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# African Journal of Biotechnology

Table of Content: Volume 17 Number 23 6 June, 2018

## ARTICLES

- Production of single cell protein (SCP) and essentials amino acids from *Candida utilis* FMJ12 by solid state fermentation using mango waste supplemented with nitrogen sources** 716  
Marius Kounbèsiounè SOMDA, Mahamadi NIKIEMA, Ibrahim KEITA, Iliassou MOGMENGA, Sonagnon H. S. KOUHOUNDE, Yerobessor DABIRE, Wahauwouélé H. COULIBALY, Essodolom TAALE and Alfred S.TRAORE
- Development and validation of analytical methodology for quantification of total flavonoids of *Morus nigra* by ultraviolet-visible absorption spectrophotometry** 724  
Pedrita Alves Sampaio, Hyany Andreysa Pereira Teixeira, Nathália Andrezza Carvalho de Souza, José Marcos Teixeira de Alencar-Filho, Grasielly Rocha Souza, Emanuella Chiara Valença Pereira, Raimundo Gonçalves de Oliveira-Júnior, Pedro José Rolim-Neto, Jackson Roberto Guedes da Silva Almeida and Larissa Araújo Rolim
- Phenotypic characterization and symbiotic effectiveness test of chickpea (*Cicer arietinum* L.) rhizobia isolated from Dejen and Aneded Districts, East Gojjam Zone, Amahara Region, Ethiopia** 730  
Birhan Aynalem, Tadele Temesgen, Yilkal Bezie and Seble W. Yohannis

*Full Length Research Paper*

# **Production of single cell protein (SCP) and essentials amino acids from *Candida utilis* FMJ12 by solid state fermentation using mango waste supplemented with nitrogen sources**

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In Burkina Faso, deficiency of amino acids in protein is becoming a major healthy public problem. This study was purposed to optimize essentials amino acids in single cell protein (SCP) by supplementing different nitrogen sources during fermentation of mango waste with *Candida utilis* FJM12. Analytical methods were used to determine biomass yield, chemical composition and amino acids profile of SCP. The principal component analysis (PCA) method was performed to identify the nitrogen source which exhibited best rate of SCP. The maximum biomass yield ( $6.48 \pm 0.03$  g/L) exhibited  $9.65 \pm 0.36\%$  (w/w) of ash, while using yeast extract. The proximate composition of SCP revealed  $56.40 \pm 1.30$ ,  $13.25 \pm 0.11$ ,  $3.80 \pm 0.10$ , and  $6.60 \pm 0.25\%$  (w/w), respectively for crude protein, lipids, carbohydrates, and nucleic acid content. PCA showed a strong correlation between yeast extract and Ammonium sulphate and demonstrated their positive influence to increase the rate of SCP and essentials amino acids as compared to Food and Agriculture Organization (FAO) recommendation. These results demonstrated that *C. utilis* FJM12 could be suitable for essentials amino acids.

**Key words:** Mango waste, nitrogen source, *Candida utilis*, single cell protein (SCP), amino acids.

## **INTRODUCTION**

Most residues could be converted into high-value materials through bio-components. Food security

problem concerns most of the developing countries (Tamrat, 2017). Fruit processing results in high amounts of by-products (peels, seeds) and represent an environmental pollution (Somda et al., 2011a). Burkina Faso and other developing countries have long been with problems of processing and preservation of locally produced agricultural food products (Somda et al., 2011a). Thus, finding new possible application area to further exploit these wastes for the production of high-value products have gained increasing interest (Malviya et al., 2010; Koubala et al., 2013). Waste products can be converted to biomass, protein concentrate or amino acids using proteases derived from certain microorganisms (Kurbanoulu, 2001). So, mango residues coming from industrial area, market and site stockage can attain 50,000 tons per year in Burkina Faso (Somda et al., 2010, 2011a). Due to its important carbohydrates rate, mango waste can be a valuable fermentation substrate for both single cell protein (SCP) and essential amino acids production using yeasts.

Yeast SCP is a high nutrient feed substitute (Burgents et al., 2004). Among these, most popular are yeast species *Candida*, *Hansenula*, *Pichia*, *Torulopsis* and *Saccharomyces* (Bozakouk, 2002). *Candida utilis* has been frequently used in biomass production because of its ability to utilize a variety of carbon sources and to support high protein yield. It has been used for production of several industrial products both for human and animal consumption (Otero et al., 1998). Amino acids are critical to life and used as food or feed additives, in parenteral nutrition or as building blocks protein or for the chemical and pharmaceutical industries (Darshan and Priya, 2013; Kumagai, 2000; Wendisch et al., 2016).

The optimization of amino acids in SCP needs to apply an efficient process of fermentation as solid state fermentation. The Solid-State Fermentation (SSF) is defined as a fermentation process, which involves solid matrix in the absence or near absence of free water (Singhania et al., 2009). It has many advantages, such as high product yield with little risk of bacterial contamination, extended stability of products, low wastewater generation and low production costs (Zhao et al., 2008; Barrios-Gonzalez, 2012). SSF has received more interest from researchers and has been applied in various areas, such as biotransformation of crops and crop residues for microbial preparation, nutritional enrichment (Singhania et al., 2009) and production of a range of high value-added products (Quevedo-Hidalgo et al., 2013; Yu et al., 2014). Fermentative production of amino acids in the million-ton-scale has shaped modern biotechnology and its markets continue to grow steadily

(Wendisch et al., 2016). The SCP production could easily be improved by supplementing using organic or inorganic nitrogen source as yeast extract, peptone, ammonium sulphate and Ammonium nitrate. The use of cheap and readily available nitrogen source should be desirable as it lower cost of production.

Hence, this study focused on investigating the production of SCP and some different amino acids using mango waste supplemented with nitrogen sources through solid state fermentation.

## MATERIALS AND METHODS

### Strain and inoculum preparation

*C. utilis* FMJ12 was obtained from the culture collection of Laboratory of Microbiology and Microbial Biotechnology in the Department of Biochemistry and Microbiology, University Ouaga1 Pr Joseph KI-ZERBO, Burkina Faso. It was maintained at 4°C on yeast peptone dextrose (YPD) agar. Inoculum was prepared by inoculating a loop full of cells from 24 h old culture slant in conical flask containing 100 mL of YPD liquid medium at 30°C and 150 rpm for 24 h (Adan et al., 2011).

### Media preparation for fermentation

An amount of 500 g of mango waste was dried at 105°C for 24 h (AOAC, 2016), then ground in a mortar and separated in a sieve shaker. The final waste particle size was approximately 2 mm in diameter. The nutrient broth liquid medium used was adapted from Adan et al. (2011) and then Darshan and Priya (2013). It contained in percentage (w/v): 0.5% MgSO<sub>4</sub>, 0.5% KH<sub>2</sub>PO<sub>4</sub>, 0.01% FeSO<sub>4</sub>, 0.12% Na<sub>2</sub>SO<sub>4</sub>, 5% glucose and was prepared in distilled water added with 0.2% of Tween-80. The medium for the fermentation procedure was prepared using mango waste 10% (w/v) of nutrients broth and final pH was adjusted to 7.00 ± 0.02.

### Fermentation process

Fermentation medium was supplemented with organic nitrogen sources (peptone, yeast extract) and inorganic sources (Ammonium sulphate and Ammonium nitrate). Each nitrogen source was added separately at 1% (w/v) in growth medium. The mixtures were autoclaved at 121°C for 15 min. An inoculum of 10<sup>5</sup> cells/mL was added at a ratio of 10 % (v/v) in to flasks and shaken at 150 rpm for 1 h before fermentation process. Flasks were kept in static incubator in solid state fermentation and maintained at 30°C for 72 h (Darshan and Priya, 2013).

### Biomass of yeast cells

After 72 h of fermentation, the concentration of yeast cells in the fermenting matter was measured using the turbidimetric (absorbance at 600 nm) method and by determining dry weight of yeast cells. Cells were harvested by centrifugation at 16,000 rpm

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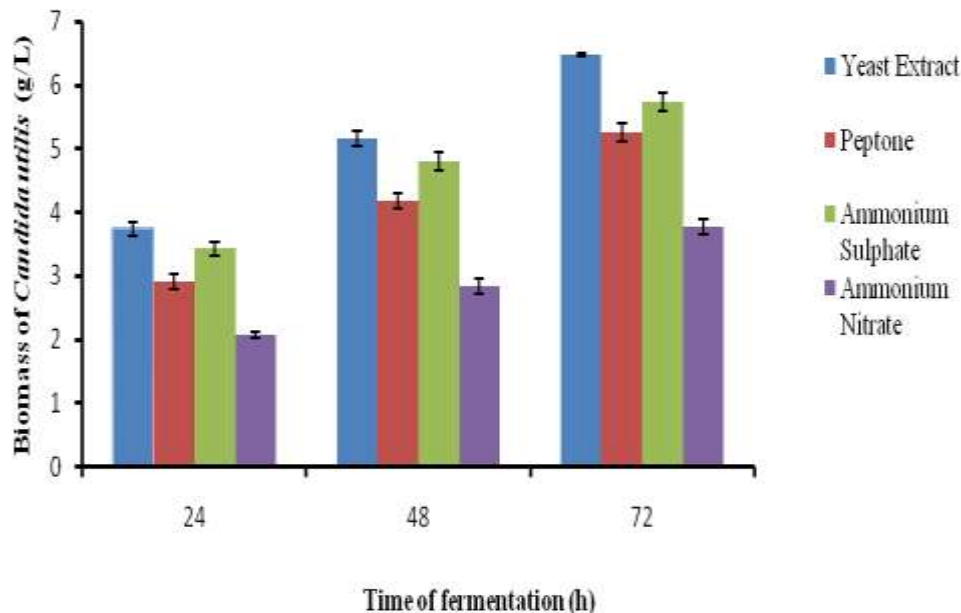


Figure 1. Biomass yield of *Candida utilis* FMJ12 according nitrogen source.

for 20 min washed twice with distilled water and dried in an oven at 50°C for 48 h. After 48 h, dry cells were weighed (Lagzouli et al., 2007b).

#### Chemical compounds of yeast cells

Total sugar content, dry matter and ash analysis were estimated by AOAC methods (2016). Total lipids were estimated by adapted methods of Kurbanoglu (2001). RNA and DNA levels were measured as described by Kurbanoglu (2001).

#### Total protein assay

Total protein content of yeast cells was measured by the micro-Kjeldahl method via multiplication of total nitrogen by 6.25 (AOAC, 2016).

#### Protein recovery by sonication

Suspension of 2.5% of dry yeast cells in 50 mL of distilled water was sonicated in a sonicator Q125 (Qsonica-LLC, USA) at a fixed power of 600 W, frequency of 20 kHz and amplitude of 50%. Total cycle time for ultrasonic treatment was 10 min. The pulse duration and pulse intervals were 1 min each. The jar was immersed in an ice-water bath to avoid a temperature increase during sonication. Cell debris and particles were removed by centrifugation at 11500xg for 10 min and crude protein was stored at -5°C (Mirzaei et al., 2015).

#### Amino acids analysis

Crude protein obtained after sonication was precipitated using ammonium sulphate and acetone. Microwave assisted acid hydrolysis was explored to speed up hydrolysis. Crude amino acids

were extracted by using centrifugation at 10,000 rpm for 15 min. Amino acid analysis was carried out after hydrolysis with 6 N HCl at 110°C for 24 h in a Biotronic LC-5001 Amino Acid Analyser (Germany) according to the method of Kurbanoglu (2001).

#### Statistical analysis

Statistical analysis was carried using Statistica V7.1. Correlation between different parameters was determined and p-value ( $p < 0.05$  or 0.0001) was considered statistically significant. Associations between nitrogen source and SCP production were performed through Pearson's correlation.

Principal Component Analysis was performed in order to identify the best nitrogen source which exhibited higher rate of SCP. Principal component analysis and principal coordinate analysis plots were generated by regrouping of variables.

## RESULTS AND DISCUSSION

Figure 1 shows the biomass yield of *C. utilis* FMJ12 when grown in different medium. The maximum biomass yield was obtained after 72 h with supplementation of yeast extract ( $6.48 \pm 0.03$  g/L) as nitrogen source followed by Ammonium sulphate ( $5.74 \pm 0.15$  g/L), Peptone ( $5.25 \pm 0.14$  g/L) and closed by Ammonium nitrate ( $3.77 \pm 0.12$  g/L). The results obtained by supplementing yeast extract were higher to those reported by Ouedraogo et al. (2017) and Jaganmohan et al. (2013) as 4.68 (g/L) but lower than those (7.23 g/L) reported by Kurbanoglu (2001). It has been remarked that biomass of *C. utilis* FMJ12 was increased with addition of yeast extract to the medium and would be strongly supporting efficient growth. The source of nitrogen plays a vital role in the improvement of

**Table 1.** Proximate composition of biomass of *Candida utilis* FMJ12.

Component (%)	Yeast extract	Peptone	Ammonium sulphate	Ammonium nitrate	P-value
Ash	9.65±0.36	7.72±0.47	8.23±0.38	6.27±0.29	<0.01
Total protein	56.40±1.30	45.12±1.19	50.76±1.17	30.84±1.15	<0.01
Total lipids	13.25±0.11	7.95±0.13	10.60±0.21	4.64±0.35	<0.01
Carbohydrates	3.80±0.10	2.28±0.13	2.85±0.12	1.14±0.10	<0.01
Nucleic Acid	6.60±0.25	4.29±0.23	5.28±0.20	3.33±0.17	<0.01

efficiency and economics of microbial fermentation (Nancib et al., 2001).

The proximate composition of biomass obtained from the fermentation of *C. utilis* FMJ12 using organic and inorganic sources of nitrogen is shown in Table 1. The percentages of ash in dry cells were significantly different ( $p < 0.01$ ) and ranged respectively as 6.27±0.29, 7.72±0.47, 8.23±0.38, and 9.65±0.36% (w/w) for ammonium nitrate, peptone, ammonium sulphate and yeast extract. These results were emphasized as found by Nasser et al. (2011) who demonstrated that ash of yeast ranged from 5 to 10%. The rate of ash obtained using Yeast extract was close to the result (8.4%) recorded by Husseiny et al. (2016).

Crude protein was produced with significant difference under influence of organic and nitrogen source ( $p < 0.01$ ). The mean values were located between 30.84±1.15 and 56.40±1.30% (w/w). Data showed that the maximum yield of protein was achieved by the addition of organic nitrogen source as Yeast extract to the production medium. The protein reached after supplementing of yeast extract was higher than the value found by Gao et al. (2007) in yeast (53%) and similar with value of Rajoka et al. (2006) in *C. utilis* (56.34%) and yet lower than that found by Rajoka (2005) in *Cellulomonas biazotea* (60%) and Somda et al. (2017) in *Saccharomyces cerevisiae* SKM10 (79.14%). The mean values of lipids content significantly ranged ( $p < 0.01$ ) from 4.64±0.35 to 13.25±0.11% (w/w). The result obtained using yeast extract as organic nitrogen source was higher than crude lipid content recorded by Parajo et al. (1995), Kurbanoglu (2001) and Husseiny et al. (2016) who reported, respectively 9, 5.4, and 5.05% in SCP of yeast.

Concerning carbohydrates content, it was found significantly different ( $p < 0.01$ ) and ranged from 1.14±0.10 to 3.80±0.10% (w/w), which are lower to the percentage obtained in *Hansenula* species (24%) by Shojaosadati et al. (1999) and *S. cerevisiae* (26%) by Husseiny et al. (2016). Nucleic acid contents of SCP was found to range from 3.33±0.17 to 6.60±0.25% (w/w) which is significantly lower than the values reported by Kurbanoglu (2001) as 7.47% and higher than Ibrahim Rajoka et al. (2005) at 2.75%. On the other hand, the high RNA contents are reported to be toxic for human consumption, while harmless for most animals (Kurbanoglu, 2001). While

most microorganisms contain nucleic acid between 6 and 15%, the low content in *C. utilis* is a very interesting result for animals feed.

The comparison of the obtained data presented in Table 1 demonstrates that the best bioconversion of mango waste to high yield of protein was obtained by supplementing Yeast extract to basic medium. The mango waste contained mineral elements which could help to increase growth and stability of fungi strains. Amino acid profile of SCP produced by *C. utilis* FMJ12, was determined and data indicated that it could be compared favourably with FAO standards (Table 2). Data recorded in Table 2 showed that, the biomass cells had 19 kinds of essential amino acids. It is apparent from the results that the addition of nitrogen sources efficiently affects the SCP and essential amino acids productivities by *C. utilis* FMJ12. Amino acid concentrations as isoleucine, leucine, lysine, phenylalanine, threonine, and Tryptohan were somewhat higher than the FAO reference protein and could be beneficial for nutritional need. Among the amino acids, glutamic acid (16.57%) and cysteine (10.16%) were the most abundant produced after supplementing with yeast extract. Addition of yeast extract and ammonium sulphate to the production medium resulted in the highest amount of SCP essential amino acids as lysine, leucine, isoleucine, phenylalanine, methionine, threonine, and valine.

The amounts of SCP and essentials amino acids obtained after optimizing the medium by yeast extract represent more 2-fold increase as compared to amounts recommended by FAO. These results are in agreement with the results by Paraskevopoulos et al. (2003) who found that the maximum SCP production by yeast and other organisms was obtained after supplementing medium with Yeast extract and Ammonium sulphate. Also, Zhang et al. (2008) and Husseiny et al. (2016) reported that using ammonium sulphate as nitrogen source give the highest yield of SCP by *A. oryzae* and *S. cerevisiae*. It was reported that the potential nutritional value of SCP is determined with amount of lysine and methionine amino acids (Kurbanoglu, 2001). In agreement with the present results, Zheng et al. (2005) and Rajoka et al. (2006) recorded that, the biomass obtained from *C. utilis* contained all the essential amino acids for human nutrition. These results show that strain



**Table 2.** Amino acids composition of SCP from *Candida utilis* FMJ12.

Amino acids (g/100 g) of SCP	Nitrogen source				FAO* standards
	Yeast extract	Peptone	Ammonium sulphate	Ammonium nitrate	
Isoleucine	5.28±0.2	0.21±0.01	3.15±0.11	1.07±0.02	2.20
Leucine	6.73±0.21	0.26±0.11	1.69±0.10	1.10±0.11	2.20
Lysine	5.81±0.21	0.20±0.02	3.76±0.22	1.17±0.03	1.60
Methionine	1.67±0.01	0.00	0.48±0.11	0.03±0.01	2.20
Phenylalanine	4.62±0.30	0.17±0.01	3.15±0.04	1.07±0.02	2.20
Threonine	4.62±0.22	0.20±0.01	1.99±0.11	0.07±0.01	1.00
Tryptophan	2.75±0.01	0.05±0.01	1.69±0.13	0.06±0.01	0.50
Valine	6.05±0.01	0.23±0.01	3.23±0.11	1.09±0.01	1.60
Cysteine	10.16±0.10	4.62±0.21	7.35±0.33	5.30±0.20	2.20
Aspartic acid	4.62±0.20	0.40±0.01	2.24±0.01	0.15±0.01	1.85
Serine	3.87±0.11	0.19±0.01	1.43±0.11	0.78±0.21	1.80
Glutamic acid	16.57±0.12	2.52±0.12	10.10±0.10	4.20±0.10	1.82
Proline	3.08±0.01	0.18±0.01	2.40±0.12	0.16±0.01	1.84
Glycine	3.92±0.22	0.25±0.01	3.39±0.13	1.10±0.02	1.85
Alanine	7.59±0.20	0.27±0.04	3.37±0.14	1.05±0.01	1.81
Tyrosine	2.64±0.11	0.10±0.01	1.59±0.23	0.06±0.01	2.80
Histidine	3.78±0.31	0.62±0.02	1.35±0.11	0.35±0.01	1.85
Glutamine	4.84±0.21	2.79±0.13	2.40±0.10	0.08±0.01	1.83
Arginine	4.82±0.13	0.13±0.02	2.00±0.01	1.05±0.02	1.78

FAO: Food and Agriculture Organization.  
Source: \*<http://www.fao.org>.

**Table 3.** Pearson's matrix correlation of nitrogen source using for amino acids production.

Nitrogen source	Yeast extract	Peptone	Ammonium sulphate	Ammonium nitrate	FAO
Yeast extract	1				
Peptone	0.628*	1			
Ammonium sulphate	0.928*	0.693*	1		
Ammonium nitrate	0.843*	0.766*	0.897*	1	
FAO	0.067	0.149	0.0721	0.195	1

\*Correlation is significant at the 0.05 level.

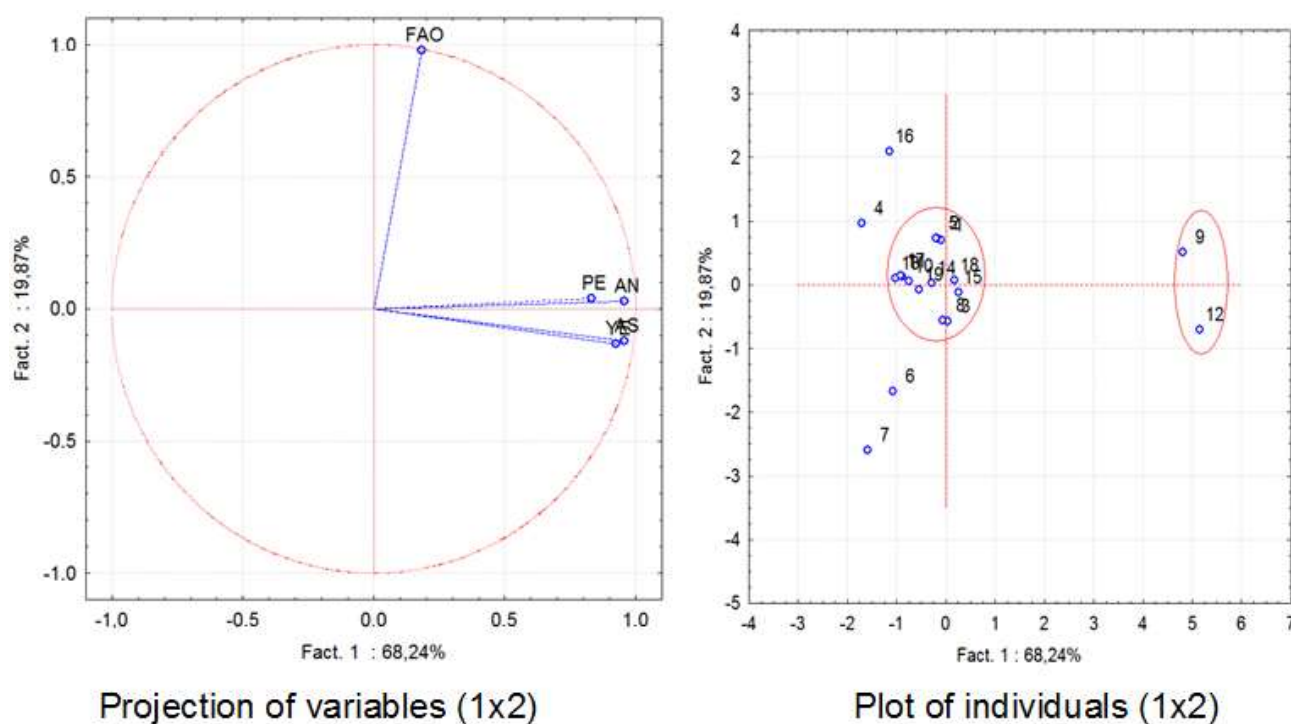
*C. utilis* FMJ12 was suitable for single-cell protein production.

The Pearson's matrix correlation of nitrogen source influencing amino acids production is shown in Table 3 and shows positive correlations ( $p=5\%$ ) among parameters studied. The highest positive correlation was observed between the presence of yeast extract and ammonium sulphate ( $r=0.928$ ) followed by ammonium sulphate and Ammonium nitrate ( $r=0.897$ ), then yeast extract and ammonium nitrate. The strong correlation between yeast extract and ammonium sulphate has demonstrated their positive influence on increase of the rate of SCP and amino acids as compared to FAO recommendation.

Table 4 contains the relevant results of coordinate of nitrogen sources influence to SCP and essential amino acids production. Analysis of principal components exhibited the variability of influence of four nitrogen sources on SCP and essentials amino acids production. The cumulative values of the variance of the first three principal components (F1, F2 and F3) for the parameters were 100%, with Eigen-values range between 0.41 and 3.41 (Table 4). Principal component F1 had an Eigen value of 3.41 and contributed to 68.24% of the variation of the parameters. This principal component (F1) is associated positively to isoleucine, leucine, lysine, and valine production. Principal components F2 and F3 had respective Eigen-values of 0.99 and 0.41, accounting for

**Table 4.** Coordinate of nitrogen source and their contribution to SCP and essentials amino acids production.

Parameter (Nitrogen source)	Principal components		
	F1	F2	F3
Yeast extract	0.92	-0.13	-0.28
Peptone	0.83	0.039	0.54
Ammonium sulphate	0.96	-0.12	-0.18
Ammonium nitrate	0.96	0.029	-0.0079
FAO	0.18	0.98	-0.082
Eigen value	3.41	0.99	0.41
Variance (%)	68.24	19.87	8.30
Cumulative (%)	96.41	98.82	100.00



**Figure 2.** Principal Component Analysis (PCA) of amino acids produced in fermentation medium supplemented with different nitrogen sources. The explained variance (%) is reported for each principal component, and dotted ellipses represent the 95% confidence limits for amino acids. AN, Ammonium nitrate; AS, ammonium sulphate; PE, peptone; YE, yeast extract. 1, isoleucine; 2, leucine; 3, lysine; 4, methionine; 5, phenylalanine; 6, threonine; 7, tryptophan; 8, valine; 9, cysteine; 10, aspartic acid; 11, serine; 12, glutamic acid; 13, proline; 14, glycine; 15, alanine; 16, tyrosine; 17, histidine; 18, glutamine; 19-arginine.

19.87 and 8.30% to the total variation and were associated positively with the rate of Cysteine and Glutamic acid.

Principal component analysis (PCA) of amino acids produced in fermentation medium supplemented with different nitrogen sources are shown in Figure 2. The results with influence of nitrogen source and amino acids data in Figure 2 confirm that PCA can find a reduced set of variables that are useful for understanding the

experiments. The projection and score-plot resulting from PCA achieved by combining F1 (68.24% explained variance) and F2 (19.87% explained variance) is as shown in Figure 2. The first two components account for over 90% of the cumulative allowed for most of the information to be visualized in two dimensions. The production of cysteine was positively correlated by supplementation of peptone or ammonium nitrate in medium of fermentation and in opposite glutamic acid it

was correlated by yeast extract and ammonium sulphate. The loading plot revealed that the variance associated to Isoleucine, Leucine, Lysine, and Valine had the largest weight in F1. It was shown that the production of Isoleucine and Leucine influenced by Peptone or Ammonium nitrate were negatively correlated with Lysine and Valine influenced by Yeast extract and Ammonium sulphate. The results demonstrated that, the maximum yield of essential amino acids was strongly correlated with the presence of Yeast extract.

## Conclusion

Finally, the possibility to use of mango waste, as a low cost agro-industrial biomass source for production of SCP by *C. utilis* FMJ12 was demonstrated in this study. This approach could also be used to minimize the environmental pollution. The amino acid profile of the produced SCP was comparable to FAO standards, therefore advocating their use in food applications. It is however, recommended that further large-scale studies be carried out in addition to extensive toxicological and acceptability tests.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# Development and validation of analytical methodology for quantification of total flavonoids of *Morus nigra* by ultraviolet-visible absorption spectrophotometry

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***Morus nigra* L.** is known in the region of Brazilian São Francisco Valley as "amora-miúra". It is widely used in traditional medicine, mainly in treatment of diabetes, hypercholesterolemia, cardiovascular problems, obesity and gout. This study proposed to develop and validate a fast, simple, efficient and low cost analytical method that could quantify total flavonoid content present in crude ethanolic extract of leaves of *M. nigra* (Mn EtOH). For this, ultraviolet-visible (UV-Vis) absorption spectrophotometry was used as the analytical tool and the complexation with aluminium chloride (AlCl<sub>3</sub>). The quantification of flavonoids by this method was based on the complexation of AlCl<sub>3</sub> with the flavonoid nucleus as a selectivity tool made by two procedures: without complexation (NCP) and with complexation (WCP), in order to evaluate the effects of the complexing agent in quantification, proving the efficiency of this technique. In this way, it became necessary to validate the method used to ensure its efficiency. The validation of the method of quantification of total flavonoid content by UV-Vis demonstrated that the method was selective, linear, precise, accurate, and robust.

**Key words:** *Morus nigra*, UV-Vis, Moraceae, total flavonoids, natural products.

## INTRODUCTION

The use of plants in the treatment of diseases often symbolizes the only therapeutic resource of several

people around the world (Maciel et al., 2002). However, many medicinal plants still require more detailed studies.

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In 2009, the Brazilian Ministry of Health published the National List of Medicinal Plants of Interest to the Unified Health System, with the purpose of guiding research on medicinal plants. Among the plants mentioned are those of the genus *Morus*, which belongs to the family Moraceae and comprises around 24 species and approximately 100 varieties (Oliveira et al., 2013).

*Morus nigra* L. originates in far East, being brought to the region of the São Francisco Valley (Brazil) by Japanese immigrants, adapting well to the climate and soil conditions of the region. In folk medicine, the population uses this species as leaf tea (decoction) for the treatment of diabetes, cholesterol, cardiovascular problems, obesity, and gout. It is popularly known as "amora-miúra", however, in other regions of this country, it is called as "amora-preta" or "blackberry" (Souza et al., 2015).

Although widely used by traditional communities, there are still few chemical and pharmacological studies. However, some important pharmacological activities have already been proven. Naderi et al. (2004) demonstrated that the compounds present in three different types of extracts obtained from *M. nigra* fruit presented protective activity against the peroxidative damage of biomembranes and biomolecules. The results show that all the three extracts inhibited haemoglobin glycosylation induced by glucose to differing degrees. Anti-inflammatory and antinociceptive activities have also been demonstrated for its fruit extract and were related to the presence of flavonoids such as rutin (RUT), the major compound in the extracts (Chen et al., 2016). Other studies revealed its antinociceptive and anti-inflammatory activities using different parts of this plant species (Souza et al., 2000; Padilha et al., 2009, 2010).

The main bioactive substances present in *M. nigra* are phenolic compounds and flavonoids are the most studied because they stand out due to their wide range of pharmacological activities that have already been demonstrated experimentally in the literature, such as antitumor, antioxidant, antiviral, photoprotective and anti-inflammatory, which gives it significant pharmacological importance (Nestel, 2003; Alencar Filho et al., 2016).

The standardization of the vegetal raw material is essential to guarantee its quality, effectiveness and safety, and to prove the therapeutic effects of the vegetal drug used and consequently of the final product (Fonseca, 2007). In order for an herbal remedy to be effective, the chemical integrity of the active ingredients must be preserved, guaranteeing the pharmacological action. In this way, the plant used requires necessarily previous studies related to the botanical, phytochemical and development of analytical methodologies, highlighting the profile of the chemical constituents of interest (Toledo et al., 2003). Analysis of the content of the main bioactive compounds in raw material of plant origin is an essential step for safety and efficacy.

Several techniques can be used to quantify flavonoids

in plant materials. Ultraviolet-visible absorption spectrophotometry (UV-Vis) is used in the analysis of these compounds as it is a simple, fast, low cost, easy to perform and with high reliability of results. This technique is recognized by the advantages of its use, being used mainly in quality control in pharmaceutical industry, which demands speed and reliability of the results (Alves et al., 2010).

Thus, for the method to be recognized, the analytical procedures require an evaluation that estimates its efficiency in the laboratory routine and its capacity to detect and quantify a particular analyte denominated validation (Brito et al., 2003). In Brazil, the National Health Surveillance Agency (ANVISA), through specific resolution (ER 899, 2003), defines that "validation must guarantee, through experimental studies, that the method meets the requirements of the applications analytically, assuring the reliability of the results" (BRASIL, 2003). Thus, it is necessary to carry out tests for the determination of the specificity, linearity, limit of detection, limit of quantification, accuracy, precision and robustness for the analysis.

Considering the medicinal interest in *M. nigra*, the aim of this study was to develop and validate a UV-Vis region absorption spectrophotometry methodology capable of quantifying the total flavonoids present in the leaves of this species.

## MATERIALS AND METHODS

### Chemicals, glassware and solvents

All solvents used were of analytical grade: aluminium chloride (Vetec®), methanol (MeOH, Synth®, Vetec®), and ethanol (EtOH, Synth®). Phox® glassware was used. As standard for flavonoids, hydrated rutin (Sigma-aldrich®); purity ≥94% was used.

### Equipment

The equipment used were EVEN® analytical balance (model FA-2204B), Cristófoli® ultrasonic bath, ETHIK TECHNOLOGY® stove with air circulation (model 420-6TD), SOLAB® knife mill (model SL-31), EVEN® UV-Vis spectrophotometer (model IL-592), and Nova Instruments® UV-Vis Spectrophotometer (Model NI-1600 UV).

### Plant

The harvest of *M. nigra* leaves were performed at Fazenda Ouro Verde, located in the municipality of Casa Nova-BA (S 9°16'15"; W 40°51'44"). The samples were identified by the botanist José Alves de Siqueira Filho from Centro de Recuperação de Áreas Degradadas da Caatinga-CRAD. A voucher specimen was deposited in the HVASF Federal University of San Francisco Valley Herbarium (voucher number 1764).

### Experimental procedures

The plant material was subjected to a drying process in an oven with circulating air at 40°C for five days. Subsequently, it was

pulverized in a knife mill, obtaining a dry and pulverized vegetable material (500 g of dry powder). Exhaustive maceration was used to prepare the crude ethanolic extract, using ethanol 95%, for 12 days, performing successive extractions every 72 h. The extractive solution was concentrated in a rotary evaporator under reduced pressure at 50°C, obtaining 57.24 g of the crude ethanolic extract of *M. nigra* (Mn-EtOH).

The stock solution was prepared from Mn-EtOH (10 mg/ml) in MeOH (99.8%) with sonication for 10 min. This solution was diluted for 1.0 mg/ml in methanol concentration for further analysis. The AlCl<sub>3</sub> solution was prepared at 5% (w/v) with EtOH (99.5%). Triplicate tests were performed with 9, 10 and 11 ml of AlCl<sub>3</sub> added to the test solution and in the time of 0, 10, 20 and 30 min to verify the complexation reaction (Marques et al., 2012). The volume of 10 ml was chosen for the next analyses.

The procedures were performed without complexation (NCP) and with complexation (WCP), in order to evaluate the effect of the complexing agent (AlCl<sub>3</sub>) on increasing the selectivity for the quantification of the flavonoids. Aliquots were withdrawn in 0.5, 1 and 1.5 ml of the stock solution and completed to a volume of 100 ml with distilled water (NCP) and adding 10 ml of 5% AlCl<sub>3</sub>, adjusting the volume to 100 ml with distilled water (WCP), obtaining the test solutions.

#### Analysis with NCP

Initially, the analysis of the three levels of the samples in NCP was performed in triplicate, with the 1.0 mg/ml solution of Mn-EtOH. The analysis was carried out by scanning the UV-Vis spectrophotometer (EVEN®, model IL -592) with glass cuvettes, varying the wavelength from 300 to 500 nm (5 in 5 nm), using distilled water as the blank of the experiment, and the absorbance measured and recorded.

#### Analysis with WCP

The samples were then analyzed in triplicate with 1.0 mg/ml solution of Mn-EtOH containing 10 ml of AlCl<sub>3</sub> (WCP), first to verify the complexation reaction with the flavonoids, at 0, 10, 20 and 30 min. With this, it was possible to observe that there was no significant difference in relation to time, noting that the complexation reaction was immediate. Then, the same analysis of the three levels of the samples was carried out, in triplicate, in a spectrophotometer scan, varying the wavelength at 300 to 500 nm (5 in 5 nm), using 10 ml of AlCl<sub>3</sub> as the blank of the experiment adjusted for the final volume of 100 mL.

#### Determination of sample concentration and wavelength for reading

After the UV scan analysis, the means of each triplicate of the test solution (0.5, 1 and 1.5 ml stock solution in the NCP and WCP), were used to obtain a scan curve of each sample. Then, the wavelength where the highest absorption of the analytes occurred (major peak) was determined (Marques et al., 2012).

#### Validation of the analytical method

The procedures were evaluated according to the norms established by ANVISA, through specific resolution (ER 899, 2003), which defines what should be considered during the validation of analytical methods. The parameters specificity, linearity, precision (repeatability, intermediate precision and reproducibility), limits of detection and quantification, accuracy and robustness were

evaluated (Brazil, 2003).

#### Linearity

To measure linearity, three different calibration curves were prepared from seven concentration levels (0.05 to 0.15 mg/ml) at a volume adjusted to 100 ml, determining the equation of the line and the coefficient of determination for NCP and WCP. The calibration curves were obtained from the mean absorbance as a function of concentration.

#### Limits of detection (LOD) and quantification (LOQ)

LOD and LOQ were calculated from the calibration curves for the NCP and WCP, according to the following equations:

$$\text{LOD} = \text{SDa} \times 3/\text{IC} \quad (1)$$

$$\text{LOQ} = \text{SDa} \times 10/\text{IC} \quad (2)$$

Where, SDa is the standard deviation of the intercept with the Y axis, obtained from the average of the three linearity curves and IC is the slope of the line of the respective calibration curves.

#### Specificity

This parameter was determined for the overlap of UV spectra of RUT (200 µg/mL) and Mn-EtOH sample (1 mg/mL) in the treatment with and without complexing with 5% (m/v) AlCl<sub>3</sub> (WCP and NCP) in the range of 300 to 500 nm.

#### Precision

Precision was evaluated by three subparameters, repeatability, intermediate precision and reproducibility. Repeatability was performed by measuring the absorbance in six-fold of each triplicate of the stock solution (10 mg/ml) by a single-day analyst, resulting in a total of 18 determinations. Intermediate precision was performed in the same way, in six replicates of each of the three test solutions and the analyses were done by two analysts on two distinct days, with a total of 18 determinations each. The reproducibility analysis of the method was carried out in another laboratory, by varying the UV-Vis spectrophotometer model (Nova Instruments®, model NI-1600 UV).

#### Accuracy

Accuracy was analyzed by the recovery test, from the addition of a known amount of RUT (200 µg/ml). For the NCP samples, 2 ml of the test solution was added to each cuvette, with which the reading was read and then 100 µl of the rutin solution was added. In the WCP samples, the same procedures were performed and the reading was taken for 5 min, a time considered satisfactory for RUT complexation. The result of the recovery was obtained by Equation 3:

$$R (\%) = \text{TFC} - \text{CFE} / \text{CFP} \times 100 \quad (3)$$

Where, R is the percent recovery, TFC corresponds to the total flavonoid concentration (RUT) added to the Mn-EtOH solution, CFE corresponds to the concentration of the RUT in Mn-EtOH and CFP concentration of the RUT.

**Table 1.** Linear equation, linearity, limit of detection (LOD) and quantification (LOQ) of rutin and Mn-EtOH (WCP and NCP).

Sample	Types	Linear equation	R <sup>2</sup>	LOD (µg/mL)	LOQ (µg/mL)
Rutin	NCP	$y = (-0.02) + 2.14x$	0.983	18.1	60.3
	WCP	$y = (-0.002) + 3.37x$	0.997	2.2	7.4
Mn-EtOH	NCP	$y = 0.02 + 2.19x$	0.997	3.6	12.1
	WCP	$y = (-0.0328) + 8.162x$	0.997	$12.2 \times 10^3$	$40.8 \times 10^3$

The total flavonoids calculated for Mn-EtOH was  $228 \pm 0.0037$  µg of rutin equivalents/mg of the extract.

### Robustness

The robustness of the method was performed by varying the manufacturer of the MeOH used, Synth® by Vetec®. The procedure was performed with three stock solutions analyzed in triplicate.

### Statistical analysis

All analyzes were performed in triplicate and the reliability of the parameters was verified by the relative standard deviation (RSD%). The results were analyzed statistically by analysis of variance (ANOVA); One-Way or Two-Way, when applicable, being considered statistically significant F calculated less than tabulated F ( $p > 0.05$ ). The statistical treatment was obtained by the software OriginPro 8®.

## RESULTS AND DISCUSSION

Triplicate tests were performed with 9, 10 and 11 ml AlCl<sub>3</sub> added to the test solution. After that, statistical treatment of the absorbances was done at 0, 10, 20 and 30 min, and it was verified that there was no significant difference between the results, that is, the complexation reaction happened immediately. The 10 ml volume was chosen to continue the next analyses, due to the critical F value (6.9427) that was lower than the calculated F (3078.587).

### Linearity

This parameter was determined by constructing calibration curves of the extracts (RUT and Mn-EtOH) in seven concentration levels (0.05 to 0.15 mg/ml), of determination for NCP and WCP. The results are shown in Table 1. The correlation coefficients (R<sup>2</sup>) were obtained from linear regression analysis and were higher than 0.98 (WCP) and 0.99 (NCP) for RUT and 0.99 (WCP and NCP) for Mn-EtOH (Table 1).

The Brazilian legislation (Brazil, 2003) allows the methods to develop complex mixtures that are validated with correlation coefficient value, since there is more difficulty in obtaining accurate results when working with this type of sample.

### LOD and LOQ

The LOD and LOQ results were obtained from three different calibration curves for each standard (RUT) and Mn-ETOH, respectively. The LOD results found were 18.1 and 2.2 µg/mL for NCP and WCP, respectively. The LOQ results found were 60.3 and 7.4 µg/mL for NCP and WCP, respectively. The LOD results for Mn-ETOH found were 3.6 and  $12.2 \times 10^3$  µg/mL for NCP and WCP, respectively. The LOQ results found were 12.1 and  $40.8 \times 10^3$  µg/mL for NCP and WCP, respectively (Table 1).

From these results, it is possible to observe that the method provides spectrophotometric responses with high sensitivity to detect and quantify RUT in extract of *M. nigra*, with expected reliability and without changes of intrinsic factors.

### Specificity

It was possible to observe in the overlap of UV spectra, RUT that presents maximum absorption in 355 nm, while Mn-EtOH presents maximum absorption band near this wavelength, confirming that in this wavelength it is possible specifically to quantify the standard for flavonoids contained in the sample, even in the presence of other components of the extract. In the spectra of RUT + AlCl<sub>3</sub> and Mn-EtOH + AlCl<sub>3</sub> samples, a shift of the absorption band was observed for a bigger wavelength and a hyperchromic effect due to the complexation of the flavonoid with Al<sup>3+</sup> leading to a wavelength energy absorption higher than other phenolic compounds, avoiding interference in absorbance measurements (Fonseca et al., 2007).

### Precision

Precision was evaluated by three sub parameters: repeatability, intermediate precision and reproducibility both performed by two different analysts using the same equipment on two consecutive days. Repeatability and intermediate precision were assessed. The results are



**Table 2.** Results for repeatability parameter.

Sample	Mean ( $\mu\text{g/mL}$ )	DP	RSD%
NCP	0.276	0.01	3.64
WCP	0.808	0.008	0.99

**Table 3.** Results for intermediate precision analysis.

Parameter	Analyst	Day 1	Day 2	F
NCP	Analyst 1	0.276	0.288	<i>F cal</i> 0.00437
	Analyst 2	0.279	0.295	<i>F tab</i> 0.99564
WCP	Analyst 1	0.807	0.823	<i>F cal</i> 0.15206
	Analyst 2	0.819	0.840	<i>F tab</i> 0.71263

**Table 4.** Results for reproducibility analysis.

Spectrophotometers UV-Vis (model)	Mean ( $\mu\text{g/mL}$ ) $\pm$ RSD%	<i>F cal</i>	<i>F tab</i>
EVEN <sup>®</sup> (model IL -592)	NCP 0.2802 $\pm$ 0.00838%	0.3333	0.6667
	WCP 0.825 $\pm$ 0.00933%	0.3333	0.6667
Nova Instruments <sup>®</sup> (model NI-1600 UV)	NCP 0.2916 $\pm$ 0.0147%	0.3333	0.6667
	WCP 0.804 $\pm$ 0.0183%	0.3333	0.6667

**Table 5.** Results for robustness analysis.

Parameter	Variables	Mean ( $\mu\text{g/mL}$ ) $\pm$ RSD%	<i>F cal</i>	<i>F tab</i>
	Synth <sup>®</sup>	NCP 0.2802 $\pm$ 0.00838%	0.3333	0.6667
		WCP 0.825 $\pm$ 0.00933%	0.3333	0.6667
Solvent acquisition	Vetec <sup>®</sup>	NCP 0.2458 $\pm$ 0.0151%	0.3333	0.6667
		WCP 0.7504 $\pm$ 0.00168%	0.3333	0.6667

shown in Tables 2, 3 and 4.

The results for repeatability (intra-day) and intermediate precision (inter-day) showed values for RSD% lower than 15%, which is the maximum value pre-acquired by ANVISA for plant material. For the repeatability parameter, RSD% values were 3.64% for NCP and 0.99% for WCP (Table 2).

For intermediate precision, calculated F was lower than the tabulated F ( $p > 0.05$ ), so, no significant statistical difference was observed when the same analyst evaluated the method on different days and when different analysts evaluated on different days (Table 3). For reproducibility parameter, the calculated F was also lower than the tabulated F, inferring that no statistical difference was observed, and therefore the reproducible method. Therefore, the results are reliable and confirm an accuracy in accordance with what is recommended by the Brazilian legislation (Table 4).

### Accuracy

This parameter was evaluated by the analyte recovery method, adding a known amount of the RUT standard (200  $\mu\text{g/mL}$ ) in test solution. The result of the recovery was obtained in percentage by the equation described on validation procedures. The experimental data obtained revealed the average standard recovery of  $118.32 \pm 1.436\%$ , coefficient of variance 1.21%, attesting that this value is acceptable for natural products. These values show that the analytical method developed is sufficiently accurate.

### Robustness

For robustness of the solvent-proof procedures of the solvent acquisition MeOH (99.8%), the obtained data

show that all the procedures (NCP and WCP) were robust regarding the parameter analyzed, since the calculated F values were lower than the tabulated F (Table 5).

## Conclusions

In this work, a spectrophotometric method was developed to be used for routine analysis of flavonoids present in ethanolic extract of leaves of *M. nigra*. The reported developed protocol is simple, fast, specific, precise, accurate, robust and inexpensive and thus recommended for quantification and quality analysis of flavonoids in plant leaves.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# **Phenotypic characterization and symbiotic effectiveness test of chickpea (*Cicer arietinum* L.) rhizobia isolated from Dejen and Aneded Districts, East Gojjam Zone, Amahara Region, Ethiopia**

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**Chickpea (*Cicer arietinum* L.) is an important leguminous crop grown in different parts of Ethiopia. It is a nutritionally valued and N<sub>2</sub>-fixing legume which forms a symbiotic association with *Mesorhizobium*. This study was conducted to characterize and evaluate symbiotic efficacy of chickpea rhizobia isolated from soil samples collected from the study area. Seventeen chickpea rhizobia were isolated by soil host plant trap method and characterized for edaphic stress tolerance. Five of the isolates were grown at high temperature of 45°C and salt concentration of 6%, whereas seven were grown at acidic pH of 4, and four were grown at basic pH of 9. All isolates were not resistant to kanamycin, while fair resistance to erythromycin and streptomycin and modest resistance to ampicillin and azithromycin were observed. Furthermore, most of the isolates showed a variation in nodulation with higher (22 NN/p) and lowest (9 NN/p) scores. Shoot dry weight (SDW) of the plant ranged from 1.18 to 1.84 g/p and isolates showed effective (67%) to highly effective (100%) N<sub>2</sub>-fixing performance. From these, four isolates showed multiple edaphic stress resistance and are recognized as promising candidate for chickpea production in stressed soil; however, further study in the field is required.**

**Key words:** Chickpea, *Rhizobium*, edaphic stress, symbiotic effectiveness.

## **INTRODUCTION**

Chickpea (*Cicer arietinum* L.) is a cool season leguminous crop commonly grown in tropical, subtropical, temperate and semi-arid regions of the world (Miller et al., 2002; Singh et al., 2014). Ethiopia is considered as the center of secondary diversity for chickpea (Van der Maesen, 1987). Chickpea production ranks third among

pulse crops grown in the country next to Faba bean (*Vicia faba*) and Field pea (*Pisum sativum*). Spatially, Amhara regional state takes the first share and is considered as a potential chickpea producer with 62% of annual production (IFPRI, 2010). Among the existing chickpea varieties, *Desi* type that was preferably grown in semi-

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arid tropics is the most dominant in Ethiopia (Naser et al., 2008). Chickpea cultivation in the country covers more than 208,388.6 ha of the land (CSA, 2011).

Chickpea is being valued for its high dietary nutrition and serves as an invaluable source of protein and other nutrients for consumers (Shiferaw and Teklewolde, 2007; Mohammed et al., 2011). Chickpea contains 29% protein, 59% carbohydrate, 3% fiber, 5% oil and 4% ash, and it is a good source of absorbable ions like Ca, P, Mg, Fe and K (Christodoulou et al., 2005). Therefore, it is commonly incorporated as part of the different Ethiopian dishes and used for balanced diet.

On the other hand, chickpea serves as cash generating crop in the country with 312,000 tons of annual production and has appreciated export markets (IFPRI, 2010). For instance, in Ethiopia from the 48% of the pulse exported volume, chickpea accounts for about 27% of the total quantity production, while the remaining is used for domestic market and household consumption (Shiferaw and Teklewolde, 2007).

Besides its nutritional quality and source of income, chickpea plays tremendous role in soil fertility by improvement of symbiotic N<sub>2</sub>-fixation in association with Mesorhizobia bacteria (Werner, 2005; Funga et al., 2016). Improved soil fertility boosts crop production and maximizes chickpea yield (Jida and Assefa, 2012). The remaining plant biomass in the soil also increases nitrogen pool and serve as a nitrogen source for succeeding crops production by crop rotation cultivation process (Keneni et al., 2011; Beyene et al., 2013). Therefore, symbiotic N<sub>2</sub>-fixation of chickpea is economically cost effective and environmentally friendly alternative to benefit farmers and help in sustainable crop production by shift cultivation of crops with limited use of synthetic fertilizer (Tena et al., 2017).

The N<sub>2</sub>-fixation efficiency of chickpea infected by *Mesorhizobium* strains was determined by soil edaphic factors (Imran et al., 2015). Thus, the chickpea *Mesorhizobium* which were isolated from the local agro-ecology were expected to infect respective host plants and fix atmospheric nitrogen in a better way (Simon et al., 2014). This is because, indigenous Mesorhizobia are expected to have better adaptation mechanism of the localized soil ecological factors of a given farmland (Beyene et al., 2013). Hence, identifying efficient and superior N<sub>2</sub>-fixing *Mesorhizobium* strain from the local agro-ecology has paramount importance to enhance chickpea production and improve soil fertility.

Therefore, this study aimed to obtain efficient N<sub>2</sub>-fixing Mesorhizobial isolates from chickpea rhizosphere soils and identify potential isolates which could be substitutes of synthetic fertilizer for chickpea cultivation.

## MATERIALS AND METHODS

### Study site and sample collection

This study was conducted in two purposely selected districts

**Table 1.** Composition of YEMA medium.

Components	Amounts used
Mannitol	10 g/l
K <sub>2</sub> HPO <sub>4</sub>	0.5 g/l
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.2 g/l
NaCl	0.1 g/l
Yeast extract	0.5 g/l
Agar	15 g/l
Distilled water	1000 ml
pH	7±0.1

Autoclaved at 121°C for 15 min.

located in East Gojjam Zone, Amahara Regional state, Ethiopia because of their dominant production of chickpea (Dejen and Aneided). From those districts, the most potential chickpea grower kebeles (smaller administrative next to district) were identified during the field survey. In each selected kebele, one chickpea farm was taken as soil sample source. From these chickpea farms, triplicated soil samples were pooled by digging at 20 to 30 cm depth. Composite soil samples were collected using ethanol sterilized (70%) plastic bags in November 2015. Collected composite samples were then taken to Microbiology Laboratory, Department of Biology, Debre Markos University (DMU) for further work.

### Nodule collection

Nodules were collected from chickpea by using soil-plant trap method in pots under greenhouse condition (Vincent, 1970). The chickpea plants grown in the collected soil samples for 45 days were uprooted and large sized, yellow colored nodules were picked and surface-sterilized (Somesagaran and Hoben, 1994).

### Rhizobium isolation

Entrapped Mesorhizobia were isolated from the collected nodules after brief surface sterilization using 70% ethanol for 10 s and 5% local bleach for 3 min (Vincent, 1970). Then, treated nodules were rinsed five times by using sterilized water. Sterilized nodules were then crushed and loop full of sap was transferred onto yeast extract mannitol agar (YEMA) containing plates (Table 1). Inoculated plates were incubated in a bacteriological incubator adjusted at a temperature of 28°C for 3-5 days (Vincent, 1970). After growth, a single colony was picked up and purified periodically by re-streaking method on the fresh YEMA medium. Then, pure isolates were preserved on YEMA slants containing 0.3% (w/v) CaCO<sub>3</sub> and stored in refrigerator adjusted at a temperature of 4°C (Vincent, 1970).

### Authentication of the isolates

Isolates infectivity of *Desi* types of chickpea was confirmed by inoculating them onto plant seedlings. Activated isolates were inoculated onto chickpea seedlings planted on sand filed plastic pots and allowed to grow for 45 days in the greenhouse. After 45 days of the growth, plant were uprooted and the existence of nodule were checked.

### Characterization of the Isolates

All isolates were checked on YEMA medium containing 25 µg ml<sup>-1</sup>

Congo red to evaluate their ability to absorb the dye. In addition, isolates were inoculated on medium containing 25 µg ml<sup>-1</sup> bromothymol blue (BTB) to determine their ability to produce acid or base and color change of medium were observed (Lupwayi and Haque, 1994). Furthermore, appearance, color and size of the grown colonies were examined on YEMA plates.

### Physiological characterization of isolates

All tests were carried out three times on YEMA plates and compared with control. The isolates growth was qualitatively determined and recorded as (+) for growth, (±) for limited growth and (-) for no growth.

### Salt tolerance

Isolates were tested for their salinity tolerance using YEMA medium supplemented with NaCl at concentrations of 0.1, 0.3, 0.5, 0.8, 1, 2, 3, 4, 5 and 6% (w/v) (Belay and Assfa, 2011).

### Temperature tolerance

The ability of isolates growth at high and low temperatures were monitored using YEMA medium incubated at 5, 10, 15, 35, 40 and 45°C (Jida and Assefa, 2012).

### pH tolerance

Isolates acid and alkaline tolerance were evaluated by growing them on the medium where pH was adjusted to 4, 4.5, 5, 5.5, 8 and 9 using sterile HCl and NaOH (Belay and Assefa, 2011).

### Carbohydrates utilization by the isolates

Carbohydrate utilization by isolates was determined using the methods described by Somasegaran and Hoben (1994) on six carbohydrates. These carbohydrates were prepared as 10% (w/v) solution in water. Carbohydrate free medium, which is essentially similar to YEMA medium were modified by reducing yeast extract to 0.05 g/L. Heat-labile carbohydrate solutions were sterilized by membrane filtration method using Millipore with a pore size of 0.22 µm and added to the autoclaved basal medium. The heat-stable carbohydrates were autoclaved together with the medium. YEMA medium without carbon source and with mannitol was used as negative and positive controls, respectively.

### Intrinsic antibiotic resistance (IAR)

The intrinsic antibiotic resistance of isolates was determined using some selected antibiotics. The tested antibiotics were ampicillin, streptomycin, kanamycin, erythromycin, azithromycin and chloramphenicol. These antibiotics were incorporated into YEMA medium after membrane filter sterilization using 0.22 µm size at the concentration of 2.5, 5 and 10 µg/ml (Beynon and Josey, 1980). Then, the isolates growth and failure were recorded.

### Evaluation of isolates N<sub>2</sub>-fixation effectiveness

The effectiveness of isolates was tested in a pot experiment conducted in greenhouse condition. 3 kg of carefully washed, sieved and HCl acid sterilized river sand were filled with alcohol-

sterilized (70%) plastic pots. Chickpea seeds of uniform size and color were surface sterilized as described before and transferred to 0.75% (w/v) of water agar plates and allowed to germinate at 25°C for 3 days. Four chickpea seedlings were transferred into each pot, which were later thinned down to three. Each isolates grown in YEMA broth medium to logarithmic phase were adjusted to 10<sup>9</sup> cells ml<sup>-1</sup>. Activated 1 ml of isolates were inoculated onto each seedling (1 ml/seedling) of the sand culture. The experiment set up was a complete randomized design (CRD) with three replicates. A plus -N with no inoculation and a non-inoculated with no N were used as the controls. The plus control contains 70 mg/L of N applied as a 0.05% KNO<sub>3</sub> (w/v) solution every week (Somasegaran and Hoben, 1994). Plants were supplied with tap water every two days and fertilized once a week with the quarter strength of N-free nutrient solution (Belay and Assefa, 2011). Plants growth were carried out in a greenhouse with a 12/12 h light/dark cycle. Finally, after 45 days of growth, all plants were harvested and the roots were scored for nodulation. The top plants and nodules were oven dried at 70°C for 48 h to determine the dry weight.

The percentage of isolates symbiotic effectiveness were calculated using equation proposed by Date et al. (1993) and indicated in Belay and Assefa (2011) with N<sub>2</sub>-fixing effectiveness classified as ineffective <35%; lowly-effective, 35 to 50%; effective, 50 to 80%; and highly effective, >80%.

$$SE (\%) = \frac{SDW \text{ of inoculated plants}}{SDW \text{ of N-fertilized plants}} \times 100\%$$

SE = Symbiotic effectiveness; SDW = shoot dry weight.

### Data analysis

Data analysis was done using one way analysis of variance (ANOVA) using version 20 SPSS statistical program. Mean separation was calculated using Tukey's HSD test when the value was significant at p = 0.05.

## RESULTS AND DISCUSSION

A total of 17 chickpea bacteria were recovered from the rhizospheric soil collected from chickpea farms of two purposely selected Districts (Dejen and Aneded) by using soil-host plant trap method. All the isolates were authenticated as chickpea rhizobia by re-inoculation test using sterilized sand-filled pot experiment.

Colony characteristics and dyes absorption ability of the isolates are summarized in Table 2. After 72 h of growth on YEMA medium, colonies were found to be large in size (3.0 to 5.0 mm), diameter, and showed large mucoid, watery, flattened and raised appearance similar to the findings obtained by Singh and Bamanian (2012). Most of them were colorless and transparent, while some became yellowish after 3 days of the growth. The staining experiment also confirmed that all bacterial cell wall were stained as pink as the color of safranin and grouped under Grahams' negative category (Agrawal et al., 2012). Furthermore, isolates showed considerable diversity on bromothymol blue (BTB) color conversion after 48 h of the growth. The isolates changed YEMA-BTB medium to yellow and deep yellow were categorized as fast growing

**Table 2.** Sample site, colony morphology and dye absorbance of isolates.

Sample collected kebeles	Designated isolates	Colonies morphological characteristics				YEMA-BTB test	YEMA-CR test	Graham reaction
		Colony size (mm)	Colony appearance	Colony structure	Colony color	25 µg/ml	25 µg/ml	
Tike	DMU-1	3.0	D	F	CL	MY	NA	-ve
Yetnora	DMU-2	3.0	LM	R	Y	MY	NA	-ve
Terch	DMU-3	1.5	LM	R	CL	Y	NA	-ve
Konkoy	DMU-4	2.5	LW	F	CL	DY	A	-ve
Koncher	DMU-5	1.5	LM	R	Y	DY	A	-ve
Koncher	DMU-6	5.0	LW	R	CL	VLY	A	-ve
Enajima	DMU-7	3.2	LW	F	CL	N	A	-ve
Gudalima	DMU-8	5.0	LW	R	CL	DY	A	-ve
Sebshengo	DMU-9	4.0	LW	F	CL	MY	A	-ve
Denbukebay	DMU-10	3.0	D	F	CL	MY	A	-ve
Zemetin	DMU-11	2.0	LM	R	CL	DY	A	-ve
Denbukebay	DMU-12	4.5	LW	F	CL	Y	A	-ve
Gudalima	DMU-13	5.2	LW	F	CL	Y	A	-ve
Yetnora	DMU-14	5.3	LW	F	CL	MY	A	-ve
Terch	DMU-15	3.5	LW	F	Y	DY	A	-ve
Zemetin	DMU-16	3.0	LM	R	Y	DY	A	-ve
Sebshengo	DMU-17	4.5	LM	R	CL	MY	LA	-ve

D, Dry; LM, large mucoid; LW, large watery; R, raised; F, flatten; Y, yellow; CL, color less; MY, moderately yellow; DY, deep yellow; VLY, very less yellow; N, not changed; NA, not absorbed; A, absorbed; -ve, negative.

and others which changed to moderate yellow and did not show any color change were considered as a slow growing rhizobia. Chickpea rhizobia was reported to have both fast and slow growing strains (Nour et al., 1994). Moreover, isolates obtained from chickpea nodules failed to grow on BTB-medium (Wei et al., 2003). Most of the isolates were Congo red dyes absorbent, except the three DMU-1, DMU-2 and DMU-3.

Edaphic condition of the soil is the most determinant factor for successful symbiotic association of *Rhizobium* with their host plants. Temperature, pH, salinity, antibiotic tolerance and carbohydrates utilization are important parameters to characterize rhizobia by consideration as a phenotypic identification marker (Maatallah et al., 2002). Temperature and pH tolerance of isolates is presented in Table 3. Chickpea rhizobia in this study showed a variation in these parameters. Almost all the isolates were grown at a temperature range of 5 to 40°C. Temperature tolerance of chickpea rhizobia ranging from 10 to 42°C has been already reported in India (Rai et al., 2012). Only five (DMU-1, DMU-2, DMU-10, DMU-14 and DMU-15) isolates were tolerant to temperature at 45°C. These isolates were expected to have high temperature resistancy and considered as an important candidate to develop inoculants as a bio-fertilizer. Furthermore, all the isolates grew well at pH range of 4.5 to 8.5 and this report is in line with findings of Kucuk et al. (2006), Baoling et al. (2007) and Singh and Bamanian (2012).

Most importantly, eight isolates (DMU-3, DMU-6, DMU-8, DMU-9, DMU-11, DMU-13 and DMU-17) showed their acidity tolerance by growing at pH 4. These isolates were considered as fast-growing strains as recognized from BTB-medium dyes conversion test and important candidate for acidic soil. This report is in agreement with findings of Gao et al. (1994) that showed that the rhizobia grown at pH as low as 4 were grouped under fast-growing strains whereas, four isolates such as DMU-6, DMU-7, DMU-10 and DMU-14 were grown at pH 9. Some chickpea rhizobial isolates grew very well at pH 10 and tolerance to alkalinity increased at pH 11 (Singh et al., 2015). These alkaline condition preferring rhizobia were reported as slow-growing strains (Anand and Dogra, 1991). However, a number of reports indicated complete growth failures of chickpea rhizobia at pH of 9 (Kucuk et al., 2006; Baoling et al., 2007; Singh and Bamanian, 2012). In this study, isolates showed edaphic factor tolerance diversity and similar with findings of Rai et al. (2012). Salinity test result also showed bacterial diversity towards different concentrations of the salt (Table 4). All the isolates were grown on the medium containing NaCl salt concentration ranging from 0.1 to 2%. Some isolates tolerated salt concentration upto 4%, while only a few isolates were grown at 5 and 6% of salt concentration. Most isolates are reported not to grow from 5% NaCl concentration and salt tolerance ability reduced with increase in salt concentration (Saraf and

**Table 3.** Evaluation of temperature and pH tolerance of the isolates.

Isolates	Temperature test (°C)						pH test					
	5	10	15	35	40	45	4	4.5	5	5.5	8.5	9
DMU-1	+	+	+	+	+	+	-	+	+	+	±	-
DMU-2	+	+	+	+	+	+	-	+	+	+	+	-
DMU-3	+	+	+	+	+	-	+	+	+	+	+	-
DMU-4	+	+	+	+	+	-	-	+	+	+	+	-
DMU-5	+	+	+	+	+	-	-	+	+	+	+	-
DMU-6	+	+	+	+	+	-	+	+	+	+	+	+
DMU-7	+	+	+	+	+	-	-	+	+	+	+	+
DMU-8	+	+	+	+	+	-	+	+	+	+	+	-
DMU-9	+	+	+	+	+	-	+	+	+	+	+	-
DMU-10	+	+	+	+	+	+	-	+	+	+	+	+
DMU-11	+	+	+	+	+	-	+	+	+	+	+	-
DMU-12	+	+	+	+	+	-	-	+	+	+	+	-
DMU-13	-	-	+	+	+	-	+	+	+	+	+	-
DMU-14	+	+	+	+	+	+	-	+	+	+	+	+
DMU-15	+	+	+	+	+	+	-	+	+	+	+	-
DMU-16	+	+	+	+	+	-	-	+	+	+	+	-
DMU-17	+	+	+	+	+	-	+	+	+	+	+	-

+, Growth of isolates; -, nongrowth of isolates; ±, growth of very few colonies.

**Table 4.** Salt tolerance and carbohydrates utilization test of isolates

Isolates	Salt concentrations (%)										Carbohydrate utilization tests						
	0.1	0.3	0.5	0.8	1	2	3	4	5	6	Fructose	Dextrose	Dextrin	Lactose	Maltose	Sucrose	Control
DMU-1	+	+	+	+	+	+	-	-	-	-	-	+	+	+	±	-	+
DMU-2	+	+	+	+	±	±	-	-	-	-	-	+	+	+	+	-	+
DMU-3	+	+	+	+	+	+	+	+	±	-	-	+	+	+	+	-	+
DMU-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DMU-5	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
DMU-6	+	+	+	+	+	+	±	±	-	-	-	+	+	+	+	+	+
DMU-7	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+
DMU-8	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	±	+
DMU-9	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+
DMU-10	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+
DMU-11	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+
DMU-12	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+
DMU-13	+	+	+	+	+	±	-	-	-	-	+	+	+	+	+	+	+
DMU-14	+	+	+	+	+	±	±	±	-	-	+	+	+	+	+	+	+
DMU-15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DMU-16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DMU-17	+	+	+	+	+	±	-	-	-	-	+	+	+	+	+	+	+

+, Growth of isolates; -, nongrowth of isolates; ±, growth of very few colonies.

Dhandhukia, 2005; Singh and Bamanian, 2012).

The isolates grown at 5 and 6% NaCl concentration were considered as salt tolerate and expected to have better adaptability to salty soil conditions. Therefore,

isolates designated as DMU-4, DMU-5, DMU-11, DMU-15 and DMU-16 were grouped as salt tolerant rhizobial groups in this study.

Similarly, the salt tolerant isolates were better utilized

**Table 5.** Intrinsic antibiotic resistance test of the isolates.

Isolates	Antibiotics concentration range ( $\mu\text{g}$ )																		
	Ampicillin			Chromaphynicol			Erythromycin			Streptomycin			Azithromycin			Kanamycin			
	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	
DMU-1	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	-	-	-
DMU-2	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
DMU-3	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
DMU-4	+	+	+	+	-	-	+	+	+	-	-	-	+	+	+	-	-	-	-
DMU-5	+	+	+	-	-	-	+	+	+	+	-	-	+	+	+	-	-	-	-
DMU-6	+	+	+	-	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-
DMU-7	+	+	+	+	-	-	+	+	-	+	-	-	+	+	+	-	-	-	-
DMU-8	+	+	+	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-	-
DMU-9	+	+	+	-	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-
DMU-10	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-
DMU-11	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-
DMU-12	+	+	+	-	-	-	+	+	-	+	+	-	+	+	+	-	-	-	-
DMU-13	+	+	+	-	-	-	+	+	-	+	+	-	+	+	-	-	-	-	-
DMU-14	+	+	+	-	-	-	+	+	-	+	+	-	+	+	+	-	-	-	-
DMU-15	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	-	-	-	-
DMU-16	+	+	+	+	-	-	+	+	-	+	+	-	+	+	+	-	-	-	-
DMU-17	+	+	+	-	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-

+, Growth of isolates; -, nongrowth of isolates.

among all tested carbohydrates. Only isolates DMU-5 and DMU-11 failed to grow on the disaccharide sugar, fructose, although tolerant to high salt concentration (Table 4). All the isolates were grown on dextrose, dextrin, lactose and maltose; however, isolates DMU-1, DMU-2, DMU-3, DMU-5, DMU-6, DMU-10 and DMU-11 failed to grow on the fructose and sucrose. With regards to this, there is well-established fact on *Rhizobium* utilization of various carbon sources for their growth and this is used as important tool to characterize the isolates (Maatallah et al., 2002). Rhizobial strains isolated from chickpea nodules were reported to utilize mannitol, lactose, sucrose, sorbitol, arabinose, galactose, mannose, maltose and raffinose as carbon sources (Singh and Bamanian, 2012). Especially, fast-growing rhizobia were broadly recognized to grow on several types of carbon substrates, whereas slow growing rhizobia were grown only on very limited types of carbon sources. However, in this study, almost all isolates were grown on the tested carbohydrates and a broad range of carbohydrates was used as sources of carbon for growth. Hence in this regard, the result of this study is in line with the results of other studies (L'taief et al., 2007; Jida and Assefa, 2012). It is very interesting to note that chickpea *Mesorhizobium* can utilize a broad spectrum of carbohydrates for their cell growth and development. Such characteristics are usually used as diagnostic features for root nodule bacteria to test different carbon sources for their survival (Kucuk and Kivanc, 2008).

On the other hand, as summarized in the Table 5,

majority of the isolates failed to tolerant several types of antibiotics in different concentrations especially isolates that were completely susceptible to kanamycin and only a few isolates were tolerant to chloramphenicol at  $2.5 \mu\text{g l}^{-1}$  concentration. However, most of the isolates were grown on different concentrations of Ampicillin, Erythromycin and Azithromycin antibiotics. Isolates grown on the Streptomycin showed very less tolerance with respect to concentration. Several studies reported the existence of broad variation among chickpea rhizobia with respect to the fate of their intrinsic antibiotics resistance (Maatallah et al., 2002; Kucuk and Kivanc, 2008). The isolates' sensitivity to antibiotics may be due to inability to resist exposed toxicity with less adaptation in natural environments (Singh and Bamanian, 2012).

The legume food crop production was expected to be boosted by use of indigenous rhizobia as biofertilizer to supplement nitrogen requirement for cultivation of plants. Symbiotic effectiveness test was carried out to select the best  $\text{N}_2$ -fixing strains among the obtained isolates (Table 6). In this greenhouse experiment, all the 17 isolates showed variation in host plant nodulation, with scores lesser (9 N/p) up to higher (22 N/p) nodules per plant. Although, plants showed a variation on nodulation,  $\text{N}_2$ -fixation efficiency were found within effective up to highly effective ranges. Especially, shoot dry weight value was proved and considered as a direct indicator of isolates'  $\text{N}_2$ -fixation efficiency. Furthermore, this study showed the maximum (1.84 g/p) and minimum (1.18 g/p) shoot dry mass. For instance, isolate DMU-5, that scored high



**Table 6.** Symbiotic effectiveness evaluation of isolates in the greenhouse condition.

Isolates	NN p <sup>-1</sup>	NDW g p <sup>-1</sup>	SFW g p <sup>-1</sup>	SDW g p <sup>-1</sup>	NFE (%)	Score
DMU-1	15 <sup>c</sup>	0.102 <sup>a</sup>	5.85 <sup>c</sup>	1.30 <sup>d</sup>	74	E
DMU-2	22 <sup>a</sup>	0.112 <sup>a</sup>	7.17 <sup>ab</sup>	1.54 <sup>b</sup>	88	HE
DMU-3	17 <sup>b</sup>	0.072 <sup>c</sup>	7.39 <sup>ab</sup>	1.54 <sup>b</sup>	88	HE
DMU-4	14 <sup>c</sup>	0.091 <sup>ab</sup>	7.12 <sup>ab</sup>	1.69 <sup>ab</sup>	97	HE
DMU-5	21 <sup>a</sup>	0.093 <sup>ab</sup>	8.31 <sup>a</sup>	1.84 <sup>a</sup>	100	HE
DMU-6	17 <sup>b</sup>	0.056 <sup>f</sup>	5.95 <sup>bc</sup>	1.48 <sup>bc</sup>	85	HE
DMU-7	15 <sup>c</sup>	0.062 <sup>e</sup>	8.33 <sup>a</sup>	1.74 <sup>a</sup>	99	HE
DMU-8	12 <sup>d</sup>	0.075 <sup>c</sup>	7.07 <sup>abc</sup>	1.49 <sup>bc</sup>	85	HE
DMU-9	16 <sup>c</sup>	0.103 <sup>a</sup>	6.92 <sup>b</sup>	1.46 <sup>c</sup>	83	HE
DMU-10	12 <sup>d</sup>	0.054 <sup>f</sup>	4.79 <sup>d</sup>	1.32 <sup>d</sup>	75	E
DMU-11	11 <sup>de</sup>	0.062 <sup>e</sup>	5.28 <sup>cd</sup>	1.22 <sup>de</sup>	70	E
DMU-12	14 <sup>c</sup>	0.095 <sup>ab</sup>	6.63 <sup>b</sup>	1.47 <sup>bc</sup>	84	HE
DMU-13	10 <sup>e</sup>	0.082 <sup>b</sup>	6.23 <sup>b</sup>	1.39 <sup>cd</sup>	79	E
DMU-14	9 <sup>e</sup>	0.076 <sup>c</sup>	4.61 <sup>d</sup>	1.37 <sup>cd</sup>	78	E
DMU-15	12 <sup>d</sup>	0.097 <sup>ab</sup>	4.02 <sup>de</sup>	1.18 <sup>e</sup>	67	E
DMU-16	14 <sup>c</sup>	0.068 <sup>d</sup>	5.31 <sup>c</sup>	1.38 <sup>d</sup>	79	E
DMU-17	13 <sup>d</sup>	0.082 <sup>b</sup>	5.21 <sup>cd</sup>	1.40 <sup>cd</sup>	80	HE
Control N+			8.33 <sup>d</sup>	1.75 <sup>a</sup>		
Control N-			3.21 <sup>e</sup>	1.15 <sup>f</sup>		

NN p<sup>-1</sup>, Nodule number per plants; NDW p<sup>-1</sup>, nodule dry weight per plant; SFW p<sup>-1</sup>, shoot fresh weight per plant; SDW p<sup>-1</sup>, shoot dry weight per plant; NFE, N<sub>2</sub> fixation effectiveness; E, effective; HE, highly effective.

SDW of 1.84 g/p was highly effective (100%) and isolate, DMU-7 that scored SDW of 1.74 g/p was very effective (70%) on N<sub>2</sub>-fixation performance. Nitrogen fixation performance was positively associated with plant SDW (Qureshi et al., 2013). Nine isolates, namely DMU-2, DMU-3, DMU-4, DMU-5, DMU-6, DMU-7, DMU-8, DMU-9, and DMU-12 were potential N<sub>2</sub>-fixing isolates with highly effective (85 - 100%) fixation performance. The other isolates showed moderate percentage of N<sub>2</sub>-fixation performance variation. Such variation in each evaluated parameters were expected to depend on the chickpea bacterial diversity in the soil (Sahgal and Johri, 2003).

Some of the isolates that showed two or more environmental stress tolerance has attracted the interest of investigators in this study. Especially, four isolates such as DMU-6, DMU-10, DMU-14, and DMU-15 showed multiple abiotic stress tolerance, namely high temperature, pH and salinity, with scoped effective (67 to 78%) to highly effective (85%) performance of N<sub>2</sub>-fixation (Table 6).

Similarly, there are chickpea rhizobia which were reported from the alkaline condition of Indian soil (Singh et al., 2015). Therefore, these isolates were expected to be used as bio-fertilizer inocula in future, particularly in stressed farmlands to boost chickpea production and improve soil fertility. Inoculation with bacterial bio-fertilizer to farmland to improve crop production is not sole target rather it can have greater role to reduce the application of synthetic nitrogenous fertilizer on the farmland there by

reducing pollution (Kennedy et al., 2004; Mia and Shamsuddin, 2010). However, biological N<sub>2</sub>-fixation (BNF) use has an incredible role in substituting commercially synthetic N-fertilizer in cereal production thereby, reducing the environmental problem (Agrawal et al., 2012). The dependency and high amounts of synthetic fertilizers application are both costly for farmers and set the hazardous problem on nature and biodiversity. Therefore, BNF provides a better alternative to chemical fertilizers as the process, besides supplying nitrogen to crop, enriches soil nitrogen content and maintains soil health and productivity (Reddy and Reddy, 2004).

## Conclusion

As shown in the study, chickpea rhizobia isolated from rhizosphere soil showed variation in agro-ecological stresses tolerance. These isolates which were tolerant to edaphic stresses could be the potential asset for an alternative source of environmentally friendly bio-fertilizer and potential resources for varied agro-ecology. Isolates from this study showed sounding tolerance to temperature, pH and salinity and could have potential to tolerate environmental toxicity and hence increase N<sub>2</sub>-fixation effectiveness to enhance soil fertility in chickpea farming, thus increasing chickpea production. On the other hand, sensitive strains are least in tolerance to environmental toxicity and hence may not improve

chickpea production. From this study, it could be deduced that nodulation performance of the rhizobia strain is positively correlated to N<sub>2</sub>-fixing effectiveness as well as higher shoots dry weight which confirms high assimilation of nitrogen of the chickpea seedlings. The isolates achievement on the N<sub>2</sub>-fixing process is very high and had better nodulation, and effective to highly effective fixation performances. Thus, this confirms the presence of potentially efficient chickpea rhizobia candidates in the rhizospheric soil of the study area although further work on field condition is needed.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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