirillum* species isolated from the pepper (*Piper nigrum L*.) rhizosphere have a strong ability to dissolve phosphate under *in vitro* condition (Ramachandran et al., 2007).

PSB make the solubilizing effect through the production of organic acids such as formic acid, propionic acid, lactic acid, glycolic acid, fumaric acid, succinic acid (Vazquez et al., 2000), gluconic acid, 2-ketogluconic, oxalic acid, citric acid, acetic acid and malic acid (Zaidi et al., 2009). These acids reduce the soil pH and cause the dissolution of insoluble phosphate. During the solubilization of rock phosphate by microorganisms, Venkateswarlu et al. (1984) observed a reduction of pH from 7 to 3. The study conducted by Wahyudi et al. (2011), revealed that all *Bacillus* isolates which have significantly improved the soybean (*Glycine max*) growth were able to solubilize the phosphate excepted CR67 isolate.

PSB are also able to mineralize the insoluble organic phosphate through the excretion of extracellular enzymes such as phosphatases (catalysts of the hydrolysis of phosphoric esters), phytases and C-P lyases (Weyens et al., 2010). It should be noted that this two mechanisms (solubilization and mineralization) can coexist within the same PBS (Tao et al., 2008). Several authors have reported the yield increase of Tea (*Camellia sinensis* L.) (Chakraborty et al., 2006), soybean (Abd-Alla, 2001), alfalfa (Medicago *sativa* L.) (Rodríguez, 1999), wheat (*Triticum aestivum* L.) (Whitelaw et al., 1997) and onion (*Allium Cepa*) (Vassilev et al., 1997) by PSB inoculation. The application of phosphate solubilizing microorganisms can reduce phosphorus application to 50% without affecting the maize seeds yield (Yazdani et al., 2009). Thus, the plants inoculation with PSB increases the availability of phosphorus in the rhizosphere and its absorption by the plant.

Iron chelation (siderophores production)

Iron is essential to the functioning of living organisms. It is essential for all life form because it is involved in diverse and essential biological functions. It is the cofactor of many enzymes involved in the electron transfer (mitochondrial respiration) or oxygen transfer (hemoglobin), and in the deactivation of radical oxygen (catalases, peroxidases) (Ganz, 2003). Iron is the fourth most abundant element in ground rock. Unfortunately, this huge quantity of iron is in the ferric ions form (Fe^{3+}) very little assimilated by living organisms (bacteria, plants, etc.) (Ammari and Mengel, 2006). To overcome this difficulty and provide iron to the plant, rhizobacteria have developed various iron uptake strategies to survive and to adapt to their environment. One of these strategies is the production of siderophores.

Siderophores are the molecules of low molecular weight (400 to 1500 Da), having an exceptional affinity for Fe^{3+} (Ka ranging from 10^{23} to 10^{52}) and membrane receptors capable of binding the complex Fe-siderophores in order to facilitate the iron absorption by micro-organisms and plant (Hider and Kong, 2010). They are used in fertilizer formulations for regulation of iron intake in plants, and thus facilitate its growth (Miller and Malouin,1994). Siderophores are produced by a wide variety of microorganisms (bacteria and fungi) and some plants (phyto-siderophores of grasses) (Van der Helm and Winkelmann, 1994). *Agrobacterium*, *Bacillus*, *Escherichia coli*, *Pseudomonas*, *Rhizobium* and many fungi are capable to produce these iron chelating compounds (Zahir et al., 2004).

According to the chemical function involved in the iron chelation, the siderophores are classified into three classes: phenol/catechol, hydroxamate and hydroxycarboxylique acid. Today, more than 500 siderophores are known and the chemical structures of 270 of them were determined (Hider and Kong, 2010).

Several studies have shown the beneficial effects of bacterial siderophores on improving of plant growth. Robin et al. (2008), using the iron-siderophore complex radioactive as the only source of iron, showed that plants are able to absorb the radioactive iron. The iron-pyoverdin synthesized by *P. fluorescens* C7 tested on *Arabidopsis thaliana* plants, has increased the iron level inside the plants and improved their growth (Vansuyt et al.,2007).The siderophores are also involved in chelation of other rhizosphere metals having a low availability to plants such as zinc and lead (Dimkpa et al., 2009).

Extracellular polysaccharides production

The ability to produce polysaccharides is one of the many benefits of rhizobacteria in promoting plant growth. These polysaccharides include structural polysaccharides, intracellular polysaccharides and extracellular polysaccharides (exopolysaccharides, EPS). The main contribution of rhizospheric microorganisms to soil stability is associated to the EPS production. These are in the form of hydrated gels around the cells. They constitute the interface between the microorganisms and their immediate environment. In the rhizosphere, EPS produced by rhizobacteria, enter aggregate soil and alter its porosity (Alami et al., 2000). Thus, the porosity of the soil, which is directly related to soil water transferred to the roots, is partly controlled by bacterial activity. EPS bacterial products on the surface of roots also help maintain the film of water required for the photosynthetic activity and growth of plants. Sandhya et al. (2009) argue that EPS participate in the formation of bacterial aggregates and consequently improve soil aeration, water infiltration and root growth. EPS cover and protect the roots against attacks by pathogenic microorganisms. They are used as delete molecules in plant defense mechanisms against pathogens.

In salt stress condition, the EPS chelate cations available in the root zone, thus contributing to reduce the salinity of the rhizosphere. The bacterial EPS in conditions of water stress in the soil can limit or delay the middle of desiccation. Conversely, in case of excess water (rain, floods), EPS contribute to avoid dispersion of

soils clayey (Henao and Mazeau, 2009).

Phytostimulation

Phytohormones are chemical messengers that influence the ability of plants to respond to their environment. They are organic compounds which are generally effective at very low concentrations. Botanists recognize five main groups of plant hormones: (i) Auxins (ii) Gibberellins, (iii) Ethylene, (iv) Cytokinins, and (v) abscisic acid. Only the first four are involved in the phytostimulation by rhizobacteria.

Indole acetic acid production

Auxin (from the Greek "auxien", which means "increase") is the first plant hormone discovered by Darwin (1880) in canary seed (*Phalaris canariensis*). Therefore, auxin was isolated from several other plants. Indole Acetic Acid (IAA) includes the plant hormones belonging to auxin group. IAA is a molecule signal, involving in the regulation of plant development, specifically in organogenesis, cell division, cell differentiation and genes regulation (Ryu and Patten, 2008).

Although the plant is able to synthesize the IAA, it responds positively to an IAA exogenous supply, at certain stages of its development cycle (Khalid et al., 2006). The stimulation of plants growth by rhizobacteria is often associated with their ability to produce IAA (Patten and Glick, 2002). Shobha and Kumudini (2012) reported that several species of *Bacillus* spp., *Pseudomonas* species, *Azotobacter* species, *Azospirillium* species, *Phosphobacteria* species, *Glucanoacetobacter* species, *Aspergillus* species and *Penicillium niger* can produce IAA. The production of IAA by PGPR depends on species and strains and is also influenced by the culture conditions, stage of development and availability of substrates in the rhizosphere (Ashrafuzzaman et al., 2009). It has been reported that the wheat inoculation with *Azotobacter* and *Bacillus* has increased its seeds yield of 30 and 43% respectively. This increase due to the production of certain growth hormones such as IAA (Kloepper et al., 1991).

The microbial production of IAA makes several metabolic pathways (Persello-Cartieaux et al., 2003). Although some pathways independent of tryptophan have been identified in some microorganisms, tryptophan remains the most common and major precursor of IAA biosynthesis by microorganisms. The four main metabolic pathways dependent of tryptophan are: tryptophol, tryptamine, indole-3-pyruvic acid and indole-3-acetamide pathway (Bartel, 1997). Gravel et al. (2007) have shown that the rhizobacteria *Pseudomonas putida* and *Trichoderma atroviride* synthesized *in vitro* IAA in the presence of L-tryptophan. When these researchers inoculated tomato plants with previous rhizobacteria in addition with several concentrations of L-tryptophan, they found that the higher concentration of L-tryptophan increased more than tomato plants developed. Let note that the tryptophan is naturally excreted by the tomato plant through the root exudates. Thus, the majority of auxins found in the tomato rhizosphere come from the microbial biosynthesis (Kamilova et al., 2006). Several studies have shown that some microorganisms produce low quantity of auxins in the absence of L-Tryptophan become strong producing of auxins in the presence of L-tryptophan (Zahir et al., 2004). Zahir et al. (2010) observed an increase up to 8 times auxin production by *Rhizobium* strains after addition of L-tryptophan to the culture medium.

The plant response to IAA addition varies according to plant species, IAA concentration, complexity of tissue and stage of plant development (Glick, 2012). IAA is strongly involved in the tomatoes fruition especially during fruit setting and its final development phase (Srivastava and Handa, 2005). It has been shown by Xie et al. (1996) that the synthesis of great quantity of IAA by rhizobacteria inhibits the development of the plant root system. Indeed, the root level of endogenous IAA can be suboptimal or optimal for plant growth (Pilet and Saugy, l987). Through the additional effect, the exogenous IAA (produced by rhizobacteria) brings the IAA levels of plant to optimum or supra optimal (Glick, 2012). In the first case, there has been an improvement of the plant growth due to the induction of a better development of the root system (initialization of root elongation and cell division), which improves the plant nutrition through a more absorption of water and nutrient. In the second case, a root inhibition will be observed. Thus, the bacterial IAA can have an inhibitory effect on root growth from a certain concentrations. Tanimoto (2005) says that the development of the root system can be greatly affected by external sources of growth regulators.

Spaepen et al. (2007) affirmed that the IAA plays a very important role in root elongation and root hairs proliferation. San Francisco et al. (2005) showed that the application of exogenous IAA increases the phosphorus level in roots of Pepper plants under hydroponic conditions. Moreover, Patten and Glick (2002) also reported that low levels of IAA can stimulate root elongation, while optimal levels of biosynthesized IAA stimulate the lateral and adventitious roots formation.

Gibberellins and cytokinins production

Rhizobacteria have the capacity to produce phyto-hormones cytokinins and gibberellins (Van Loon, 2007). The evaluation of ability to produce plant hormones of 24 *Streptomyces* strains in broth medium, revealed that all strains synthesized cytokinins and gibberellins (Mansour et al., 1994). The improvement of plant growth by some PGPR producing cytokinins or gibberellins was reported (Kang et al., 2009). The mechanisms used by cytokinins and gibberellins synthesized by rhizobacteria to promote plant growth are still not well understood. The assumptions

so far are based on the conventional knowledge on the role of cytokinins and gibberellins in the plant physiology and those relating to the plant response to the addition of purified hormones. Among other effects, cytokinins and gibberellins are involved in plant morphology modifying and in the stimulation of development of the plant aerial part (Van Loon, 2007).

Ethylene regulation

Ethylene is one of the small bioactive molecules known as a plants growth inhibitor. At low concentrations, ethylene can promote the growth of several plant species, including *Arabidopsis* (Pierik et al., 2006) by the stimulation of seed germination, initiation of root growth, fruit ripening and activation of other phytohormones synthesis. However, the moderate or high levels of ethylene induced the inhibition of root elongation, *Rhizobium* species nodulation and plant-mycorrhiza interactions, the wilting flowers, the falling leaves, and disruption of plant response to biotic and abiotic stress (Abeles et al., 1992). Thus, the elevation of ethylene concentration (> 25 µg/L) under stress conditions caused by heavy metals (Belimov et al., 2005), pathogens (Wang et al., 2000), drought (Mayak et al., 2004a), salinity (Mayak et al., 2004b) and organic contaminants (Reed and Glick, 2005) induces the inhibition of hair formation and root elongation and therefore a reduced vegetable growth.

The decrease of the high levels of ethylene in the plant can be performed through the degradation of its direct precursor 1-aminocyclopropane-1-carboxylic acid (ACC) using the ACC-deaminase enzyme. This enzyme is expressed in several rhizobacteria (e.g. *Alcaligenes* species, *Bacillus pumilus*, *Burkholderia cepacia*, *Enterobacter cloacae*, *Methylobacterium fujisawaense*, *Ralstonia solanacearum*, *Pseudomonas* spp. and *Variovorax paradoxus*). These rhizobacteria through the ACC-deaminase can degrade plant ACC to α-ketobutyrate and ammonium (Glick et al., 2007). The consequence of this degradation is the reduction of ethylene produced by the plant. Through this mechanism, the PGPR producing ACC-deaminase regulates the ethylene level in the plant and prevents the growth inhibition caused by high levels of ethylene.

BIOCONTROL OF SOIL-BORNE PHYTOPATHOGENIC MICROORGANISMS

PGPR involved in the biological control of soil-born phytopathogenic organisms through certain mechanisms such as: production of antagonistic metabolites (antibiotics, lytic enzymes, hydrogen cyanide, volatile compounds and siderophores), induction of systemic resistance and nutrients and space competition. In study conducted by Noumavo et al. (2015), *Streptomyces hygroscopicus*, *Ectocarpus fasciculatus*, *Pseudomonas aeruginosa*, *P. putida*, *P. fluorescens* and *Azospirillum lipoferum* inhibited mycelial growth of *Fusarium verticillioides* and *Aspergillus ochraceus* pathogens of maize plants. *P. fluorescens* and *P. aeruginosa* were highly antagonistic against *F. verticillioides* (52.24% of mycelial growth inhibition) and *A. ochraceus* (58.33% of mycelial growth inhibition).

Antibiotic production

The antibiosis is probably the best known and perhaps the most important mechanism used by PGPR to limit the pathogens invasion in the plant tissue. It consists to inhibit the development of plant pathogenic microorganisms through the production of secondary metabolites of low molecular weight, possessing antifungal and/or antibiotics properties. *Bacillus*, *Streptomyces*, and *Stenotrophomonas* strains produce a wide range of potent antifungal metabolites such as oligomycin-A, xanthobaccin (Compant et al., 2005), zwittermicin-A, kanosamine and lipopeptides of the surfactins, iturins and fengycin family (Ongena and Thonart, 2006). *Pseudomonas* strains are known for the production of amphisin, 2,4-diacetylphloroglucinol (DAPG) oomycin-A, phenazine, pyoluteorin, pyrrolnitrin, tensin, tropolone, and the cyclic lipopeptides (Loper and Gross, 2007). It was recently demonstrated the role of these lipopeptides in protective effect of a particular *B. subtilis* strain against *Pythium ultimum* pathogen of bean plants (Ongena et al., 2005) and against mould gray of apple after harvesting (Touré et al., 2004).

This metabolites production is influenced by abiotic factors (oxygen, moisture, temperature, pH and soil nitrogen, micronutrients and organic matter content), biotic factors (vegetable specie, pathogen organisms, native microflora, and density of strains producing metabolites) and some other.

Lytic enzymes production

Some PGPR strains have the ability to degrade fungal cell walls through the production of hydrolytic enzymes such as chitinases, dehydrogenases, β-glucanases, lipases, phosphatases, proteases, hydrolases, exo and endo-polygalacturonases, pectinolyases and cellulases (Joshi et al., 2012; Whipps, 2001). Various *Pseudomonas* strains showed *in vitro* antifungal activity against three zoospores fungi (Sharma et al., 2009). These authors proved that the antifungal activity is due to the production of rhamnolipid causing the lysis of plasma membrane of zoospores fungi. This PGPR lytic activity allows to protect the plant against biotic stress through the pathogens elimination.

Hydrogen cyanide and volatile compounds production

The antagonistic activity of PGPR also results in the

production of volatile compounds. The best known compound is hydrogen cyanide (HCN). Devi et al. (2007) reported the excretion of HCN by rhizospheric strains. *Pseudomonas* strains producing HCN are used in biological control against bacterial canker of tomato (Lanteigne et al., 2012). *P. corrugata* showed antagonistic activity against *Alternaria alternata* and *Fusarium oxysporum* pathogen microorganisms of several cultures such as maize (Trivedi et al., 2008). This antagonism has been associated with the production of volatile compounds, although *P. corrugata* also produced some hydrolytic enzymes. *Bacillus subtilis* strains isolated from tea, producing volatile antifungal compounds induced structural defects on six pathogenic fungi under *in vitro* culture conditions (Chaurasia et al., 2005). *B. megaterium* inhibits the growth of two plant pathogens *A. alternata* and *F. oxysporum* through the production of volatile compounds (Trivedi and Pandey, 2008).

Induction of systemic resistance

PGPR can trigger the plants inducible defense mechanisms, phenotypically similar to normal defense reaction of plants, when attacked by a pathogen (Pieterse et al., 2009). This phenome non called Induced Systemic Resistance (ISR) can make the plant much stronger hardy against future aggression of pathogens (Van Loon, 2007). This phenomenon of systemic resistance induction by rhizobacteria is considered as a promising strategy for biological control of plant disease (Ramos Solano et al., 2008). The ISR can be induced by a wide range of microorganisms included Gram-positive bacteria such as *B. pumilus*, or Gram-negative bacteria belonging to the genus *Pseudomonas* (*P. fluorescens, P. putida*, *P. aeruginosa*), and enterobacteria such as *Serratia* (*Serratia marcesens*, *Serratia plymuthica*) or *Pantoea agglomerans* (Jourdan et al., 2009). The IRS protects the plants against a large spectrum of pathogens not only fungal, bacterial and viral, but also against certain diseases caused by insects and nematodes (Durrant and Dong, 2004). Several bacterial metabolites can induce an IRS. These metabolites include lipopolysaccharides (LPS), siderophores, cyclic lipopeptides, 2,4-diacetylphloroglucinol, homoserine lactones, and volatile compounds such as acetoin and 2,3-butanediol (Doornbos et al., 2012).

Competition for space, nutrients and iron

Although it is difficult to directly demonstrate, the indirect evidences showed that the competition between pathogens and PGPR may limit the incidence and severity of plant pathology. The rapid and abundant root colonization by PGPR, which occupies the infection sites of plant pathogens and uses most of the available nutrients, makes difficult the development of pathogens. Lemanceau and Heulin (1998) affirmed that high and active microbial biomass reduces the probability of pathogen to infect the plant. This makes the nutrient competition an important mean of biological control (Benitez et al., 2004). Beside the intrinsic growth capacity of PGPR, the other properties enhancing the root colonization are mobility (presence of flagellum), chemotaxis, lipopolysaccharide (LPS), the ability to synthesize vitamins and macromolecules and the capacity to use the compounds excreted by the roots (Lugtenberg and Kamilova, 2009). In a series of experiments, researchers have shown that the treatment of tomato leaves with *Pseudomonas syringae* pv. prevented *Sphingomonas* species to cause the disease symptoms (Innerebner et al., 2011).

Another form of competition is established between the pathogens and PGPR. This is the struggle for iron. Indeed, iron is an essential element for the growth and survival of most phytopathogenic fungi. Then, some PGPR synthesize siderophores that chelate iron in the rhizosphere and thus inhibiting the pathogens growth.

CONCLUSION

This paper showed the beneficial effects of PGPR. PGPR improve soil fertility through increase plant nutrients (nitrogen, phosphorus and iron) available in soil. The phytohormones produced by PGPR are assimilated by plant for best growth. Also, PGPR inhibit plant pathogens growth through the production of antagonistic metabolites, induction of systemic resistance and nutrients and space competition. Additionally, PGPR polysaccharides alter soil porosity and consequently improve soil aeration. It is therefore clear that the objectives of chemical fertilizers and pesticides use can be reached with PGPR use. These rhizobacteria are the best alternatives to use of chemical fertilizers and pesticides that generate many problems such as groundwater and crop products contamination by heavy metals from there, interruption of the natural ecological cycle of nutrients, destruction of the soil biological communities and physical and chemical deterioration of agricultural soils. Thus, this technology based on the PGPR use, should be integrated into agricultural production strategies of all countries to a healthy and sustainable agriculture.

Conflict of Interests

The authors have not declared any conflict of interests.

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Whole genome homology-based identification of candidate genes for drought tolerance in sesame (*Sesamum indicum* L.)

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Sesame (*Sesamum indicum* L.) is one of the most important oilseed crops. It is mainly grown in arid and semi-arid regions with occurrence of unpredictable drought which is one of the major constraints of its production. However, the lack of gene resources associated with drought tolerance hinders sesame genetic improvement towards this osmotic stress. The present research aimed at identifying candidate genes associated with drought tolerance in the whole genome of sesame through homology search of known drought associated genes from three relative species, viz., potato, tomato and the well-described model plant *Arabidopsis*. Based on 2,495 sequences including 1,150 from *Arabidopsis*, 1,075 from potato and 270 from tomato, comparative analysis against sesame genome led to the identification of a set of 75 candidate genes (42, 22 and 11 from *Arabidopsis*, potato and tomato, respectively). Mapping results showed that the candidate genes were distributed on the 16 sesame linkage groups. Wide range of genes with various functions identified in this study confirmed that drought tolerance in sesame is under the control of several genes. Based on their functional classification, 2 groups of candidate genes were identified: (a) genes which protect the plant against drought effect; (b) signal transduction genes and transcription factors. Many transcription factors were retrieved including 6 AP2/ERF genes among which 3 were more expressed in drought tolerant material compared to the sensitive one and might play some important roles in drought tolerance in sesame. Our results provided genomic resources for further functional analysis and genetic engineering towards drought tolerance improvement in sesame.

Key words: *Sesamum indicum*, candidate genes, drought tolerance, orthologous gene, whole genome.

INTRODUCTION

Sesame (*Sesamum indicum* L., 2*n* = 2x = 26) is one of the most commonly grown oilseed crops a seed production of more than 4.8 million tons worldwide in year 2013 (FAOSTAT, 2013) and has been suggested as the

most ancient oil crop (Nayar and Mehra, 1970). Its seeds are an important source of high-quality oil and contain natural antioxidants such as sesamin and sesamol (Zhang et al., 2013). Sesame is a good source of vitamins (pantothenic acid and vitamin E), minerals such as calcium (1.450 mg/100 g), phosphorous (570 mg/100 g) for human consumption and the seed cake is also an important nutritious livestock feed (Balasubramaniyan and Palaniappan, 2001).

It is mainly grown in tropical and subtropical regions of Asia, Africa and South America, in marginal lands or under very difficult conditions with drought, high temperatures, high solar radiation and high evaporation demand which make sesame a drought tolerant plant (Langham, 2007; Witcombe et al., 2007). Despite its tolerance, drought is one of the most important environmental factors that limit sesame production by affecting the number of capsules per plant, grain yield as well as oil yield and quality depending on the genotypes and drought intensity (Betram et al., 2003; Hassanzadeh et al., 2009; Bahrami et al., 2012). Recently, drought will be a serious threat in the coming decades as the Intergovernmental Panel on Climate Change (IPCC) has concluded that elevated greenhouse gas concentrations are likely to lead to a general drying of the subtropics by the end of this century, creating widespread drought stress in agriculture (IPCC, 2007). Therefore, improvement of drought tolerance in sesame genotypes is one of the major objectives of sesame breeding programs which can be achieved by integrating new approaches (Pathak et al., 2014). In the past years, many investigations have been carried out to enhance our understanding on the genetic basis of drought tolerance by using the genomics, transcriptomics and transgenesis approaches in the model plant *Arabidopsis thaliana* (Shinozaki et al., 2003; Jang et al., 2004, 2007; Ramirez et al., 2009; Lata et al., 2011; Harshavardhan et al., 2014). These studies showed that the main genes involved in drought tolerance were transcription factors (TFs). In *Arabidopsis*, about 1,500 TFs are considered to be involved in stresses response (Riechmann et al., 2000) including drought. So far, many drought associated genes have been identified including TFs belonging to basic leucine zipper (bZIP), AP2/EREBP, ABA-binding factor (ABF), MYC, MYB, NAM, ATAF1-2, NAC, CCAAT-binding and zinc-finger families and have been characterized in detail (Abe et al., 1997; Bartels and Sunkar, 2005; Sakuma et al., 2006; Nakashima et al., 2014; Mondini et al., 2012, 2015).

The availability of genome sequences in a number of plant species combined with comparative genomics analysis can improve our understanding of the fundamental aspects of plant biology including the identification and analysis of genes involved in adaptive traits of crops (Foucher et al., 2003). In fact, plant genomes share extensive similarities known as synteny, even between distantly related species (Guyot et al., 2012). Through comparative analysis against the *Arabidopsis* genome, many functional genomics regions and candidate genes such as flowering time *FLC* genes (Schranz et al., 2002), clubroot resistance genes (Suwabe et al., 2006), aliphatic glucosinolate biosynthetic pathway (Bisht et al., 2009) and genes for male fertility (Ashutosh et al., 2012) have been identified in *Brassica*. Similar strategies have been used to predict stress-responsive TFs in soybean, maize, sorghum, barley and wheat based on *Arabidopsis* and rice genome analyses (Mochida et al., 2009; Tran and Mochida, 2010b). In addition, alike analyses have been performed in tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*) two economically important and naturally drought sensitive crops (Li et al., 2013; Obidiegwu et al., 2015) belonging to the *Asteridae* subclass which includes sesame, leading to the identification of drought tolerant genes in these crops (Reiter and Vanzin, 2001; Vasquez-Robinet et al., 2008; Evers et al., 2010; Anithakumari et al., 2011, 2012; Solankey et al., 2014). Thus, it is well documented that using synteny approach in closely related species is suitable for the identifying orthologous genes (Rubin, 2001).

The identification of drought related candidate genes in sesame will provide useful information for its improvement. In the best of our knowledge no data have been reported regarding drought tolerance genes identified in sesame. Based on the sesame genome sequence recently released by Wang et al. (2014), a set of candidate genes in whole genome of sesame were identified in this study through homology search of known drought associated genes from three relatives species, viz. tomato, potato, and *Arabidopsis* and these genes were analyzed for further functional and validation experiments.

MATERIALS AND METHODS

Data resources

Arabidopsis thaliana genomic and annotation data were downloaded from the TAIR (http://www.arabidopsis.org) (Huala et al., 2001) while the potato and tomato genomes were downloaded from the Sol Genomics Network (http://www.solgenomics.net) (Fernandez-Pozo et al., 2015). In addition, the publicly known drought related

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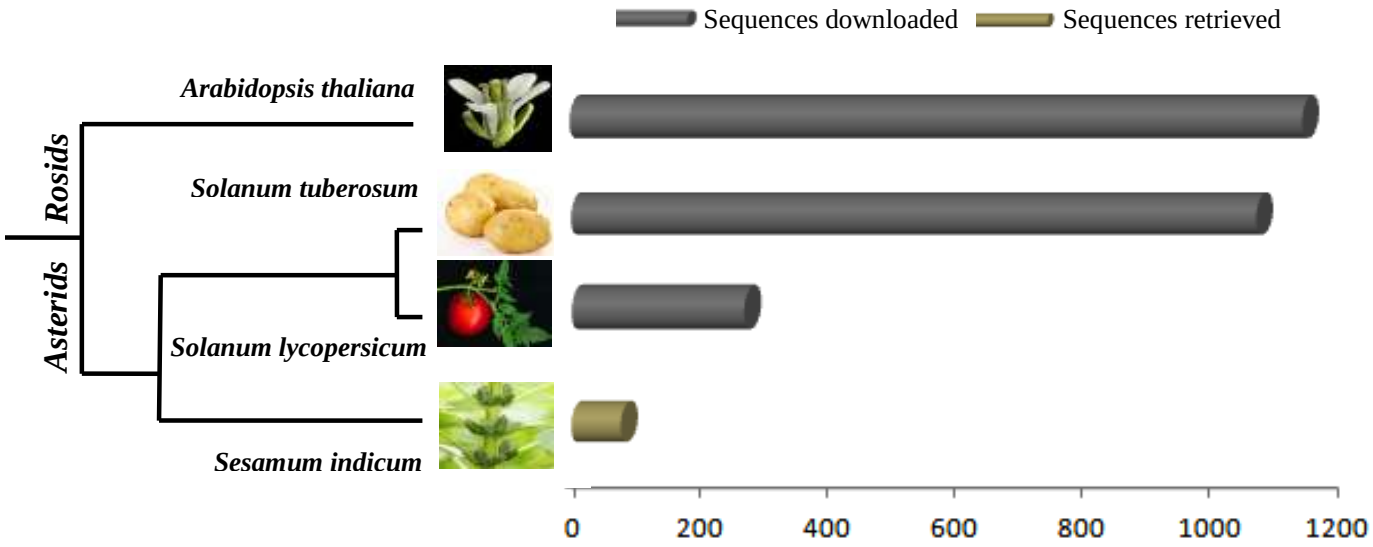

Figure 1. Phylogenetic relationships of the species studied and number of sequences downloaded.

genes and proteins sequences of these three species were downloaded from the Sol Genomics Networks, TAIR, NCBI database (http://www.ncbi.nlm.nih.gov) (Pruitt et al., 2007), and Drought Stress Gene Database (http://pgsb.helmholtz-muenchen.de/droughtdb/) (Alter et al., 2015). A total of 2,495 sequences including 1,150 from *Arabidopsis*, 1,075 from potato and 270 from tomato were downloaded. The phylogeny data of the four species were downloaded from NCBI Taxonomy common tree (http://www.ncbi.nlm.nih.gov/Taxonomy/CommonTree/wwwcmt.cgi) and a tree was constructed by MEGA 6.0 software (Tamura et al., 2013) as shown in Figure 1.

Comparative genomics and genes expression assay

A local bank with the retrieved sequences was generated in order to make searches for similar sequences against the sesame genome (Wang et al., 2014) using the BLASTn and tBLASTn algorithms (Altschul et al., 1990) for DNA and protein sequences, respectively with a cut-off of $1e^{-30}$. A threshold value of 70% identity was considered as significant level (Roy et al., 2011). After removing redundant genes, analyses of the candidate drought related genes in the whole genome of sesame were carried out including their identification, classification in functional groups, sequences analysis and chromosomal location.

The sesame genes with unknown functions were submitted to the AutoFACT program (Koski et al., 2005), and annotated according to the data available in the largest functional annotation databanks (KEGG, PFAM, SMART). The homologous genes found in the sesame genome were mapped onto the 16 Linkage Groups (LGs) according to their physical positions using MapChart 2.3 (Voorrips, 2002). The comparative orthologous relationships of the candidate drought associated genes among sesame and *Arabidopsis* were illustrated using Circos program (Krzywinski et al., 2009). To find out the whole genome AP2/ERF genes, the Hidden Markov Model (HMM) profile of the AP2/ERF domain (PF00847) obtained from Pfam v28.0 database (http://Pfam.sanger.ac.uk/) (Finn et al., 2014) was searched against the sesame proteome using Unipro UGENE (Okonechnikov et al., 2012).

Furthermore, a drought stress experiment was carried on to assess the expression of 6 AP2/ERF genes retrieved. For that, two contrasting sesame accessions (LC164-drought tolerant) and (hb168-drought sensitive), previously studied by Boureima et al. (2012), were sown in pots (25 cm diameter and 30 cm depth) filled with a mixture of soil, sand, and compost (5:2:2, v/v/v). The seedlings were grown and watered normally during 21 days before applying drought stress by withholding water for 5 days. At this stage, all plants were transferred under a plastic rain shelter. Total RNA of drought-stressed sesame seedlings were extracted from leaves using Trizol Reagent (Invitrogen, USA) according to the manufacturer's protocol and digested with DNase I (MBI, USA) to remove the genomic DNA contamination. One microgram RNA was reverse transcribed using the Reverse Transcription System (Promega). The semi quantitative reverse-transcription PCR (RT-PCR) amplification was carried out using gene specific primers (Table 1) and the cDNA libraries synthesized by using the following protocol: 4-min incubation step at 95°C for complete denaturation, followed by 30 cycles consisting of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and the final cycle at 72°C for 5 min. RT-PCR products were run on 2.0% (w/v) agarose gel, stained with ethidium bromide (5 µg ml^{-1}) and the expression of each gene was qualitatively evaluated after imaging under UV light (Kodak EDAS 290).

RESULTS AND DISCUSSION

The identification of drought tolerance candidate genes which have a high potential to be used for breeding drought tolerant crops presents a challenge (Krannich et al., 2015). For species with largely unexplored genomes such as sesame, comparative genomics is a promising tool to gain information by utilizing the conservation between closely related plant species (Akpinar et al.,

Table 1. Primers used for the RT-PCR.

Locus	Primer code	Forward sequence (5'-3')	Reverse sequence (5'-3')
LOC105155867	SSAp1	ATTTCCATCATCTAACCCTAAC	CCTCATCCGTATCCCTCTAT
LOC105165339	SSAp2	GAAAGCAAAGGCAACA	TATCTCGGCTACCCAC
LOC105160523	SSAp3	AGGGAGCCCAACAAGA	TTAGCATTCGCAGACG
LOC105162917	SSAp4	GTCGCCTTGTCCTTCTGT	TTCCTCGTTGTTGTCTGG
LOC105157874	SSAp5	CGATGACTGACGACGGAATG	GGTGAGGGTGCCCAACAA

2013). Hence, comparative genomics will broaden the ability to transfer information from model plants to other species that are fundamental to food production or as a source of alternative energy (Ma et al., 2012). From a total of 2,495 sequences downloaded from *Arabidopsis*, tomato and potato genomes, 75 candidate genes were found in the whole genome of sesame with high identity (Figure 1). Among these candidate genes, 42, 22 and 11 genes were homologous to *Arabidopsis*, potato and tomato genes, respectively. *Arabidopsis*, potato, tomato and sesame belong to the Asteridae subclass which includes nearly 60,000 species. Lee et al. (2005) reported that genes involved in adaptive processes tend to be highly conserved. Therefore, interspecies sequence comparison is a powerful tool to extract functional or evolutionary information from the genomes of organisms (Chiba et al., 2008). After functional annotation and classification, only 2 candidate genes with unknown functions remained (Table 2). The whole set of candidate genes could be classified into 2 main categories according to earlier reports on osmotic stress responsive genes in *Arabidopsis* (Seki et al., 2002, 2003): (a) genes which protect the plant against drought effect and (b) signal transduction genes and TFs. Drought tolerance is a quantitative trait that exhibits complex genetic control (Mc William, 1989). It greatly affects the plant both at the micro (that is, membrane structure), and at the macro level (that is, the physiology of the whole plant), with results that reflect the variety of responses involved in the acquisition of tolerance (Soares et al., 2012). The complexity of this trait explains why there is slow progress in crop improvement in drought-prone areas (Cattivelli et al., 2008).

The candidate genes were mapped onto the 16 Linkage Groups (LGs) in the sesame genome (Wang et al., 2014) (Figure 2). All LGs were represented and the distribution of these genes was globally uneven. However, some gene clusters existed on LGs 1, 3, 5, 6, 7, 8, 10 and 14. Recent works of Wei et al. (2015) on MADS gene family in sesame also find similar clustering patterns of some MADS genes along 14 LGs. In fact, there is evidence that functionally related genes tend to cluster more commonly than expected by chance (Boutanaev et al., 2002; Cohen et al., 2002). Our results suggested that these clustering regions of the genome might be highly active in drought tolerance in sesame. The maximum number of genes (10; 13.16%) was localized on LG 3, whereas LGs 13 and 16 have the lowest number of genes (1; 1.3%).

To trace orthologous relationships of the candidate genes associated to drought, the physically mapped candidate genes of sesame were compared with those of *Arabidopsis* since most of the genes in *Arabidopsis* have been functionally characterized (Figure 3). According to Wang et al. (2014), sesame and *Arabidopsis* share more than 2,200 homologous genes. Forty three orthologous gene pairs were detected between *Arabidopsis* and sesame, including 43 sesame candidate genes and 32 *Arabidopsis* drought associated genes. Most of the candidate genes in sesame showed syntenic bias towards the chromosomes 1, 2 and 4. The comparative mapping information offers a useful preface for understanding the evolution of genes among different species.

Many drought stress associated genes encode TFs that in turn control other various genes involved in diverse physiological and molecular responses to drought stress. TFs are therefore good candidates for genetic engineering to improve crop tolerance to drought because of their role as master regulators of clusters of genes (Rabara et al. 2014). AP2/ERF transcription factors are reported to be involved in drought stress in many plants (Licausi et al., 2010; Lata et al., 2014; Rabara et al., 2014). Whole genome scanning of AP2/ERF genes resulted in 132 putative AP2/ERF genes. Regarding the importance of this gene superfamily in abiotic stress tolerance in plants, many AP2/ERF genes were expected to be found among the set of candidate genes identified in this study. Only five candidate AP2/ERF genes (3.8%) were retrieved as probably associated to drought tolerance in sesame (Figure 4), suggesting that these AP2/ERF genes should be targeted for drought research in sesame. According to the classification of Sakuma et al. (2002), the AP2/ERF genes found in this study, were classified into the ERF (2), AP2 (2) and DREB (1) subfamilies, respectively.

Although, the power of similarity-based gene discovery at a genome scale has been demonstrated in many works and partially reviewed by Windsor et al. (2006), the importance of functional characterization cannot be

Table 2. List and functions of orthologous genes retrieved from sesame genome.

Homologous species	Gene ID (Genbank)	Linkage group (LG)	Start (bp)	End (bp)	Predicted Function
Arabi-dopsis	LOC105166387	7	5906451	5910956	ATPase 8, plasma membrane-type
	LOC105162305	5	10204583	10209025	ATPase 8, plasma membrane-type
	LOC105167840	8	7731490	7737270	protein cellulose synthase A catalytic subunit 8
	LOC105172646	10	15149055	15149891	Squalene monooxygenase-like
	LOC105162306	5	10212601	10213008	WD repeat-containing protein 76
	LOC105158687	3	18059219	18059416	Importin-7 homolog
	LOC105163505	6	3064706	3064831	U6 snRNA-associated Sm-like LSm5
	LOC105166431	7	6449340	6449752	Pyrophosphate-energized vacuolar membrane proton pump-like
	LOC105172598	10	14826310	14826859	Chlorophyll a-b binding CP24 10A
	LOC105169208	8	18023545	18024092	Chlorophyll a-b binding CP24 10A
	LOC105165339	1	12753457	12753552	AP2/ERF
	LOC105155867	2	8573570	8573654	AP2/ERF
	LOC105155595	2	8022269	8022013	Zinc finger protein ZAT10-like
	LOC105159020	3	19550162	19550800	Annexin D1-like
	LOC105157342	3	5932731	5932993	Protein argonaute 1
	LOC105178119	15	6916417	6916015	RING-H2 finger protein ATL78-like
	LOC105156611	2	17750487	17750029	Galactinol synthase 1-like
	LOC105163012	6	238678	238355	Galactinol synthase 1-like
	LOC105172447	10	13338815	13338497	Galactinol synthase 1-like
	LOC105159079	3	21325879	21325607	Squalene epoxidase 1-like
	LOC105162196	5	7272028	7271749	Squalene monooxygenase-like
	LOC105164049	6	7911616	7911192	Respiratory burst oxidase protein A
	LOC105167614	8	3228940	3229962	LRR receptor-like serine/threonine-protein kinase RPK2
	LOC105177329	15	1389614	1390636	LRR receptor-like serine/threonine-protein kinase RPK2
	LOC105176001	2	867511	868042	ABC transporter G family member 25
	LOC105165477	6	22166960	22167350	Alpha,alpha-trehalose-phosphate synthase
	LOC105176909	14	3817870	3818260	Alpha,alpha-trehalose-phosphate synthase
	LOC105164867	1	12396709	12395749	Glycerol kinase
	LOC105162838	5	17176580	17177344	Plasma membrane ATPase 4-like
	LOC105164940	6	18440702	18441474	Plasma membrane ATPase 4-like
	LOC105176433	13	4333541	4333880	Homeobox-leucine zipper protein ATHB-6
	LOC105163763	6	4928626	4929458	Indole-3-acetaldehyde oxidase
	LOC105165510	6	22408587	22407052	9-cis-epoxycarotenoid dioxygenase NCED1, chloroplastic
	LOC105176883	14	3512386	3513850	9-cis-epoxycarotenoid dioxygenase NCED1, chloroplastic
	LOC105178586	16	2456138	2458102	Protein far-red elongated hypocotyl 3 isoform X2
	LOC105167986	8	8723674	8724107	Calcium-dependent protein kinase-like

Table 2. Contd.

	LOC105175346	12	3311638	3311229	Calcium-dependent protein kinase-like
	LOC105167437	8	2135795	2137643	Far-red impaired response 1
	LOC105175707	12	5079580	5079898	Potassium channel KAT3-like
	LOC105165006	6	18910517	18910130	Abscisic acid 8'-hydroxylase 1-like
	LOC105157462	1	530090	530409	Abscisic acid 8'-hydroxylase 1-like
	LOC105160523	4	10290113	10291144	DREB
	LOC105158557	3	16161829	16162134	Phosphoenolpyruvate carboxykinase [ATP]-like
	LOC105156478	2	16843822	16844116	Alpha-1,4-glucan-protein synthase
	LOC105177713	15	3453387	3454069	Oxygen-evolving enhancer protein 1
	LOC105167477	8	2684789	2685468	Oxygen-evolving enhancer protein 1
	LOC105165268	1	12691700	12692388	Heat shock 70 kDa protein-like
	LOC105155749	2	10818136	10818702	40S ribosomal protein S2-4-like
	LOC105162725	5	16732816	16733391	40S ribosomal protein S2-4-like
	LOC105171901	10	4259842	4260638	Heat shock cognate 70 kDa
	LOC105177235	15	867494	868130	Tubulin alpha chain
	LOC105170695	9	5296418	5296884	Elongation factor 1-alpha
	LOC105160899	4	13022275	13022673	60S acidic ribosomal protein P0
Potato	LOC105174800	12	387677	387970	60S ribosomal protein L8-1
	LOC105176744	14	1569814	1570107	60S ribosomal protein L8-1
	LOC105170962	9	7060127	7060576	Ethylene insensitive 3
	LOC105157101	3	2740513	2741378	Geranylgeranyl diphosphate reductase
	LOC105168390	8	12427153	12427919	NADH dehydrogenase Flavoprotein 1
	LOC105167993	8	8776046	8776600	Aminomethyltransferase
	LOC105168993	8	16465020	16465871	S-adenosylmethionine synthase 1
	LOC105172459	10	13464015	13464776	S-adenosylmethionine synthase 1-like
	LOC105174259	11	13028743	13029504	S-adenosylmethionine synthase 3
	LOC105174436	11	13956861	13957724	heat shock cognate 70 kDa protein 2
	LOC105161464	5	777968	778829	heat shock cognate 70 kDa protein 2
	LOC105176719	14	1410275	1412327	Polyubiquitin
	LOC105165880	7	15613	16461	Unknow function
	LOC105165878	7	5147	6771	Unknow function
Tomato	LOC105158900	3	19877583	19881182	Protein hypoxanthine-guanine phosphoribosyl transferase
	LOC105161893	5	3608867	3609445	Senescence-specific cysteine protease SAG39-like
	LOC105166264	7	4914769	4915824	Abscisic acid-insensitive 5
	LOC105159163	3	22078807	22079856	Abscisic acid-insensitive 5

Table 2. Cont.

LOC105174582	11	14789561	14790058	Monothiol glutaredoxin-S16
LOC105165958	7	2367785	2368681	Thioredoxin-like protein CDSP32
LOC105157874	3	9547915	9547019	ERF RAP2-12-like
LOC105162917	1	11232516	11233406	ERF RAP2-12-like

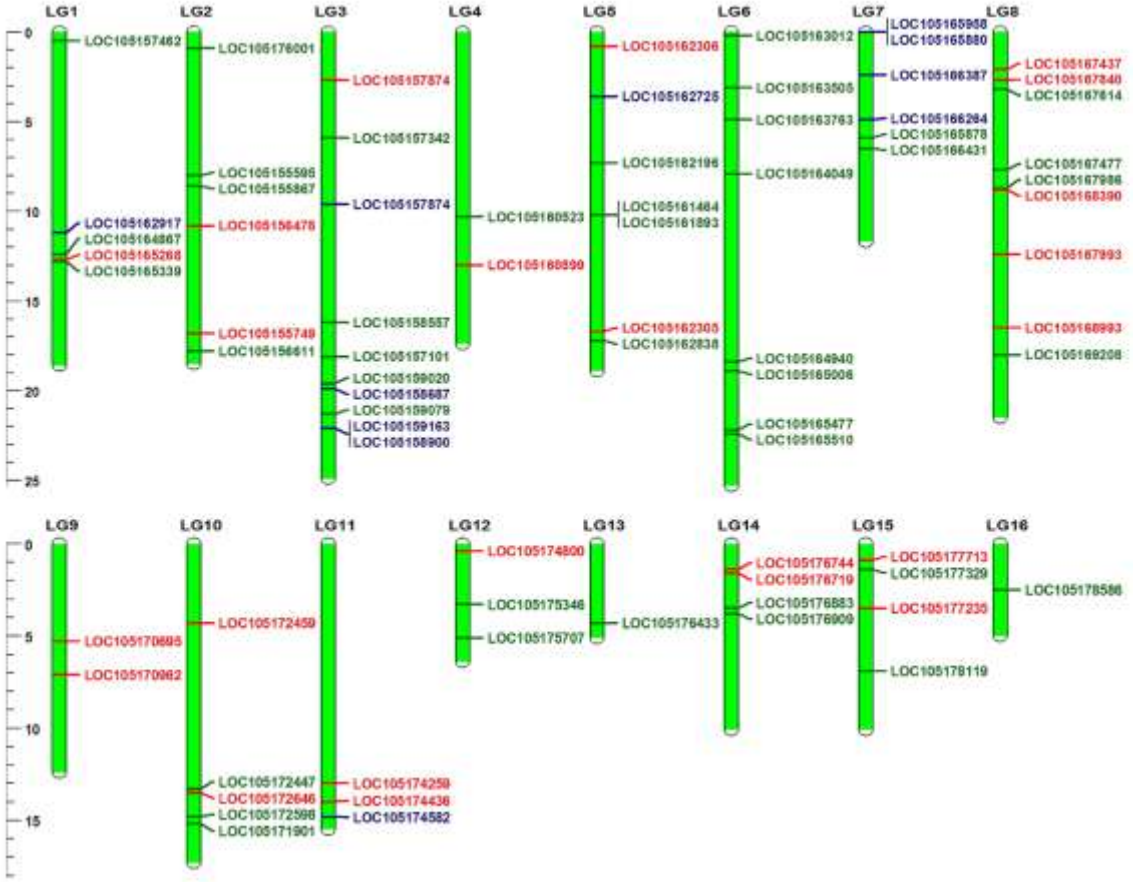

Figure 2. Distribution of the candidate genes on the sesame linkage groups. LG1~LG16 represent linkage groups of the sesame genome. Locus names in green, blue and red indicate orthologous genes of *Arabidopsis*, tomato and potato, respectively.

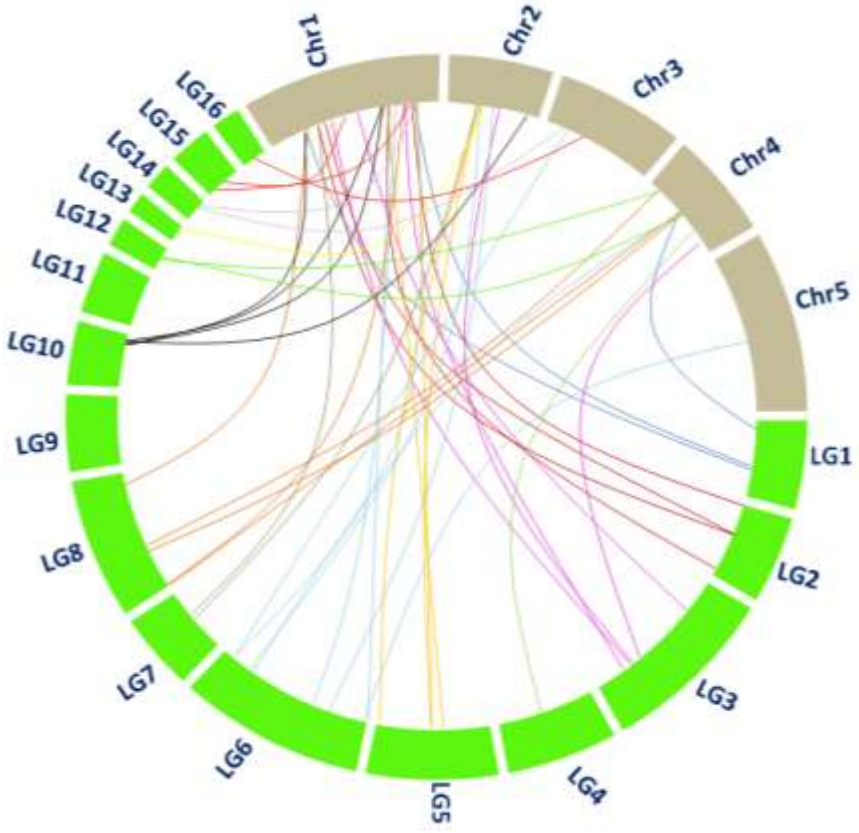

Figure 3. Syntenic relationships of drought associated genes between *Arabidopsis* and sesame genomes. Chr1~Chr5 represent pseudo-chromosomes of *Arabidopsis* genome and represented by gray bars. LG01~LG16 represent linkage groups of sesame genome and drawn in green bars. Colorful lines stand for the relationships of orthologous gene pairs between the two species.

ignored. An expression profiling of these AP2/ERF genes under drought stress in two contrasted sesame lines through RT-PCR were further performed. Gene expression patterns are usually closely correlated with their functions (Peng et al., 2015). One primer pair (SSAp5) designed for the gene LOC105157874 did not amplify any of the two accessions suggesting probably an inadequate primer. Three out of the four remaining genes expressed highly in the drought tolerant material compared to the sensitive one under drought stress (Figure 5). The expression level of the gene LOC105160523 was more striking in drought tolerant material compared to the sensitive one whereas the gene LOC105162917 showed similar expression pattern under both water regimes. The BLASTp search against *Arabidopsis* genome showed that the highest expressed gene LOC105160523 is the orthologs of CBF4 (*AT5G51990*) described as regulator of drought adaptation in *Arabidopsis* (Haake et al., 2002) suggesting that this gene plays a pivotal role in drought tolerance in sesame. Since, few sesame accessions have been used in this study, it was proposed that these genes should be more deeply studied on a large sample of contrasted materials to uncover their biological roles in drought tolerance in sesame. Our results corroborate well with that of Kamvysselis (2003), who reported that comparative genomics analysis can reveal biological findings that could not have been discovered by traditional genetic methods, regardless of the time or effort spent.

Sesame is an oil crop that contributes to the daily oil and protein requirements of almost half of the world's population (Wei et al., 2015). One of the major constraints for its production is drought as it is mainly grown in semi-arid areas. Functions of most sesame genes are still uncharacterized. Hence, the identification and functional analysis of valuable genes in sesame genome is necessary for its improvement. Since reports on drought associated genes in sesame are lacking, this study provided, a set of candidate genes spanning the whole genome and including different functional genes for drought research in sesame using comparative

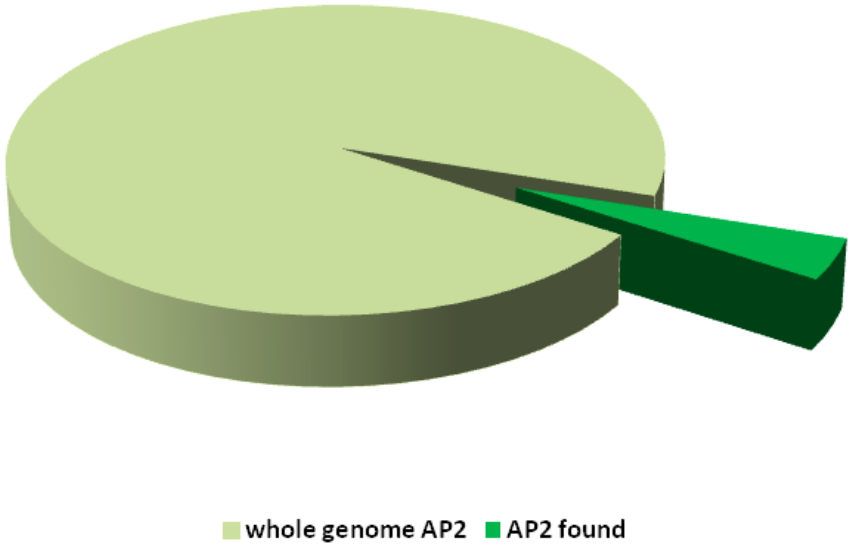

Figure 4. AP2/ERF transcription factor genes found within the candidate genes compared to whole genome AP2/ERF genes.

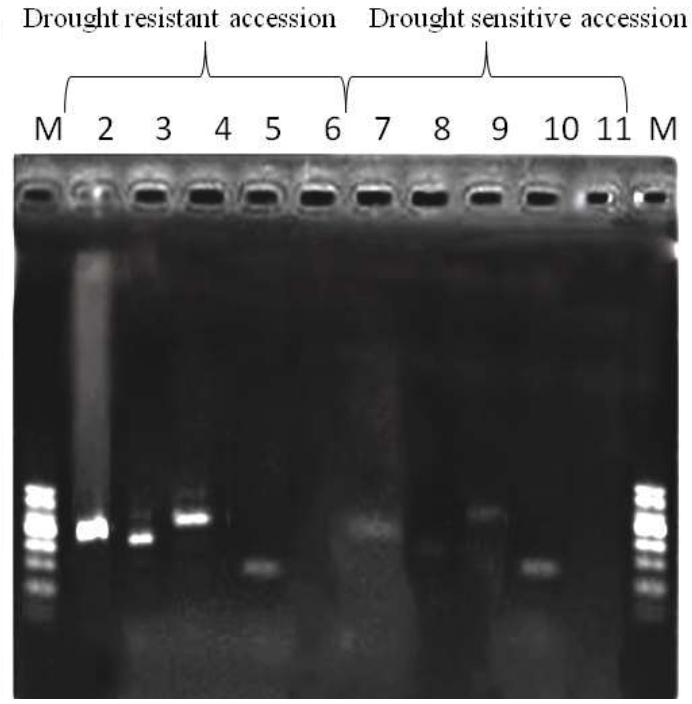

Figure 5. Expression profile of five AP2/ERF genes in two contrasted sesame accessions under drought stress. M represents the migration profile of the ladder; 2 and 7- SSAp3; 3 and 8- SSAp2, 4 and 9- SSAp1, 5 and 10- SSAp4, 6 and 11- SSAp5.

genomic approach. Further and thorough functional experiments including transgenic studies could rely on these gene resources to validate their functions and decipher the mechanisms of drought tolerance in sesame.

Conflict of interests

The authors have not declared any conflict of interests.

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Effect of substrate on the growth, nutritional and bioactive components of *Pleurotus ostreatus* and *Pleurotus florida*

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Mushrooms are increasingly being recognized as important food products for their significant role in human health, nutrition and disease. This study was carried out with the aim of comparing the effect of substrate on growth, determining nutritional and bioactive components of two oyster mushroom, *Pleurotus ostreatus* and *Pleurotus florida*. A completely randomized block design with two treatments replicated three times was done and a laboratory analysis was carried out on the nutritional and bioactive components. The results obtained indicated that the growth and yield of *P. ostreatus and P. florida* varied widely depending on the kind of substrate used. It was observed that sawdust had the greatest influence on both growth and total yield because it had mean height, weight and pileus length significantly higher than those cultivated on corn cobs. It took least days for pin head formation and maturity period but had the highest number of fruiting bodies produced. *P. ostreatus* had the highest weight, height and biological yield while *P. florida* had the least pin head formation, maturity period and number of fruiting bodies. The biological efficiency and moisture content was the highest for sawdust substrate as a whole. The substrate had an effect on the nutritional value as maximum protein (29.45%) was observed on sawdust while minimum (25.12%) on corn cobs. Maximum lipid (4.62%) was observed on corn cobs while minimum (1.97%) was still on corn cobs. Regarding substrate, maximum ash (8.67%) was observed on sawdust while minimum (8.215%) on corn cobs; also maximum crude fibre (16.69%) was observed on corn cobs while minimum (5.08%) was on sawdust. The bioactive components analysis revealed the presence of major bioactive compounds such as flavonoids, polyphenols, saponins, triterpenoids and steroids. Oyster mushroom cultivated on sawdust possesses better growth and nutritional properties than those cultivated on corn cobs.

Key words: Bioactive components, cultivation, nutritional analysis, oyster mushroom.

INTRODUCTION

Mushroom has been defined as a macro-fungus with a distinctive fruiting body, which can be hypogenous or epigeous, large enough to be seen with the naked eye and to be picked by hand (Chang and Miles, 1989).

Oyster mushroom (*Pleurotus* species) belongs to the family of Tricholomataceae and is usually found clustering naturally on dead trees at spring season (Lee, 1993). Among all species of mushroom, the oyster mushroom is the second widely cultivated mushroom worldwide following the *Agaricus bisporus* (Kües and Liu, 2000). *Pleurotus* spp. are popular and widely cultivated throughout the world mostly in Asia, America and Europe because of their simple, low cost production technology and high biological efficiency (Mane et al., 2007).

Mushrooms are increasingly being recognized as important food products for their significant role in human health, nutrition and disease. Several species of mushrooms are of great importance because of their medicinal properties, for example, they are active against hypercholesterolemic conditions, hypertension, diabetes, cancer and other infections (Alam et al., 2007). The nutritional and chemical compositions of mushroom are responsible for their medicinal values. However, nutritional composition of mushroom is affected by many factors among which the composition of growth substrate and the method of cultivation are of major importance (Benjamin, 1995). During an investigation of the cultivation of mushroom on agricultural residues, it was found that rice husk, sorghum stover, saw dust, cotton waste, cocoa bean shell, and saw dust such as *Gliricidia* mixture were suitable substrates for the cultivation of edible mushroom (Belewu, 2003). Various substrates have different effects on the growth, yield and quality of mushrooms (Ponmurugan et al., 2007).

The genus *Pleurotus* is a heterogeneous group of economic importance. Several species are of nutritional and/or medicinal importance (Cohen et al., 2002). *Pleurotus* spp. have the ability to absorb microelements from different cultivation media and thus they may present an excellent dietary source (Stajic et al., 2002). Fungi of the *Pleurotus* genus have an important place among the commercially cultivated *Basidiomycetes*, because they have gastronomic, nutritional and medicinal properties and can be easily cultivated on a large range of substrates (Kumari and Achal, 2008).

Mushrooming in Cameroon rainforest zones is often possible only during the rainy season and is usually inefficient in terms of time spent to collect sufficient mushroom. Most edible species rot quickly and collector must be at the right time at the right place. Hence, there is a need for a cultivation of mushroom for lasting availability all year round. Also, there is an inadequate food supply in most rural areas, diminishing quality of health and increasing environmental deterioration. Many children and people in Cameroon are malnourished and most families cannot afford meat in their daily meal, there is also high prevalence rate of HIV/AIDS, malaria, and tuberculosis amongst others. Hence, mushrooms can help to improve health and nutrition. When used as food, mushrooms promote good human health, being rich sources of protein and vitamins (Kinge et al., 2014).

Also, there is a very high incidence of malnutrition, especially of protein deficiency in most developing countries. This study would help to provide the community with an additional vegetable of high quality and enrich the diet with high quality proteins, minerals and vitamins which can be of direct benefit to the human health and fitness. The extractable bioactive compounds from medicinal mushrooms would enhance human's immune systems and improve quality of life. This study will serve as means of generating employment, particularly for rural women and youths in order to raise their social status. The harvested fruiting bodies can be sold in local markets for additional family income or exported for an important source of foreign exchange that will definitely improve the economic standards of the people in and around the study area. Hence, this study is very important because it takes little time, less energy and the use of wastes to provide a good and nutritious mushroom that is also medicinal. The aim of this research was to investigate the effect of two substrates on the growth, nutritional and bioactive components of two oyster mushroom species.

MATERIALS AND METHODS

Study area

This work was carried out in the Mbeng Adio Mushroom Farm, Banjah village; in Bamenda III sub division of the Mezam division in the North West Region of Cameroon. It is located between latitude 5° 57'23.72 and longitude 10° 13'06.86. The climatic variations of this area fit into two seasons (the dry and rainy season), and experience the tropical rain forest climate in general. Thus this area has normal rainfall like all other villages in Bamenda III sub division as a whole which the rains begin in February or March ending up to October and the dry season which runs from October to February or March ending. Although places are normally wet during this period, the temperatures are relatively warmer (28 to 30°C) favouring the cultivation of mushroom. People in this area carry out agricultural activities in which most of them carry subsistence agriculture, cultivating crops like cocoyam, Irish potatoes, corn, beans, huckleberry and some engaged in the mushroom cultivation with little or no knowledge on the agro waste that would give the best quality and productivity. Since most of the people in this area are farmers, mushroom should be encouraged, since the main substrates needed in the cultivation of mushroom is agriculture wastes. The map of the study area is as shown in Figure 1.

Collection and preparation of samples

The spawn was bought from the Mbeng Adio Mushroom farms, Banjah village, Bamenda of the North West Region of Cameroon.

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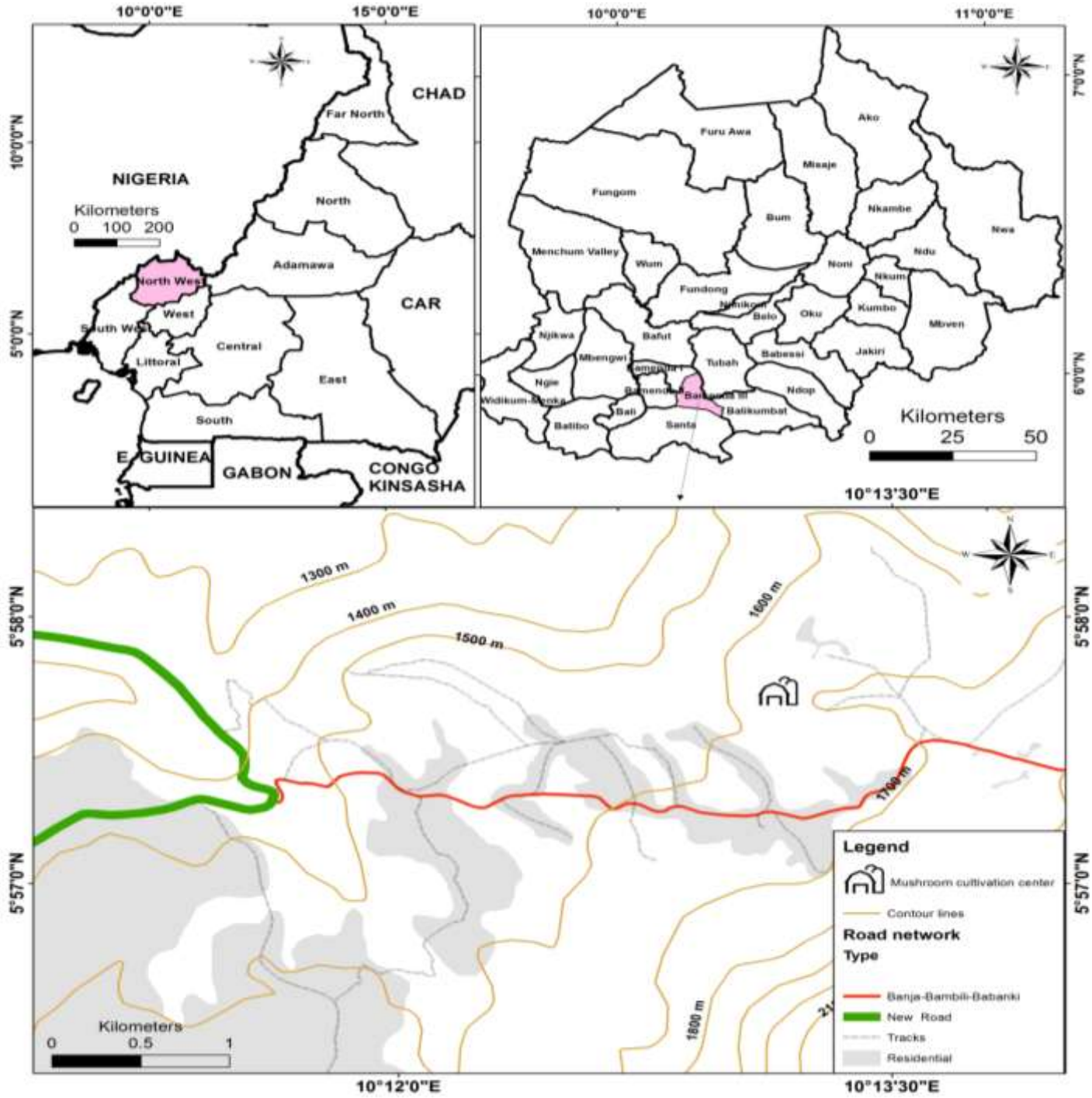

Figure 1. Locational map of Mushroom Research Center in Banjah, Cameroon.

The sawdust was collected from a saw mill in Nkwen, Bamenda and was identified to be from Eucalyptus. Corn cobs were collected from household in Banjah village. The rice husk was collected from church centre rice mill while the corn flour was collected from a milling machine in Bamenda town. Cultivation bags, slake lime were bought from a provision store in Bamenda. The sawdust and corncobs were well selected to remove physical contaminants such as sticks, stones, plastic paper and rotten corn cobs. The corn cobs were further crushed into smaller pieces using a crusher. The materials were further weigh and taken to the mixing room.

Spawn preparation

Two buckets of sawdust, dried corn flour, rice husk and slake lime were mixed using a cleaned spade. Water was added to 65%

Table 1. Treatments, substrates and their composition used in the cultivation *of P. ostreatus* and *P. florida*.

S/N	Treatment	Substrates and composition
1	Treatment-1	Corn cob+corn flour = 3:1
2	Treatment-2	Sawdust + rice husk + corn flour = 6:3:1

moisture content. The mixture was put in bottles, wiped, covered and sterilized for 2 h in an oil drum. The bottles were removed from the drums after 6 h and allowed to cool. The bottles were then transferred to the inoculation room where they were sent to the inoculation box and they were inoculated with the mother culture bought from Mbeng Adio Mushroom farms Bamenda. The inoculated bottles were then transferred to a room for maturity. It took the spawn 25 days to mature. The spawn were then used in the inoculation of the substrates.

Mushroom cultivation

Cultivation of the two species of oyster mushrooms *Pleurotus ostreatus* and *Pleurotus florida* on two different substrates was done following the procedure of Anagho (2008). The cultivation experiment was laid out in a completely randomized design with two treatments replicated three times. The composition of the different treatments is shown in Table 1.

Shavings and pieces of wood that were too big or too sharp were removed by hand from the saw dust because these pieces absorb water poorly and easily pierce plastic bags during handling. Rice husk and corn flour wastes were supplemented as organic nitrogen sources. Slake lime was diluted with water, and then showered onto the sawdust to adjust pH. The following are the cultivation of the different treatments.

Treatment 1

In treatment 1, three 15 L buckets of corn cob was measured using a scale. The weight of each bucket of crushed corn cobs was 3 kg (total of 9 kg). One bucket of corn flour was measured using 15 L bucket and weight as 8 kg. 30 g of powdered calcium carbonate was measured and added to the mixture. The three ingredients were then mixed dry using a spade until a homogenous mixture was obtained to about 65% moisture content. The moisture content was tested by squeezing the mixture in the palms of the hands. After testing the moisture content, calcium carbonate was then applied on the hand to avoid microbial contamination and substrates was then filled in black polythene bags (25 × 18 cm) using the hand. The substrate was pressed in the bag while filling. The bag was filled such that each bag weight as 2 kg. The bag was tight with a knob so that it could be easily untied later. These bags were then transferred to the sterilization unit.

Treatment 2

In treatment 2, six buckets of volume 15 L sawdust of fine type was measured using a measuring scale balance; the weight of each of the bucket of saw dust was 4 kg (total 24 kg). Three buckets of rice husk was measured in 15 L buckets, each weigh 1.8 kg (total of 5.4 kg); one bucket of the corn flour was also measure using 15 L bucket and weight of bucket was 8 kg. The measured corn flour and rice husk was added to the measured sawdust. 30 g of calcium carbonate was measured and added to the mixture of sawdust, rice husk and corn flour. The four components were homogenously mixed using a spade. The mixture was done on a cemented floor that has been well cleaned. Water was then added to the homogenous mixture to about 65% moisture content. The moisture content was tested by squeezing the mixture in the palms of the hands. After testing the moisture content, calcium carbonate was then applied on the hand to avoid microbial contamination and substrates was then filled in black polythene bags (25 × 18 cm) using the hand. The substrate was pressed in the bag while filling. The bag was filled such that each bag weight as 2 kg. The bag was tight with a knob so that it could be easily untied later. These bags were then transferred to the sterilization unit.

Sterilization of substrates

A tripod was placed in the 1000 L oil drum such that it occupied 1/5 of the height of the drum. This was done to prevent direct contact of substrates and water. Water (15 L) was then poured into the drum and jute bags were placed on top of the tripod and around the walls of the drum. The polythene bags containing the substrates were then paced in the drum. Twelve substrates were placed round the drum and three each in the middle up till the drum got full. Each drum carried a total of 60 polythene bag substrates for sterilization. The drum was then covered on the top with jute bags and plastic papers to prevent heat from escaping. Wood was used to set up heavy flames on which the substrate was sterilized (at about 100°C) for 4 h. Sterilization of substrate bags was done under high temperature using an oil drum with fuel from wood. The substrate had enough heat and started boiling after 30 min. From the boiling point, the substrate was sterilized (at about 100°C) for 4 h in order that all other microorganisms were destroyed and the substrate well cooked. The sterilization took place in the evening and bags were allowed to cool overnight and removed the next day ready for spawning.

Spawning or inoculation

The bags were then removed from the oil drum and transferred to the spawning room. The first step of spawning was washing of hands and knives to be used. The bags were then untied and allowed to cool. The spawns were introduced using a knife to avoid contamination. 20 bottles of spawns weighing 750 g each were used to plant 60 substrates. The substrates were planted at a ratio of 1 bottle to 3 substrates of 2 kg (1:3). The spawn was introduced deep into the substrates and was well mixed with the help of the knife such that the spawn covered almost all the whole substrates. The bags were then tied and transferred to a room with shelves.

The mushroom substrates were stored in a dark room for colonization to take place. The substrates were placed on shelves that had been painted with calcium carbonate and the window was covered with black polythene bag to reduce the light in the room. The substrates were left in this dark room for 21 days at 25°C. Spawn run for mushroom differ from species to species, the size of the polythene bag, the colour of the bag and the nature and compactness of substrates. After the incubation period of 21 days, the windows were open for proper ventilation. At the beginning of fruiting, the substrates were transferred to a fruiting room. The mushroom basidiocarps were seen to shoot in different direction from each of the substrates. The number of days taken for the initiating of primordial and harvesting was noted for the different substrate mixture and species. To maintain proper temperature, moisture and humidity, the room was watered daily and by watering the bags one after each harvesting by pouring water on the bags.

Harvesting

Harvesting was carried out when the fruiting bodies were matured.

Harvesting was made three times for each bag of substrate. The process of harvesting involves the removal with the hand of the matured fruiting bodies from their substrate without any destruction on the substrate bag. The mature mushroom was held on their stipe below the pileus and close to the substrate level and was gradually pulled out. All fruiting bodies of a particular substrate bag were harvested at the same time since each bag had to be watered after harvest. Watering was done by immersing the bags in a bowl of water for 5 s. This is to enable the substrate to have moisture that enables fruiting to occur again for harvest.

Morphological data collection

The growth and yield of *P. ostreatus* and *P. florida* on the different substrate was determined by recording the number, weight and size of the fruit bodies after sprouting. The measurements from the various replicates were added and their mean value calculated.

The following parameters of growth and yield were measured. From three bags of each treatment and replicates of the two species were sampled by measuring the growth and yield parameters thus.

Biological yield

The biological yield was obtained by taking the total fresh weight of the fruiting bodies per bag. The average for the three bags per treatment and per replicate were calculated and recorded.

Biological efficiency

It was calculated as the weight of fresh mushroom as a percentage of the dry weight of the substrate.

Time required for primordial initiation

Three bags for a particular treatment were sampled at random after the incubation process completion. These three bags were observed on daily basis to note the number of days it took after incubation to the formation of the first primordial.

Time required for harvest

Time taken for harvest was done for the three sample bags from initiation stage to the time of maturation of fruiting body. The average of the time taken for primordial initiation and the time taken for harvesting were calculated and recorded.

Number of total primordial

After the primordial formation, the number of primordial was counted for each of the three sample bags and the average for the three bags were calculated and recorded.

Number of total effective fruiting body

The number of effective mature fruiting body was counted just before harvesting was done for the three sample bags. The average number of fruiting bodies for the three bags were calculated and recorded.

Weight of individual fruiting body

The weight of the individual fruiting bodies per bag for the three sample bags were measured using a scale balance. The average of the three bags were calculated and recorded. The weight taken was the fresh weight.

Height of fruit bodies

This entails measuring the distance from the substrate where the stalk starts growing. The height was measured in centimeters using transparent ruler from the base of the stipe to the pileus. The height of the stalk was carried out for at least three fruiting bodies for each of the three bags of the particular treatment. The average height was recorded and calculated. Morphological data were subjected to student t test to see if there is any significant difference in the growth substrates used or the species used.

Nutritional analysis

A laboratory analysis (AOAC) was done in the Nutritional Laboratory in the University of Dschang, Cameroon to compare composition of nutrient in *P. ostreatus* and *P. florida* cultivated on the two substrates. The data was recorded on moisture, crude protein, crude fiber, crude fat, ash, organic matter and dry matter according to Raghuramulu et al. (2003).

Moisture analysis

Twenty gram of fresh mushroom was weighed into a weighed moisture and dried in an oven at 100 to 105°C and cooled in a dessicator. The process of heating and cooling was repeated till a constant weight was achieved. The moisture content of mushroom was also expressed in percent and calculated by the formula;

Moisture content (%) = Weight of fresh sample – Weight of dry sample × 100 Weight of fresh sample

Determination of total protein

Five grams of ground mushroom was taken with 50 ml of 0.1 N NaOH and boiled for 30 min. The solution was cooled at room temperature and centrifuged at 1000 × g by a DSC-200T tabletop centrifuge. The supernatant was collected and total protein content was measured according to the method of Lowry et al. (1951).

Determination of total lipid

Total lipid was determined by slightly modifying the method of Folch et al. (1957). Five grams of ground mushroom was suspended in 50 ml of chloroform: methanol (2: 1 v/v) mixture then mixed thoroughly and let stand for 3 days. The solution was filtrated and centrifuged at 1000 g by a table centrifuge machine. The upper layer of methanol was removed by Pasteur pipette and chloroform was evaporated by heating. The remaining was the crude lipid.

Determination of crude fiber

Ten grams of moisture and fat-free sample were taken in a beaker and 200 ml of boiling 0.255 N H_2SO_4 was added. The mixture was boiled for 30 min keeping the volume constant by the addition of water at frequent intervals. The mixture was then filtered through a muslin cloth and the residue washed with hot water till free from acid. The material was then transferred to the same beaker, and 200 ml of boiling 0.313 N NaOH was added. After boiling for 30 min

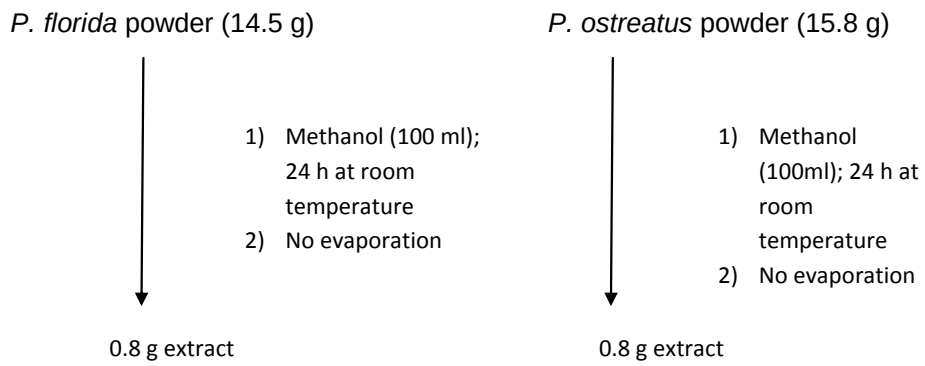

Figure 2. The extraction method.

(keeping the volume constant as before), the mixture was filtered through a muslin cloth and the residue washed with hot water till free from alkali, followed by washing with some alcohol and ether. It was then transferred to a crucible, dried overnight at 80 to 100^{o}C and weighed (We) in an electric balance (Keyi: JY-2003; China). The crucible was heated in a muffle furnace (Nebertherm: Mod-L9/11/c6; Germany) at 600°C for 5 to 6 h, cooled and weighed again (Wa). The difference in the weights (We - Wa) represents the weight of crude fiber. Crude fiber (g/100 g sample) = [100 − (moisture + fat)] × (We - Wa)/Wt of sample (Raghuramulu et al., 2003).

Determination of total ash

One gram of the sample was weighed accurately into a crucible. The crucible was placed on a clay pipe triangle and heated first over a low flame till all the material was completely charred, followed by heating in a muffle furnace for about 5 to 6 h at 600°C. It was then cooled in a dessicator and weighed. To ensure completion of ashing, the crucible was then heated in the muffle furnace for 1 h, cooled and weighed. This was repeated till two consecutive weights were the same and the ash was almost white or grayish white in color. Then total ash was calculated as: Ash content (g/100 g sample) = Weight of ash × 100/Weight of sample taken (Raghuramulu et al., 2003).

Total carbohydrate estimation

The content of the available carbohydrate was determined by the following equation:

Carbohydrate (g/100 g sample) = 100 − [(moisture + fat + protein + ash + crude fiber) g/100 g] (Raghuramulu et al., 2003).

Detection of bioactive components

The bioactive component analysis was done using standard procedures (AOAC, 1984).

Preparation of extracts

After collection, the mushrooms samples were wrapped in newspaper and stored in moisture free open places. The removal of all foreign matters was done. Thereafter they were cut in small pieces of around 2 to 3 cm across using a knife. They were incubated for 2 days at a temperature of 50°C. Then, they were ground using metal mortar and pestle. The powder was collected and ground again at the end. The extraction was done as shown in Figure 2. The bioactive components of oyster mushroom were determined using standard procedures (Sofowora, 1982; Trease and Evans, 1983; Sofowora, 1993).

Test for saponin

Sample (0.5 g) was weighed in a beaker; 5 ml of distilled water was added and heated to boil. Persisted foaming on warming was taken as an evidence for the presence of saponin.

Test for alkaloid

Sample (0.5 g) was weighed in a beaker and it was extracted with 10 ml of 2% hydrochloric acid (HCl) by heating gently for about 5 min. The HCl extract was filtered with Whatman No.1 filter paper to have a clear solution and prevent false result; 2.5 ml of the filtrate was treated with few drops of Dragendoff's reagent. Appearance of precipitate indicated the presence of alkaloid in the extract.

Test for flavonoids

This was done using the Shinoda test (Magnesium Hydrochloride reduction test). To the test solution, few fragments of magnesium ribbon were added and concentrated HCl was added drop wise, pink scarlet, crimson red or occasionally green to blue colour appears after few minutes indicated the presence of flavonoids.

Test for triterpenoids and steroids

The Libermann Burchard's test was used. The extract was treated with few drops of acetic anhydride, boiled and cooled. Concentrated sulphuric acid was added from the sides of the test tube, showing a brown ring at the junction of two layers and the upper layer turning green shows the presence of steroids and formation of deep red colour indicated the presence of triterpenoids.

Test for phenolic compound and tannins

Ferric chloride test

To 5 ml of the extract, few drops of neutral 5% ferric chloride

Table 2. Biological yield determinants on three flushes of *Pleurotus ostreatus* and *Pleurotus florida*.

Growth parameters and substrate	Flush 1 on P. os	Flush 1 on P.fl	Flush 2 on P.os	Flush 2 on P.fl	Flush 3 on P.os	Flush 3 on P.fl	Mean on P.os	Mean on P.fl	t value and significance
Mean height on sawdust	39.1	22.5	38.2	22.0	31.3	19.7	36.3	21.3	9.226*
Mean height on corn cobs	25.2	19.8	18.0	19.2	15.3	16.0	19.5	18.3	0.5499^{ns}
t value and significance	-	-	-	-	-	-	9.017*	9.6442*	-
Mean weight on sawdust	410.0	238.4	316.7	211.6	176.6	200.0	301.1	216.7	1.4752^{ns}
Mean weight on corn cobs	106.6	133.3	133.3	106.6	76.7	76.4	98.9	98.8	0.00642^{ns}
t value and significance	-	-	-	-	-	-	3.3113^{ns}	17.989**	-
Mean pileus on sawdust	42.7	20.5	37.7	16.3	29.3	14.0	36.6	16.9	9.0104*
Mean pileus on corn cobs	32.5	14.5	11.7	13.8	9.8	11.1	18.0	13.1	0.7406^{ns}
t value and significance	-	-	-	-	-	-	4.049^{ns}	3.4356^{ns}	-

^{ns}P > 0.05; *P < 0.05; **P < 0.01.

solution were added. A dark green colour indicated the presence of phenolic compounds.

Test for glycosides

Sample (0.5 g) was stirred with 10 ml of boiling distilled water. This was filtered and 2 ml of the filtrate hydrolyzed with a few drops of concentrated HCL and the solution rendered alkaline with a few drops of ammonia solution. 5 drops of this solution was added to 2 ml of Benedict`s qualitative reagent and boiled. Appearance of reddish brown precipitate showed the presence of glycosides.

Test for resins

To 0.5 g of each sample was added 5 ml of boiling ethanol. This was filtered through Whatman No.1 filter paper and the filtrate diluted with 4 ml of 1% aqueous HCL. The formation of a heavy resinous precipitate indicated the presence of resins.

Test for tannins

Sample (0.5 g) was stirred with 10 ml of boiling distilled water. This was filtered and a few milliliters of 6% ferric chloride added to the filtrate. Appearance of deep green coloration indicated the presence of tannins. The second portion of the filtrate was treated with a few milliliters of iodine solution. Appearance of a faint bluish coloration confirmed the presence of tannins.

RESULTS AND DISCUSSION

Effect of two substrates on the growth and yield of *P. ostreatus* and *P. florida* is shown in Tables 2 and 3. There was a significant difference between mean height of *P. ostreatus* and *P. florida* cultivated on sawdust and corn cobs. Sawdust as a substrate had mean height significantly higher than those cultivated on corn cobs. The mean weight of the fruiting bodies of *P. florida* cultivated on sawdust was highly significantly higher than *P. florida* cultivated on corn cobs. Also, there was a significant difference in pileus length for *P. ostreatus* and *P. florida* cultivated on sawdust.

The growth and yield of *P. ostreatus* and *P. florida* varied widely, depending on the kind of substrate used. It was noted that sawdust had the greatest influence on both growth and total yield. It demonstrated excellent biological yield, greater height, and pileus size. There was a significant difference on pin head formation for *P. florida* cultivated on sawdust and corn cobs. There was no significant difference in maturity period for the two species and the two substrates. For the number of fruiting bodies, there was a highly significant difference in the number of fruiting bodies which sprouted from sawdust for *P. ostreatus* and *P. florida*.

Various substrates have different effects on the growth, yield and quality of mushroom (Zhang et al., 2002). Cultivation of oyster mushroom is becoming popular throughout the world, because of their abilities to grow at a wide range of agricultural wastes. The development of oyster mushrooms depends on the specific environment, nutritional and genetic factors according to species. The two species of oyster mushroom cultivated on two substrates (sawdust and corn cobs) gave the following growth outcomes. The

Table 3. Mean pin head formation, maturity period and number of fruiting bodies of *Pleurotus ostreatus* and *Pleurotus florida* for three flushes grown on sawdust and corn cobs.

Growth parameters	Species	Flush 1 on sawdust	Flush 2 on sawdust	Flush 3 on sawdust	Mean flushes on sawdust	Flush 1 on corn cobs	Flush 2 on corn cobs	Flush 3 on corn cobs	Mean flushes on corn cobs	t value for media
Pin head formation (Days)	*P. ostreatus*	10.3	11.7	11	11	14	12.3	14.3	13.5	- 2.60^{ns}
	P. florida	9.4	9.6	10.4	9.8	11.4	12.4	12	11.9	- 6.05*
	t value Species	-	-	-	2.62ns	-	-	-	1.87^{ns}	-
Maturity period (Days)	*P. ostreatus*	5.7	5.7	6	5.8	6	5.7	6.3	6	- 2^{ns}
	P. florida	5.4	6	6	5.8	5.3	5.7	6	5.7	1.51^{ns}
	t value Species	-	-	-	0.0	-	-	-	1.64^{ns}	-
Number of fruiting body	*P. ostreatus*	54	59	63	58.7	44	28	21	31	2.95^{ns}
	P. florida	37	43	45	41.7	30	20	15	21.7	2.94^{ns}
	t value Species	-	-	-	29.44**	-	-	-	3.88^{ns}	-

^{ns}P > 0.05; *P < 0.05; **P < 0.01.

biological efficiency varied significantly due to the effect of different substrate composition on the different flushes. *P. ostreatus* growth was better in sawdust than in corn cobs as it had the highest height, weight, and pileus length. This study is in the same line with the study of Shah et al. (2004) who reported that sawdust gave maximum yield of *P. ostreatus.* Overall, on the two species of mushroom cultivated on sawdust and corn cobs, *P. florida* on sawdust gave lesser days for pin head formation, maturity period and number of fruiting body. The least results was indicated by *P. ostreatus* on corn cobs which took the longest time for pin head formation, longest maturity days and the average number of fruiting body. This variability in pin head formation, maturity period and number of effective fruiting body has been reported by Shah et al. (2004). They reported that significant variability on the number of days of pin head formation, maturity period and number of effective body is due to presence of different composition of substrate. The number of days for pin head formation is supported by Bughio (2001) who stated 25 to 50 days for pinhead formation and reported that maturation of fruiting bodies took 5 to 6 days after pinhead formation as in the same line with this study.

The biological efficiency and moisture content was the highest for sawdust substrates as a whole. Biological efficiency was the highest on *P. ostreatus* cultivated on sawdust with 50.2% and least on corn cobs substrates of both *P. ostreatus* and *P. florida* with 16.5%. However, moisture content was also the highest for *P. ostreatus* cultivated on sawdust with 90% and least for *P. florida* on corn cobs with 80% as shown in Table 4.

Fully and broadly opened basidiocarps were obtained from *P. ostreatus* than for *P. florida*. This is shown in Figure 3. It can be concluded that *P. ostreatus* cultivated on sawdust is the best species of oyster mushroom which can be cultivated for commercial purposes as it had the highest biological yield value than those grown on corn cobs. The substrates used in this study can be considered practical and economically feasible due to their availability throughout the year at little or no cost in large quantities. Utilization of these agro-wastes for the production of oyster mushrooms could be more economically and ecologically practical. The effect of two substrates on nutritional content of *P. ostreatus* and *P. florida* is shown in Table 5.

Nutritional composition of mushroom is affected by many factors among which the composition of the substrate is of major importance. This can be supported by the results in the findings where *P. florida* and *P. ostreatus* on sawdust gave a significant nutritional value than that cultivated on corn cobs Shah et al. (2004). The nutritional properties of mushroom are also influenced by the species. This is supported by the fact that *P. florida* gave the best nutritional properties than *P. ostreatus* in terms of amount of proteins, lipids, fibre and ash content. The protein content ranged from 25.12 to 29.45% in 100% dry matter. Crude

Table 4. Culmulative *Pleurotus ostreatus* and *Pleurotus florida* biological efficiency (BE) and mean moisture content on two substrates.

Species and substrates	Biological efficiency (%)	Mean moisture content (%)
P. ostreatus on sawdust	50.2	90
P. ostreatus on corn cobs	16.5	82
P. florida on saw dust	36.1	86
P. florida on corn cobs	16.5	80

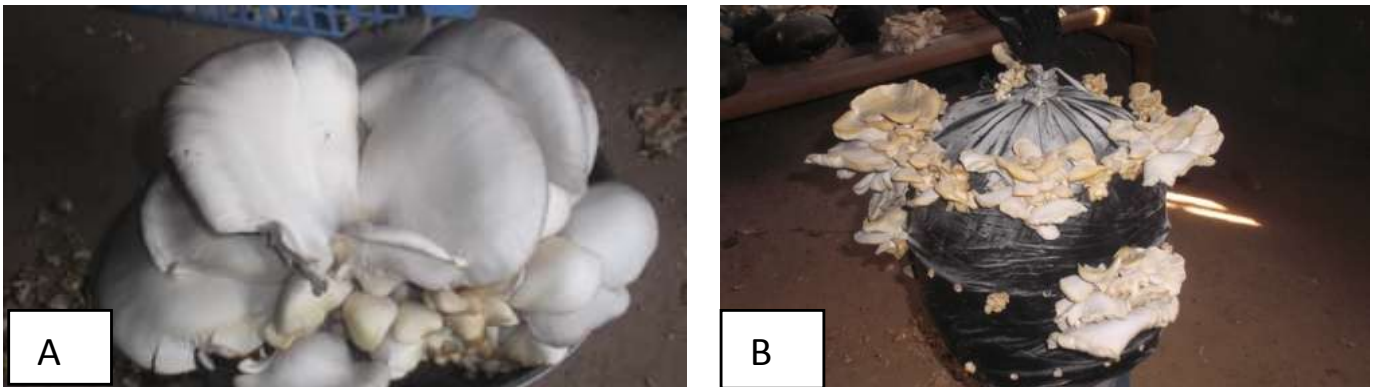

Figure 3. Fully opened Basidiocarps of *Pleurotus ostreatus* and *Pleurotus florida*.

Table 5. Nutritional analysis of *Pleurotus ostreatus* and *Pleurotus florida* on different substrate.

Species and type of substrate	DM (%)	Ash (%DM)	Organic matter (%DM)	Crude protein (%DM)	Lipids (%DM)	Crude fibre (%DM)
P. florida Corn cobs	91.1	7.0	93.0	25.1	4.6	16.7
P. florida Sawdust	90.0	9.0	90.9	29.5	2.2	5.1
P. ostreatus sawdust	88.7	8.3	91.7	26.0	3.8	12.5
P. ostreatus corn cobs	90.9	9.5	90.5	25.8	2.0	16.0

protein percentage of oyster mushroom was affected by different substrates. Regarding substrate, maximum protein (29.45%) was observed on sawdust while minimum (25.12%) on corn cobs. Genotypic variable was observed in *Pleurotus* spp. to protein percentage, the highest percentage (29.45%) was observed in *P. florida*, whereas *P. ostreatus* showed the lowest amount (25.78 %). The percentage content of protein was similar as reported in earlier studies (Patil et al., 2010). Mushroom protein is intermediate between that of animals and vegetables and it is of superior quality because of the presence of all the essential amino acids. The value of lipid ranged from 1.97 to 4.62% in 100% dry matter. Lipid percentage of oyster mushroom was affected by different substrate. Regarding substrate, maximum lipid (4.62%) was observed on corn cobs, while minimum (1.97%) was still on corn cobs. Genotypic variable was observed in *Pleurotus* spp. to lipid percentage, the highest percentage (4.62%) was observed in *P. florida*, whereas *P. ostreatus* showed the lowest amount (1.97%).

Lipid value is contrary to that of Wang et al. (2001) as lipid range from 2.5 to 2.8%. Linoleic acid makes up to 76% of unsaturated fatty acids and 90% of polar lipids. It is the presence of linoleic acid, one of the omega 6 fatty acid that contributes to mushrooms being a healthy food. The crude fibre ranged from 5.08 to 16.69% and the ash content range from 6.94 to 9.46. Regarding substrate, maximum ash (8.67%) was observed on sawdust, while minimum (8.215%) on corn cobs. *Pleurotus* spp. contain high rate of potassium to sodium, which makes it an ideal food for patients suffering from hypertension and heart diseases (Purkayastha and Nayak, 1981). Regarding substrate, maximum crude fibre (16.69%) was observed on corn cobs, while minimum (5.08%) was still on sawdust. Genotypic variable was observed in *Pleurotus* spp. to crude fibre percentage, the highest percentage

Table 6. Bioactive components of *P. florida* and *P. ostreatus*.

Bioactive components	*Pleurotus florida*	*Pleurotus ostreatus*
Alkaloids	–	–
Flavonoids	+	+
Polyphenols	+	+
Triterpenoids	+	+
Steroids	+	+
Saponins	+	+
Glycosides	–	–
Resins	–	–
Tannins	–	–

The -sign indicates the absence of the compounds while the + sign indicates the presence of the compound.

(16.69%) was observed in *P. florida*, whereas *P. ostreatus* showed (16.01%).

The nutritional composition of edible mushrooms is affected by many factors among which the composition of substrate is of major importance also mentioned by Belewu (2003). Nutritional properties also differ according to species, but this difference also depends on the substrates. These results also indicate that the studied oyster mushroom species have good nutritive value for human. Protein is an important nutritional component and protein deficiency is the world's most serious human nutritional problem, especially in third world countries like Cameroon. So oyster mushroom is a promising food that may overcome protein energy malnutrition problem and mineral deficiency in the third world. While the protein content is lower than that found in eggs, meat and fish, it is adequate to be used as a substitute in the diet of the general public. These species of oyster mushroom contain low fats of unsaturated fatty acids which makes it a healthy food for all type of people.

As concerns nutritional properties of *P. florida* on sawdust which had the highest amount of protein of 29.5%. This protein is of great importance to health as they help the body to build, repair and maintain body tissue. On the other hand, *P. florida* cultivated on corn cobs had the highest amount of fibre which aids in digestion, weight management and has the highest amount of lipids that protect the vital organs. It has been reported that not only the protein content in fruiting body but also the nature of protein depends on used substrate (Wang et al., 2001). *P. florida* cultivated on sawdust and corncobs are the most preferred species according to nutritional properties because of the presence of essential nutrients such as proteins, ash, lipids and fibers. Effect of two substrates on the bioactive components of *P. florida* and *P. ostreatus* is shown in Table 6.

Lindequist et al. (2005) stated that the nutritional and chemical compositions of mushroom are responsible for their medicinal values. The bioactive component analysis of edible mushrooms *P. ostreatus*, and *P. florida* revealed the presence of major bioactive components such as flavonoids, polyphenols, saponins, triterpenoids and steroids. This result is similar to that of (Iwalokun et al., 2007). The compound alkaloids, glycosides, resins and tannins were absent in the extracts. Bioactive compounds found in edible mushroom are known to play a vital role in promoting health. The absence of alkaloids and glycosides confirms the report of Hamzah et al. (2014). These phytochemicals play a vital role in medicinal properties of plants. Saponins for instance comprise a large family of structurally related compounds containing steroids or triterpernoid. They are reported to have a wide range of pharmaceutical properties, such as anti-inflammatory and anti-diabetic effects. Thus these mushrooms can be used in the management of diabetes and inflammation related diseases. Terpenoids have been reported to show a wide range of pharmacological benefits that include anti-malarial, anti-inflammatory and anti-cancer effects among others. Phenolic compound are antioxidant and exhibit a wide range of spectrum medicinal properties such as anti-cancer and anti-inflammatory. These mushrooms can therefore be harnessed in the management of oxidative stress induced disease since phenol and flavonoids have been shown to possess various antioxidant functions (Pandimeena et al., 2015).

Flavonoids have been isolated from hundreds of species of mushroom that are effective against many chronic diseases and the bioactive constituents of *P. florida* confirmed it to be one among these. The medicinal value of the mushroom may be due to the presence of secondary metabolites (Pandimeena et al., 2015). These metabolites present in the human diet possess a number of beneficial effects on human health such as anti-oxidant, anti-allergic, anti-viral, anti-diabetic and anti-carcinogenic. The triterpenoid in *P. florida* and *P. ostreatus* might be responsible for its anti-oxidant and anti-inflammatory activity (Price et al., 1987). Saponins cause cytotoxic effect and are beneficial in lowering

cholesterol and blood sugar levels (Price et al., 1987).

Conflict of interests

The authors have not declared any conflict of interests.

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