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ISOLATION AND IDENTIFICATION OF A CELLULOLYTIC FUNGUS FROM ANIMAL MANURE.

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Fungi are considered the best cellulose decomposers in nature. More than 80% of cellulose degradation in nature is accomplished by fungus. The most know fungal species that degrade cellulose are members of the Basidiomycota (Baldrian and Valášková, 2008), Ascomycota (Hernández *et al.*, 2018), and Chytridiomycota encountered in the rumen of some animals (Gleason *et al.*, 2011). Fungi are recognized for their ability to secrete large quantities of extracellu-

lar cellulase making them preferable for industry. Cellulases have been successfully produced in diverse industrial sectors from different fungal species such as *Aspergillus* (Bansal *et al.*, 2012), *Trichoderma* (Ellilä *et al.*, 2017), and *Penicillium* (Prasanna *et al.*, 2016). A various number of cellulase enzymes have been produced from fungal species such as *Aspergillus niger*. (Mrudula and Murugammal, 2011), *Aspergillus ornatus* (Toor and Ilyas, 2014), *Aspergillus terreus* (Gao *et al.*, 2008).

The cellulase production from *Trichoderma* species are widely known, but it lacks significant levels of β-glucosidase. As a result, the enzyme cocktails from *Aspergillus* species are used instead though with lower titers but contains higher activities of β-glucosidase (Gusakov, 2011). Cellulose degrading enzymes isolated from *Aspergillus* genus members have been extensively studied and the genomic organization of many of the enzymes has been reported (de Vries and Visser, 2001).

Agricultural waste can be an economically sustainable and renewable source of energy. The achievability of agricultural waste as a resource of energy mainly depends upon its hydrolysis of the lignocellulosic biomass for the production of desired metabolites and biofuels. The effective converting of agricultural wastes to fermentable sugars offers outstanding benefits such as improving strategic security, decreasing the trade deficits, better environmental quality, and securing a sustainable energy resource supply (Kamm and Kamm, 2004). Cellulose is one of the most abundant polysaccharides and the key component of plant biomass. Cellulose biodegradation by cellulases and cellulosomes is preferred over chemical degradation due to the absence of sugar degradation in the enzymatic process (Sari *et al.*, 2017).

Three major components constitute the cellulosic enzyme system. First, the endo-ß-glucanase (EC 3.2.1.4) breaks internal glycosidic linkages of the amorphous region of cellulose, yielding glucose and cello-oligo saccharides. Second, the exo-ß-glucanase (EC 3.2.1.91) catalyzes the production of either cellobiose or glucose units from the reducing and non-reducing ends of the cellulosic fibril. Finally, the ß-glucosidase (EC 3.2.1.21) hydrolyze cellobiose into glucose. These enzymes work synergistically to degrade cellulose to glucose units that can be used in many applications (Lynd *et al.*, 2002).

Solid-state fermentation (SSF) is a favored method for the growth of microorganisms on moist solid supports, including inert carriers and insoluble substrates. This technology has been long-established for the efficient production of cellulase enzymes using cultivating conditions of fungi that simulate their natural environment (Sukumaran *et al.*, 2009). SSF is preferred over the submerged fermentation (SmF), because of the higher concentration of products, decreased production cost, simpler fermentation equipment, minor effluent production, and less waste

output. Many reports studied the use of cheap and easily available substrates like bagasse and lignocellulose waste materials, for reducing the cost of cellulases production (Yoon *et al.*, 2014). Other studies tested the solid-state fermentation of *Aspergillus terreus* using agricultural waste (Gao *et al.*, 2008; Ismail and Hassan, 2020).

The current report describes the isolation of cellulose-degrading fungal strain, AGE-1, from cow manure. The strain was molecularly identified using 18S rRNA gene and ITS as *Aspergillus terreus*. AGE-1 fungal strain was tested for the cellulases induction under various parameters such as pH, incubation temperature, incubation time, carbon sources. Moreover, its ability to cellulases production using solid-state fermentation was optimized.

MATERIALS AND METHODS

Isolation and qualitative screening of cellulolytic activity of isolated fungal strain

In this study, cellulose-degrading fungal isolates were collected from cow rumen. Serial dilutions of the sample were cultured onto potato dextrose agar plates and incubated for 7 days at 30ºC. Single colonies were picked and sub-cultured to obtain a pure isolate. Stock cultures were maintained on potato dextrose agar slants at 4ºC (Devi and Kumar, 2012). Cellulase producing fungi were screened on selective carboxymetyl cellulose agar plates containing (g/L): $NaNO_3$ 2.0, KH_2 PO_4 1.0, $MgSO_4$ $7H_2O$ 0.5, KCl 0.5, carboxymethyl cellulose sodium salt 10.0, peptone 0.2, agar 17.0. Plates were spot inoculated with a spore suspension of pure culture and incubated at 30ºC. After three days, plates were flooded with 1% Congo red solution for 15 minutes then destained with 1M NaCl solution for 15 minutes. The diameter of the zone of decolorization around each colony was measured (Teather and Wood, 1982).

Quantitative screening of cellulolytic activity of isolated fungi

Purified fungal isolates were tested for cellulases production on Sugar cane Bagasse as a sole carbon source. Sugar cane Bagasse was washed, dried then milled to small particle sizes; about 2 mm long. The milled bagasse was mixed with 0.12% NaOH and autoclaved at 121ºC for 20 min. The autoclaved bagasse was washed again with tap water to remove the excess of NaOH till it becomes neutral and dried at 80ºC (Gutierrez-Correa and Tengerdy, 1997).

One gram of treated Sugar cane Bagasse (in 250 mL Erlenmeyer flask) was supplemented with 100 ml basal mineral medium contained (g/L): (KH_2PO_4, 2g; $(NH_4)_2SO_4$, 1.4 g; Urea, 0.3g; $CaCl_2 \cdot 2H_2O$, 0.3g; $MgSO_4 \cdot 7H_2O$, 0.3g; peptone, 1.0g; Tween 80, 0.2%; $CoCl_2 \cdot 6H_2O$, 2.0 mg; $FeSO_4 \cdot 7H_2O$, 5.0mg; $MnSO_4 \cdot 2H_2O$, 1.6 mg; $ZnSO_4 \cdot 7H_2O$ 1.4 mg., Two ml of fungal spore suspension (10^6 spores/ml) were inoculated into a 100 ml of the cellulases

production medium (pH 5.5) and incubated at 30ºC and agitated at 170 rpm in a rotary shaking incubator. After seven days, the culture filtrate was collected by centrifugation (6000 rpm for 15 min) and the supernatant was used as a source of crude cellulase enzyme (Vega *et al.*, 2012).

Enzymes assay

The fungal isolates were assayed for CMCase and FPase activities according to Ghose, (1987). One milliliter of diluted crude enzyme extract was mixed with one milliliter of the substrate (1% CMC prepared in 100 mM sodium acetate buffer, pH 4.8) to determine CMCase activity. One milliliter of diluted crude enzyme extract was mixed with one milliliter of 100 mM sodium acetate buffer (pH 4.8) containing 50mg (1x6cm strip) of Whatman filter paper no.1 for the determination of the FPase activity. The reactions were incubated at 50ºC for 30 min and 60 min in the case of CMCase and FPase assay, respectively. The released reducing sugar was estimated spectrophotometrically at 540nm by the addition of 3,5-dinitrosalicylic acid (DNS) with glucose as standard. The activity of the enzyme was expressed in IU/ml; one unit (IU) of enzyme activity is defined as the amount of enzyme required to release 1 µmol of glucose in the reaction mixture per minute under the specified conditions.

Effect of initial medium pH, temperature and incubation temperature on cellulase production.

Different pHs ranged from 4-10, varying temperatures ranged from 30ºC - 37ºC, and different incubation periods ranged from 4-10 days, were tested for determining the best conditions for cellulases induction. Each parameter was tested individually while all other parameters were kept constant (Vega *et al.*, 2012). Sugar cane bagasse (SCB) and carbon methylcellulose (CMC), were tested as a sole carbon source in a basal mineral media at 30ºC and 170 rpm. The production media was used and inoculated by 3 ml (1 x 10^7 spores/ml) and all the flasks were incubated in a shaking incubator at 170 rpm (New Brunswick, USA). Enzyme assays of both FPase and CMCase were determined as described above.

Molecular identification of fungal strain

For molecular identification, genomic DNA was extracted using GeneJET Genomic DNA Purification Kit (ThermoFisher®, California, USA). Two specific primers ITS5 (5-TCCTCCGCTTATTGATATGC-3), ITS4 (5-GGAAGTAAAAGTCGTAACAAGG-3) (Zheng *et al.*, 2009) in the Internal Transcribed Spacer (ITS) region of the rRNA were used for PCR amplification of a 600 bp. The *18S rRNA* gene was amplified using this pair of forward and reverse primer NS1 (18S) (5-GTAGTCATATGCTTGTCTC-3) and NS4 (18S) (5-CTTCCGTCAATTCCTTTAAG-3)

(Panzer *et al.*, 2015). PCR was performed using the standard reaction mixture (50 µl) containing; 1× PCR buffer, 1.5 mM MgCl2, 200 mM of each dNTPs, 15 pmol of each primer, 1 U of Taq polymerase enzyme (Promega® Corporation, Madison, USA), and 50 ng of DNA template. The PCR was carried out as follows: primary denaturation for 5min at 94ºC; 30 cycles of denaturation at 94ºC for 30 s; annealing at 58°C for 30 s, and extension at 72ºC for 60s; and an additional extension for 5min at 72°C. DNA sequencing of the purified PCR products was performed using an ABI BigDye Terminator V3.1 cycle sequencing kit and 3130xl genetic analyzer (Applied Biosystem, USA).

Phylogenetic analysis

The raw sequences (forward and reverse) *18S rRNA* gene and ITS region of the rRNA gene were assembled with Sequencher version 4.0.5 (Gene (Gene Code Corporation, Ann Arbor, MI, USA). The 18S rRNA and ITS assembled contigs were blasted for similarities to DNA sequences deposited in the GenBank using BLASTN program and submitted to the GenBank nucleotide sequence database (NCBI). The sequences were aligned using ClustalW version 1.8 (Altschul *et al.*, 1997). Further phylogenetic analysis was carried out using Molecular Evolutionary Genetics Analysis (MEGA) version7 (Kumar *et al.*, 2016), using the maximum likelihood methods (ML) and 1000 bootstrap replication to assess branching confidence. The Jones-Taylor-Thornton (JTT) model was selected as the substitution model.

Solid-state fermentation

A 7 days fungus maintained on potato dextrose agar (PDA) slants and incubated at 30ºC was used to prepare spore suspension by scratching the growing fungus in each slant with 10 ml distilled water containing 0.1% tween 80. Spores of fugal strain (5.33×10^7, total amount) were used to inoculate 100 mL of modified Mandels and Weber medium (Szijártó *et al.*, 2004) in 500 mL conical flasks, incubated at 30ºC and shaken 200 rpm for fungus propagation. After 3 days, 10 mL of culture containing the growing fungus mycelia (3.8 g/L) was transferred into 1 L Erlenmeyer flasks containing 200 mL of media supplemented with Avicel CE-15 (10 g/L, microcrystalline cellulose, (FMCBiopolymer, Philadelphia, USA).

For testing the carbon sources, five grams of the dry carbon source was mixed with the mineral solution (gram per liter distiller water: $(NH_4)_2SO_4$ 3.5 g, KH_2PO_4 3 g, $MgSO_4$ $7H_2O$ 0.5 g, $CaCl_2$ 0.5 g) in 250 ml Erlenmeyer flasks, which were all sterilized for 30 min at 121ºC in succession (Juhász *et al.*, 2005).

RESULTS AND DISCUSSION

Isolation, and screening of cellulase producing fungal isolates

Twelve fungal isolates were obtained based on their ability to grow on basal mineral medium containing sugar-

cane bagasse pulp as the sole carbon source. These fungal isolates were endoglucanase producers judged by showing hydrolyzing clear zones on agar plates containing CMC-Na and iodine staining (Kasana *et al.*, 2008). The hydrolyzing zone diameter and colony diameter were recorded for all tested isolates (Table 1) which confirmed that all isolated fungal isolates were able to grow and secrete endoglucanase.

The quantitative screening using (3,5-dinitrosalicylic acid) DNS method (Adney and Nrel, 2008) showed that all twelve fungal isolates produced exoglucanase (FPase) and endoglucanase (CMCase) (Table 1). The Fungal isolates showed FPase activity ranged from 0.006 to 0.41 U/ml, while the CMCase activity ranged from 0.007 U/ml to 0.142 U/ml. Fungal isolate AGE-1 showed the highest CMCase activity with 0.143 IU/ml in addition to 0. 41 U/ml for FPase, and was further characterized.

Some studies propose a direct relation between log enzyme concentration and the diameter of the cleared zone in the Congo-red staining method (Teather and Wood, 1982; Florencio *et al.,* 2012). Other studies showed that the isolated microorganisms did not follow this pattern (Liang *et al.*, 2014). In the present study, the isolates showed a direct relationship between the enzyme concentration and the diameter of the cleared zone in agreement with Teather and Wood, (1982) and Florencio *et al.,* (2012).

Optimization of Culture Conditions for Enzyme Production

Cellulases production or induction of FPase and CMCase from AGE-1 isolate were assessed at different pH values (Fig. 1A). The results clearly showed that the optimum pH for FPase and CMCase activity were pH 6.0. The enzyme activity gradually increased when increasing the pH up to the optimum followed by a gradual fall in the activity. The maximum values at pH 6.0 were 1.44 U/ml, and 0.88 U/ml for CMCase and FPase, respectively. Our results are in the same range as those reported for FPase and CMCase produced by *Aspergillus terreus* (Shahriarinour *et al.*, 2011). Whereas, the FPase and CMCase production by *Aspergillus niger* MS82 were maximal when the initial culture pH was between 6.0 or 7.0 (Sohail *et al.*, 2009).

The results illustrated by Fig.(1) clearly show that CMCase production, expressed as enzyme activity, gradually increased as the incubation period increased reaching the maximum at the seventh day, 1.44 U/ml ±0.02 when incubated at 30ºC. The maximum activity for CMCase was 1.30 U/ml ± 0.03 on day 7 when the fungal isolate incubated at 34ºC, and 0.966 U/ml ± 0.05 when incubated at 37ºC. A slow decrease of the CMCase was observed on the following days (Fig. 1B-D). The FPase gradually increased as the incubation period increased reaching the maximum on the seventh day, 0.88 U/ml ±0.03 when incubated at ºC. The maximum activity for FPase was on day 7 and

it reaches 0.783 U/ml ± 0.03 when the isolate incubated at 34 °C and reached 0.482 U/ml ± 0.09 when incubated at 37ºC (Fig. 1 B-D). Our results are in accordance with Sohail *et al.,* (2009); and Shahriarinour *et al.,* (2011), which confirmed the decrease of FPase and CMCase production with the increase of temperature.

Sugarcane bagasse and carboxymethyl cellulose were tested for their effects on cellulases production. Data presented in Fig. (2) showed that Bagasse was the better sole source of carbon for cellulases production as the activity was 0.74 U/ml for FPase and 1.29 U/ml for CMCase (Fig. 2).

Identification of AGE-1 using ITS and *18S rRNA*

The sequence of Internal Transcribed Spacer (ITS) region of *rRNA* gene on the AGE-1 genome was deposited in NCBI under the Accession No LC593154.1. The molecular analysis of the ITS region of the AGE-1 fungal isolate exhibited maximum homology with *A. terreus* strain SF 981 (accession number MT530257.1), *A. terreus* strain SF 938 (accession number MT530214.1), *A. nomius* strain F_1 (accession number MN700028.1) and *A. niger* strain AY01 (accession number MT771988.1). Maximum likelihood (ML) phylogenetic tree based on ITS region was generated and determined the evolutionary relatedness of the fungal isolates AGE-1 (Fig. 3). Phylogenetic analysis showed that the AGE-1 isolate was grouped with type strains belonging to *A. terreus*, *A. nomius* and *A. niger* (Fig. 3).

For more accurate molecular identification, the *18S rRNA* gene of the AGE-1 isolate was amplified and sequenced. The sequence was deposited in NCBI with Accession No LC593155.1. Nucleotide BLAST of the *18S rRNA* of the AGE-1 fungal isolate showed the highest similarity with *A. terreus* strain 23A (accession number MN995500.1), *A. terreus* strain AZM03 (accession number MH550051.1), *A. terreus* strain 8F (accession number MN995485.1), and *A. terreus* strain 6Fb (accession number MN995482.1). Maximum likelihood (ML) phylogenetic tree based on 18S rRNA was generated and confirmed the identification of the AGE-1 isolate as *A. terreus* strain (Fig. 4). Phylogenetic analysis showed that the AGE-1 isolate grouped with strains belonging to *A. terreus*. The AGE-1 was grouped in a separate clade with *A. terreus* strains, which is separated from the *A. nomius* strains. The phylogentic tree showed that *A. niger* strains, *A. fumigatus* and *A. flavus* form a separate clades that are separated from the AGE-1 strain (Fig. 4).

The use of *18S rRNA* genes, *26S rRNA* genes, *5.8S rRNA* genes, *28S rRNA* genes and ITS sequences of different fungi for the study of their genetic relationship is a well-established tool (Sugita *et al.*, 2002; Woo *et al.*, 2010). In our report, the use of ITS phylogeny could not differentiate between the closely related *Aspergillus* strains, while the use of *18S rRNA*

phylogeny resolved this tack. This is contrary to another study that suspected the ability of *18S rRNA* gene to identify *Aspergillus* down to the species and suggested the use of ITS region for this purpose (Henry *et al.*, 2000).

Optimization of FPase and CMCase production using solid-state fermentation.

SSF process is a very important fermentation strategy in countries that have huge plant biomass wastes. *Aspergillus* spp. and *Rhizopus* spp were frequently used for the production of α-amylase, lipase, caseinase, and cellulases. The two species were preferred due to their ubiquity and modest nutritional requirements. Lignocellulosic waste such as banana peels, date wastes, rice straw, sugarcane bagasse and many others were used for the biosynthesis of the aforementioned enzymes (Acourene and Ammouche, 2012 and Aliyah *et al.*, 2017). AGE-1 fungal strain was tested for the FPase and CMCase production using SSF (Table 2). The results are illustrated in Table. (2) which showed that CMCase production reached the maximum expression, 235 U per one gram of dry carbon source when incubated at^{o}C and pH7. The same condition showed the maximum expression of FPase at 92 U per one gram of dry carbon source (Table 2).

Testing carbon sources for Fpase and CMCase production by AGE-1 Fungal strain using solid-state fermentation.

Cellulase production is usually dependent on the nature of the carbon source in the culture medium. The utilization of agricultural wastes as a carbon source for the production of cellulase instead of using the refined cellulosic carbon source can reduce the cost of the production and make the enzyme production economically feasible. The ability of *A. terreus* AGE-1 for the cellulases production in the SSF using four different relatively cheap components as carbon source was investigated. The effect of sugarcane bagasse (SCB), wheat straw, ground sesame and rice hey as carbon source on the cellulase biosynthesis on A. *terreus* AGE-1 was recorded (Fig. 5). Results showed that sugarcane bagasse (SCB) is the best sole source of carbon for cellulase production as cellulase activity was 62 U for FPase and 288 U per one gram of dry carbon source for CMCase (Fig. 5). Wheat straw showed similar values for the cellulases production, as it produced 58 U for FPase and 274 U per one gram of dry carbon source for CMCase (Fig. 5).

The productivity of the CMCase and FPase using the isolated strain is comparatively high, indicating the efficiency of the isolated strain to utilize bagasse as a carbon source for the production of cellulase. Our results are in accordance with the results of Gao *et al.*, (2008), which shows similar values for CMCase and FPase using bagasse in the solid-state fermentation of *Aspergillus terreus* M11. Various reports favored the utilization of rice straw as the fermentation carbon source in fungal production (Aggarwal *et al.*, 2017). Sugar cane bagasse is stored in a huge amount in one place in Egypt, the sugar company. The utilization of sugar cane bagasse as the fermentation carbon source in the fungal production of cellulases will be a reliable

source and can reduce its disposal of environmental pollution hazards.

SUMMARY

Successful conversion of lignocellulose waste to fermentable sugars should open the door for the production of many desired metabolites. Fungi are the best cellulose decomposers in nature. The present study describes the isolation of cellulose-degrading fungal strain, AGE-1, from cow manure. AGE-1 fungal strain was tested for the cellulases production under various pHs, incubation temperatures, incubation time, and carbon sources. The AGE-1 isolate showed the maximum activity for CMCase of 1.44 U/ml ± 0.02 on day 7 when the fungal isolate was incubated at 30 °C. The maximum activity for FPase was on day 7 and it reaches 0.88 U/ml ± 0.03 when the isolate incubated at 30ºC. The strain was molecularly identified using the 18S rRNA gene and ITS as *Aspergillus terreus*. The strain was tested for cellulases production using solid-state fermentation and showed maximum expression of FPase and CMCase at 30ºC and pH7. Various agricultural wastes were tested as substrates for cellulase production by solid-state fermentation, and sugarcane bagasse (SCB) was the best sole source of carbon for cellulase production followed by wheat straw. The isolated strain can be used as a starting step for degrading agricultural waste that can be used for the production of biofuel and the production of desired metabolites from cellulose.

REFERENCES

Acourene S., and Ammouche A., (2012). Optimization of Ethanol, Citric Acid, and α-Amylase Production from Date Wastes by Strains of *Saccharomyces cerevisiae*, *Aspergillus niger*, and *Candida guilliermondii*. Journal of Industrial Microbiology and Biotechnology 39, no. 5 (May 2012): 759-66. https://doi.org/10.1007/s10295-011-1070-0.

Adney B., and Nrel J. B., (2008). Measurement of Cellulase Activities Laboratory Analytical Procedure." Renewable Energy, no. January: 8.

Aggarwal Kumar Neeraj, Goyal Varsha, Saini Anita, Yadav Anita, and Gupta R., (2017). Enzymatic Saccharification of Pretreated Rice Straw by Cellulases from Aspergillus Niger BK01. 3 Biotech 7, no. 3 (July 1, 2017): 1-10. https://doi.org/10.1007/s13205-017-0755-0.

Aliyah Andi, Alamsyah Gandhi, Ramadhani Rizky, and Hermansyah H., (2017). Production of α-Amylase and β-Glucosidase from Aspergillus Niger by Solid State Fermentation Method on Biomass Waste

Substrates from Rice Husk, Bagasse and Corn Cob. In Energy Procedia, 136:418-423. Elsevier Ltd, 2017. https://doi.org/10.1016 /j.egypro.2017.10.269.

Altschul Stephen F., Alejandro Madden Thomas L., Schäffer A., Zhang Jinghui, Zhang Zheng, Miller Webb, and Lipman D. J., (1997). Gapped BLAST and PSI-BLAST: A New Generation of Protein Database Search Programs. Nucleic Acids Research. Oxford University Press, September 1, 1997. https://doi.org /10.1093/nar/25.17.3389.

Baldrian Petr, and Valášková V., (2008). Degradation of Cellulose by Basidiomycetous Fungi. FEMS Microbiology Reviews. Oxford Academic, May 1. https://doi. org// 10.1111/j.1574-6976.2008.00106.x

Bansal Namita, Tewari Rupinder, Soni Raman, and Soni S. K., (2012). Production of Cellulases from Aspergillus Niger NS-2 in Solid State Fermentation on Agricultural and Kitchen Waste Residues. Waste Management 32, no. 7 (July 1, 2012): 1341-46.https: // doi.org/10.1016/j.wasman.2012.03. 006.

Devi Mc., and Kumar M., (2012). Production, Optimization and Partial Purification of Cellulase by Aspergillus Niger Fermented with Paper and Timber Sawmill Industrial Wastes. Journal of Microbiology and Biotechnology Research 2, no. 1: 120-28.

Ellilä Simo, Lucas Fonseca, Uchima Cristiane, Cota Junio, Goldman Gustavo Henrique, Saloheimo Markku, Sacon Vera, and SiikaAho M., (2017). Development of a Low-Cost Cellulase Production Process Using Trichoderma Reesei for Brazilian Biorefineries. Biotechnology for Biofuels 10, no. 1: 30. https://doi.org/10.1186 /s13068-017-0717-0.

Florencio Camila, Couri Sonia, and Farinas C. S., (2012). Correlation between Agar Plate Screening and Solid-State Fermentation for the Prediction of Cellulase Production by Trichoderma Strains. Enzyme Research 2012. https://doi.org/ 10.1155/2012/793708.

Gao Jianmin, Weng Haibo, Daheng Zhu, Yuan Mingxue, Guan Fangxia, and Xi Y., (2008). Production and Characterization of Cellulolytic Enzymes from the Thermoaci-dophilic Fungal Aspergillus Terreus M11 under Solid-State Cultivation of Corn Stover. Bioresource Technology 99, no. 16: 7623-29. https://doi.org/10.1016/j.biortech.2 008.02.005.

Ghose T. K., (1987). Measurement of

Cellulase Activities. Pure and Applied Chemistry 59, no. 2: 257-68. https://doi.org/10.1351/pac198759020257.

Gleason F. H., Marano A. V., Digby A. L., Al-Shugairan N., Lilje O., Steciow M. M., Barrera M. D., Inaba S., and Nakagiri A., (2011). Patterns of Utilization of Different Carbon Sources by Chytridiomycota. Hydrobiologia 659, no. 1:55-64. https://doi.org/10.1007/s10750-010-0461-y.

Gusakov Alexander V., (2011). Alternatives to Trichoderma Reesei in Biofuel Production. Trends in Biotechnology. Elsevier. https://doi.org/10.1016/j.tibtech.2011.04.004.

Gutierrez-Correa Marcel, and Tengerdy R. P., (1997). Production of Cellulase on Sugar Cane Bagasse by Fungal Mixed Culture Solid Substrate Fermentation. Biotechnology Letters 19, no.7:665-67. https://doi.org/10.1023/A:1018342916095.

Henry Travis, Iwen Peter C., and Hinrichs S. H., (2000). Identification of Aspergillus Species Using Internal Transcribed Spacer Regions 1 and 2. Journal of Clinical Microbiology 38, no.4:1510-15. https://doi.org/10.1128/jcm.38.4.1510-1515.2000.

Hernández Christian, Milagres Adriane M. F., Vázquez-Marrufo Gerardo, Muñoz-Páez Karla María, José Antonio García-Pérez, and E. Alarcón (2018). An Ascomycota Coculture in Batch Bioreactor Is Better than Polycultures for Cellulase Production. Folia Microbiologica 63, no. 4: 467-78. https://doi.org/10.1007/s12223-018-0588-1.

Ismail Shaymaa A., and Hassan A., (2020). Optimizing the Production of Rice Straw Hydrolytic Cellulase under Solid-State Fermentation Using Aspergillus Terreus RS2. Egyptian Pharmaceutical Journal 19, no. 1 (2020):7. https://doi.org/10.4103/epj.epj_44_19.

Juhász T., Szengyel Z., Réczey K., Siika-Aho M., and Viikari L., (2005). Characterization of Cellulases and Hemicellulases Produced by Trichoderma Reesei on Various Carbon Sources. Process Biochemistry 40, no. 11: 3519-25. https://doi.org/10.1016/j.procbio.2005.03.057.

Kamm B., and Kamm M., (2004). Principles of Biorefineries. Appl Microbiol Biotechnol, April 2004. https://doi.org/10.1007/s00253-003-1537-7.

Kasana Ramesh Chand, Salwan Richa, Dhar Hena, Dutt Som, and Gulati A., (2008). A Rapid and Easy Method for the Detection of

Microbial Cellulases on Agar Plates Using Gram's Iodine. Current Microbiology 57, no. 5: 503-7. https://doi.org/10.1007/s00284-008-9276-8.

Kumar Sudhir, Stecher Glen, and Tamura K., (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular Biology and Evolution 33, no. 7: 1870-74. https://doi.org/10.1093/molbev/msw054.

Liang Yan Ling, Zhang Zheng, Wu Min, Wu Yuan, and Feng J. X., (2014). Isolation, Screening, and Identification of Cellulolytic Bacteria from Natural Reserves in the Subtropical Region of China and Optimization of Cellulase Production by Paenibacillus Terrae ME27-1. BioMed Research International 2014. https://doi.org/10.1155/2014/512497.

Lynd Lee R., Weimer Paul J., Van Zyl Willem H., and Isak S., (2002). Microbial Cellulose Utilization : Fundamentals and Biotechnology Microbial Cellulose Utilization : Fundamentals and Biotechnology. Microbiology and Molecular Biology Reviews 66, no. 3: 506-77. https://doi.org/10.1128/MMBR.66.3.506.

Mrudula Soma, and Murugammal R., (2011). Production of Cellulase by Aspergillus Niger under Submerged and Solid State Fermentation Using Coir Waste as a Substrate. Brazilian Journal of Microbiology 42, no. 3 (2011): 1119-27. https: // doi. org/10.1590/S1517-83822011000300033.

Panzer Katrin, Yilmaz Pelin, Weiß Michael, Reich Lothar, Richter Michael, Wiese Jutta, Schmaljohann Rolf, *et al*., (2015). Identification of Habitat-Specific Biomes of Aquatic Fungal Communities Using a Comprehensive Nearly Full-Length 18S RRNA Dataset Enriched with Contextual Data. PLoS ONE 10, no. 7 (2015): 1-20. https://doi.org/10.1371/journal.pone.0134377.

Prasanna H. N., Ramanjaneyulu G., and Reddy B. R., (2016). Optimization of Cellulase Production by Penicillium Sp. 3 Biotech 6, no. 2. https://doi.org/10.1007/s13205-016-0483-x.

Sari, Wenny Novita, Safika Darmawi, and Fahrimal Y., (2017). Isolation and Identification of a Cellulolytic Enterobacter from Rumen of Aceh Cattle. Veterinary World 10, no. 12: 1515-20. https://doi.org/10.14202/vetworld.2017.1515-1520.

Shahriarinour Mahdi, Abdul Wahab Mohd Noor, Mohamad Rosfarizan, Mustafa Shuhaimi, and Ariff A. B.,

(2011). Effect of Medium Composition and Cultural Condition on Cellulase Production by Aspergillus Terreus. African Journal of Biotechnology 10, no. 38: 7459-67. https://doi.org/10.5897/AJB11.199.

Sohail Muhammad, Siddiqi Roquya, Ahmad Aqeel, and Khan S. A., (2009). Cellulase Production from Aspergillus Niger MS82: Effect of Temperature and PH. New Biotechnology 25, no. 6: 437-41. https://doi.org/10.1016/j.nbt.2009.02.002.

Sugita Takashi, Nakajima Masamitsu, Ikeda Reiko, Matsushima Toshiharu, and Shinoda T., (2002). Sequence Analysis of the Ribosomal DNA Intergenic Spacer 1 Regions of Trichosporon Species. Journal of Clinical Microbiology 40, no. 5: 1826-30. https://doi.org/10.1128/JCM.40.5.1826-1830.2002.

Sukumaran Rajeev K., Rani Reeta Singhania, Mathew Gincy Marina, and Pandey A., (2009). Cellulase Production Using Biomass Feed Stock and Its Application in Lignocellulose Saccharification for Bio-Ethanol Production. Renewable Energy 34, no. 2: 421-24. https://doi.org/10.1016/j.renene.2008.05.008.

Szijártó Nóra, Szengyel Zsolt, Lidén Gunnar, and Réczey K., (2004). Dynamics of Cellulase Production by Glucose Grown Cultures of Trichoderma Reesei Rut-C30 as a Response to Addition of Cellulose. In Proceedings of the Twenty-Fifth Symposium on Biotechnology for Fuels and Chemicals Held May 4-7, 2003, *in Breckenridge, CO*, 115-24. Humana Press, 2004. https://doi.org/10.1007/978-1-59259-837-3_10.

Teather R. M., and Wood P. J., (1982). Use of Congo Red-Polysaccharide Interactions in Enumeration and Characterization of Cellulolytic Bacteria from the Bovine Rumen. Applied and Environmental Microbiology 43, no. 4: 777-80. https://doi.org/10.1128/aem.43.4.777-780.1982.

Toor Yasmin, and Ilyas U., (2014). Optimization of Cellulase Production by *Aspergillus ornatus* by the Solid State Fermentation of Cicer Arietinum. Vol. 2. www.usa-journals.com.

Vega Karin, Gretty Villena K., Victor Sarmiento H., Ludeña Yvette, Vera Nadia, and Gutiérrez-Correa M., (2012). Production of Alkaline Cellulase by Fungi Isolated from an Undisturbed Rain Forest of Peru. Biotechnology Research

International 2012: 1-7. https: //doi.org/10.1155/2012/934325.

Vries Ronald de P., and Visser J., (2001). Aspergillus Enzymes Involved in Degradation of Plant Cell Wall Polysaccharides. Microbiology and Molecular Biology Reviews 65, no. 4: 497-522. https://doi.org/ 10.1128/mmbr.65.4.497-522.2001.

Woo Patrick C. Y., Shui Yee Leung, Kelvin K. W. To, Jasper F. W., Chan, Ngan Antonio H. Y., Vincent Cheng C. C., Lau Susanna K. P., and Yuen K. Y., (2010). Internal Transcribed Spacer Region Sequence Heterogeneity in Rhizopus Microsporus: Implications for Molecular Diagnosis in Clinical Microbiology Laboratories. Journal of Clinical Microbiology 48, no. 1: 208-14. https://doi.org/10.1128/ JCM.01750-09.

Yoon Li Wan, Nam Ang Teck, Ngoh Gek Cheng, and Chua A. S. M., (2014). Fungal Solid-State Fermentation and Various Methods of Enhancement in Cellulase Production. Biomass and Bioenergy 67: 319-38. https://doi .org/10.1016/j.biombioe.2014.05.0 13.

Zheng Linyong, Jia Dinghong, Fei Xiaofan, Luo Xia, and Yang Z., (2009). An Assessment of the Genetic Diversity within Ganoderma Strains with AFLP and ITS PCR-RFLP. Microbiological Research 164, no. 3: 312-21. https://doi.org/ 10.1016/j.micres.2007.02.002.

Table (1): Evaluation of Exoglucanase (FPase) activity and Endoglucanase (CMCase) activity of the cellulose-degrading fungal isolates.

Isolate code	D/d	FPase (U/ml)	CMCase (U/ml)
AGE-1	37/3.1	0.41 ± 0.002	0.143 ± 0.002
AGE-2	28/2.4	0.038 ± 0.001	0.0.75 ± 0.003
AGE-3	21/1.9	0.024 ± 0.002	0.061 ± 0.004
AGE-4	24/2.2	0.024 ± 0.01	0.062 ± 0.001
AGE-5	11/2.1	0.016 ± 0.003	0.034 ± 0.003
AGE-6	17/3.0	0.018 ± 0.002	0.031 ± 0.002
AGE-7	14/2.4	0.010 ± 0.003	0.026 ± 0.004
AGE-8	25/2.5	0.045 ± 0.001	0.085 ± 0.004
AGE-9	21/2.1	0.028 ± 0.001	0.071 ± 0.007
AGE-10	10/2.5	0.012 ± 0.002	0.029 ± 0.002
AGE-11	9/2.0	0.01 ± 0.001	0.012 ± 0.002
AGE-12	7/2.1	0.006 ± 0.002	0.007 ± 0.005

"*D*/*d*": hydrolyzed zone diameter/colony diameter on agar media containing CMC as sole carbon source;

Table (2): Evaluation of Exoglucanase (FPase) activity and Endoglucanase (CMCase) activity of the cellulose degrading fungal isolates using SSF.

Trial	pH (Fermenter)	Temp	Humidity / moisture	Cmcase	FPase
1	6.5	30	75%	215	78
2	6.5	35	75%	200	75
3	7.0	30	75%	235	92
4	7.0	35	75%	220	76
5	7.5	30	75%	222	68
6	7.5	35	75%	210	67
7	8.0	30	75%	195	61
8	8.0	35	75%	189	60

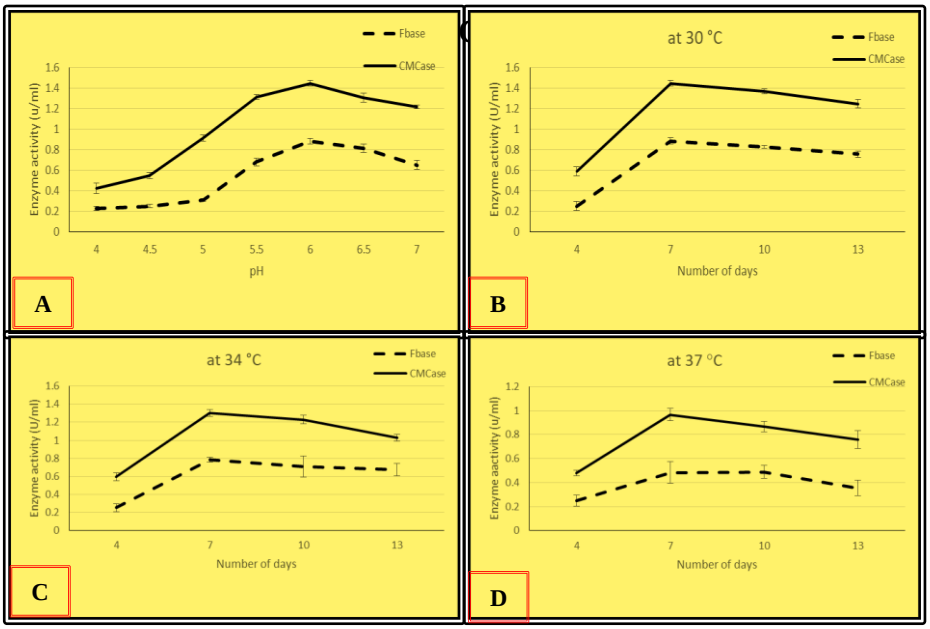

Fig. (1): Effect of pH and incubation time at different incubation temperatures on the activity of FPase and CMCase for *A. terreus* AGE-1. (**A):** Effect of different pH, at 30ºC, on the production of cellulases by *A. terreus* AGE-1. Effect of incubation period on the production of cellulases at incubation temperature30ºC (**B**), 34ºC (**C**) andº37ºC (**D**).

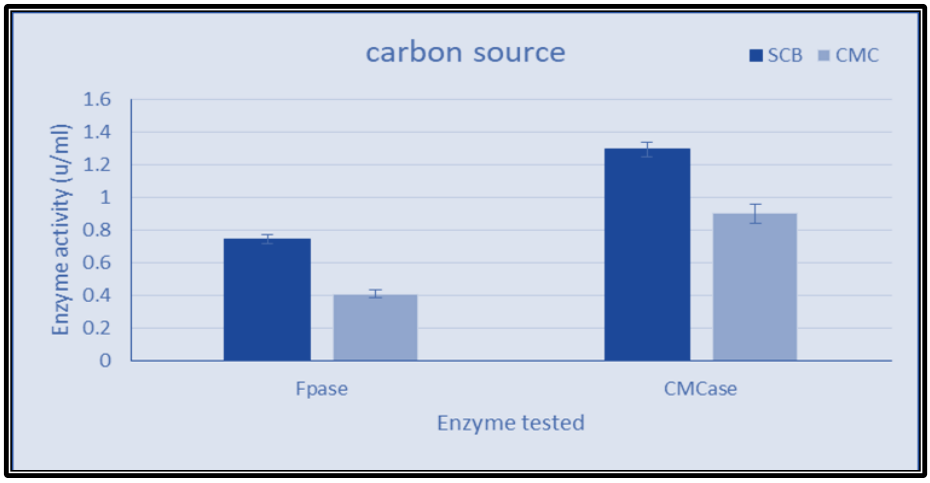

Fig. (2): Effect of SCB and CMC as sole carbon sources for the induction of FPase and CMCase.

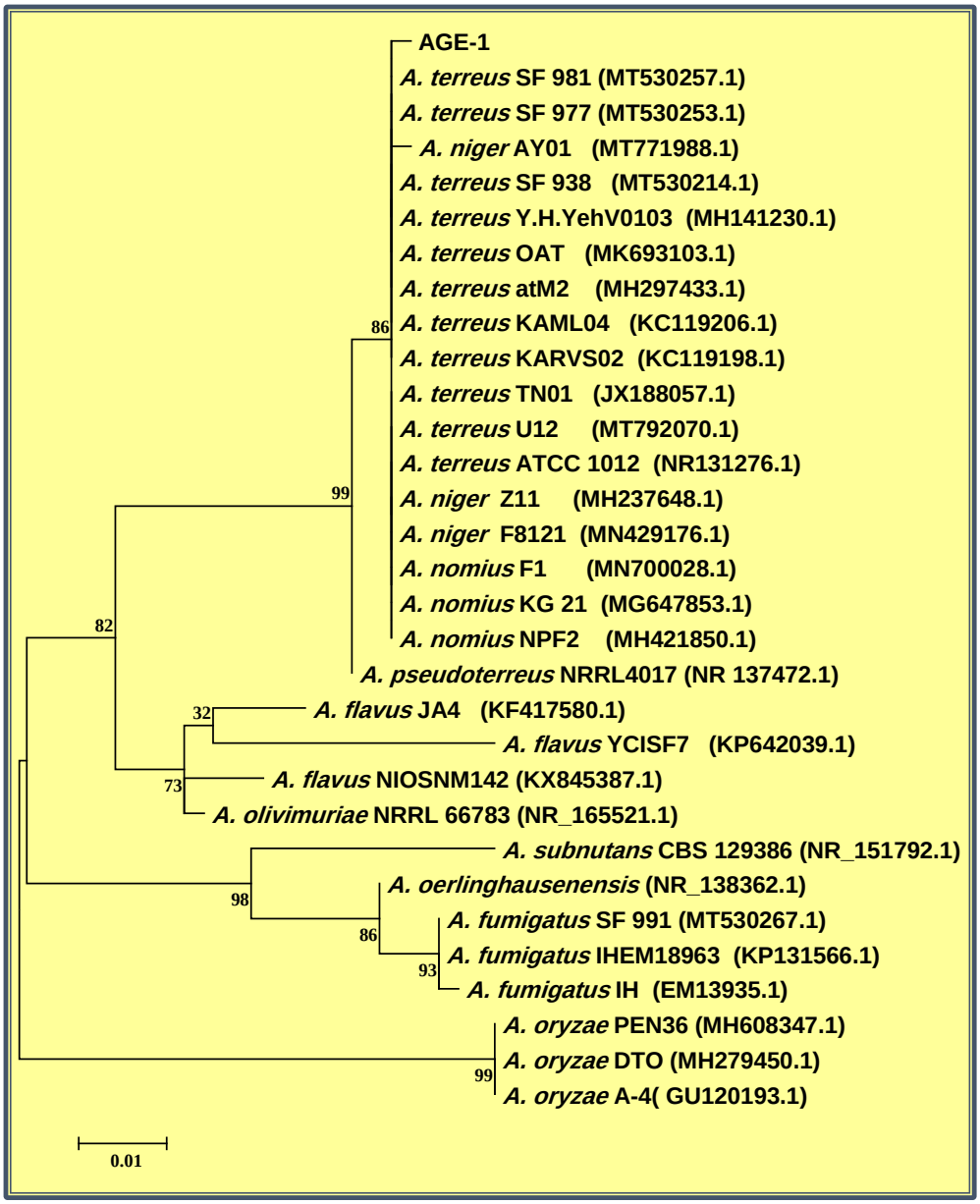

Fig. (3): Phylogenetic tree based upon the Internal Transcribed Spacer (ITS) region of *rRNA* gene of the AGE-1obtained by maximum likelihood (ML) method. GenBank accession numbers are indicated next to the strain name. Bootstrap values were calculated from 1000 trees and shown at the branching points.

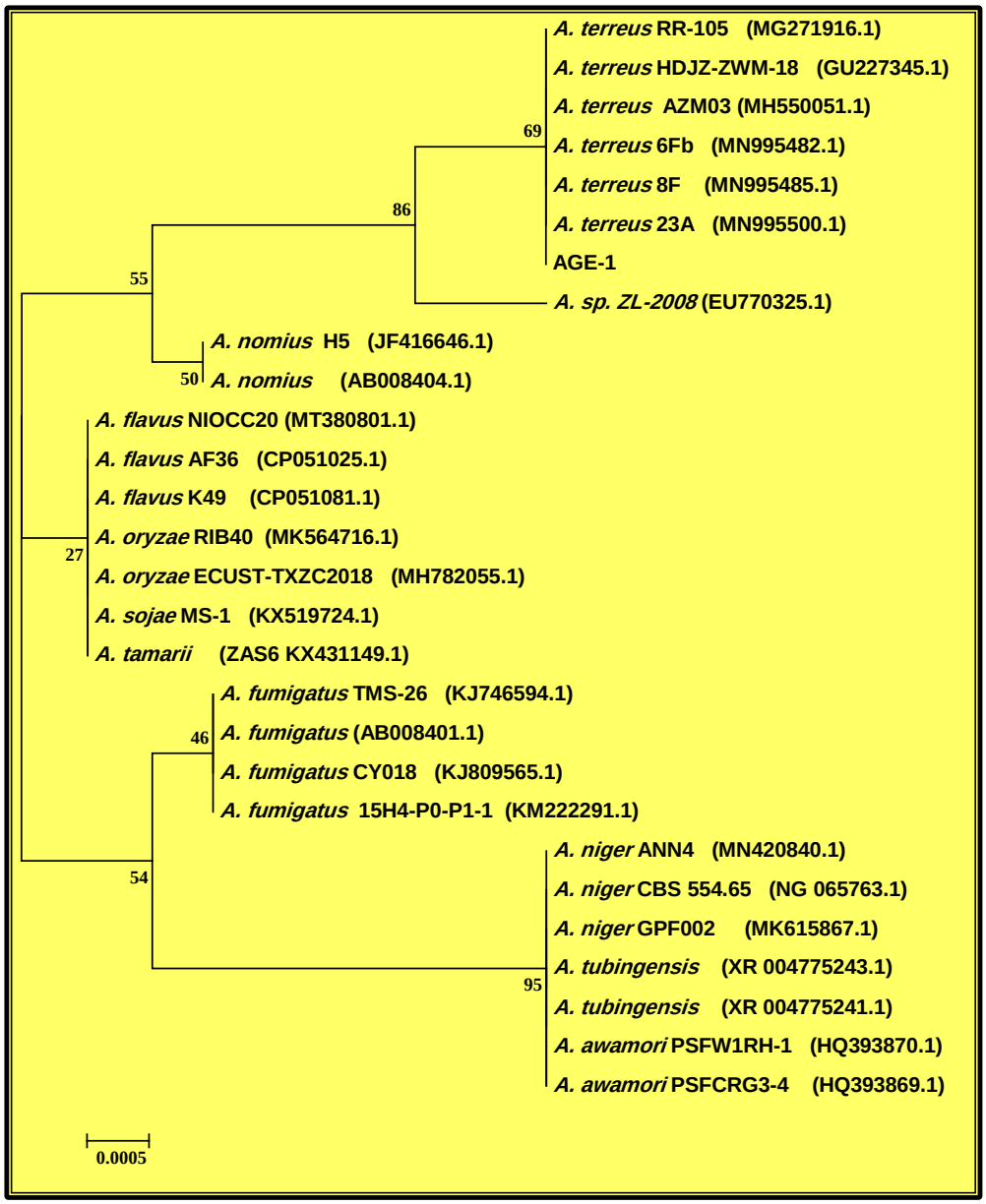

Fig. (4): Phylogenetic tree based upon the *18S rRNA* gene of the AGE-1obtained by maximum likelihood (ML) method. GenBank accession numbers are indicated next to the strain name. Bootstrap values were calculated from 1000 trees and shown at the branching points.

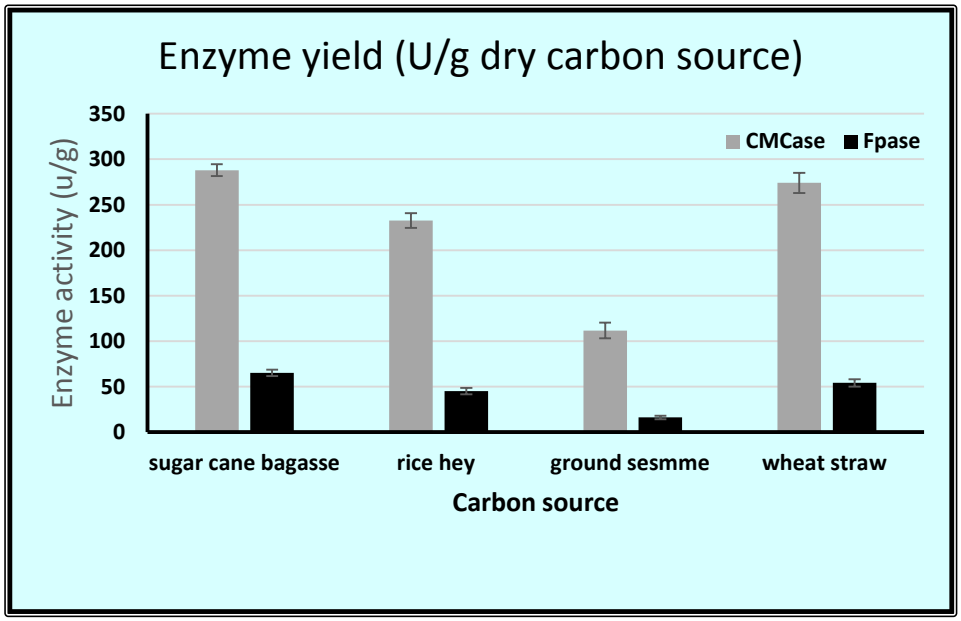

Fig. (5): Effect of sugarcane bagasse, wheat straw, ground sesame and rice hey as sole carbon sources for the induction of FPase and CMCase using SSF.

WHOLE GENOME SEQUENCING OF *Bacillus altitudinis* AGE-B8, A POTENTIAL CELLULOLYTIC BACTERIUM ISOLATED FROM ROTTED SUGAR CANE BAGASSE

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Keywords: lignocellulosic degrading, *B. altitudinis*, Phylogenetic analysis, CMCase, FPase, Whole-genome sequencing, genome annotation,

List of **Abbreviations**

B. altitudinis: *Bacillus altitudinis*; CMC carboxymethyl cellulose; CMCase: carboxymethyl cellulase; FPase: filter-paper enzyme; *gry*B: gyrase B subunit; *rpo*B: RNA polymerase β subunit; SCB: Sugarcane Bagasse; *pyc*A: pyruvate carboxylase.

Biofuel produced from agricultural wastes can be an important renewable and sustainable source to replace the depleting fossil fuels (Luo *et al.*, 2019). Successful conversion of lignocellulose waste to fermentable sugars involves three successive steps: (1) size reduction, (2) pretreatment/fractionation, and (3) enzymatic hydrolysis (Kucharska *et al.*, 2018). Enzymatic hydrolysis of natural lignocellulosic materials is considered the most difficult technological challenges that needed to be overcome (Mosier *et al.*, 2005). Due to the limited success of protein engineering to enhance the performance of existing lignocellulose degrading enzymes, retrieving enzymes from naturally evolved biomass-degrading microbial communities offers a promising strategy for the identification of new lignocellulolytic enzymes with potentially improved activities (Rubin, 2008).

Cellulase enzyme cocktails from fungus species are favored over bacteria due to the ability of the former to produce abundant amounts of cellulolytic enzymes and often less complex than bacterial cellulase (Gusakov, 2011). Compared to its counterparts, bacteria have many favored characteristics such as i) it has a higher growth rate than fungi that led to higher

recombinant production of enzymes, ii) bacterial cellulases are more complex that provide increased function and synergy iii) bacteria inhabit a wide variety of environments, which results in cellulolytic strains that are extremely resistant to environmental stresses, and hence able to produce cellulolytic enzymes stable under extreme conditions (Maki *et al.,* 2009; Pham *et al.,* 2019).

Bagasse has various higher-value uses as a second-generation biofuel, fibers for paper, xylan-based products such as xylooligosaccharides, substrate for single-cell protein, and enzymes, or other high-value microbial products (Gebbie *et al.*, 2020; Pandey *et al.*, 2000). Culturing, and metagenomics analysis of bagasse samples has discovered a unique microbial community compared to other lignocellulosic environments, which lead to the identification of new biomass-degrading microbes and enzymes (García-Huante *et al.*, 2017; Mhuantong *et al.,* 2015; Rattanachomsri *et al.,* 2011).

Whole-genome sequencing is an innovative technology to analyze all DNA sequence of an organism efficiently. Whole-genome sequencing technology was used to investigate several potential lignocellulose degrading bacteria during recent years. Full genome sequencing was performed for various bacterial strains such as *Amycolatopsis* sp. strain ATCC 39116 (Davis *et al.,* 2012), *Novosphingobium* sp. strain MBES04 isolated from sunken wood (Ohta *et al.*, 2015), *Pseudomonas* sp. strain YS-1p (Prabhakaran *et al.,* 2016), the *β-proteobacterium Cupriavidus basilensis B-8* (Shi *et al.*, 2013), *Burkholderia* sp. Strain LIG30 isolated from wet tropical forest soil (Woo *et al.,* 2014b), and *Klebsiella* sp. strain BRL6-2 (Woo *et al.,* 2014a). These studies reveal a tremendous amount of high-quality information about the agricultural waste degrading mechanisms adopted by several microorganisms.

The current report describes the isolation of cellulose-degrading bacterial isolate, AGE-B8, isolated from rotted sugar cane bagasse. Various parameters such as pH, incubation temperature, incubation time, and different carbon sources were tested for identifying the best cellulases induction conditions for the isolate. A draft assembly of the bacterial isolate genome was performed. The isolate was molecularly identified using multilocus sequence analysis as *B. altitudinis*. A total of 3,540 genes were identified in the produced bacterial genome assembly.

MATERIALS AND METHODS

Bacterial isolation and qualitative screening of their cellulolytic activity.

In this study, cellulose-degrading bacteria were collected from rotted bagasse. Twenty grams of the rotted bagasse were added to 180 ml sterilized distilled water in a 250 ml Erlenmeyer flask and shooked for one hour at 30ºC. One ml of sample suspension was transferred into 9 ml of sterilized distilled water; serial di-

luted to 10^{-7}, and one hundred µl were plated on nutrient agar plates then incubated at 30ºC for four days. Bacterial colonies were picked and sub-cultured to obtain a pure culture. Cellulase producing bacteria were screened on selective carboxymethyl cellulose agar containing (g/L): $NaNO_3$ 2.0, KH_2 PO_4 1.0, $MgSO_4$.$7H_2O$ 0.5, KCl 0.5, carboxymethyl cellulose sodium salt 10.0, peptone 0.2, agar 17.0. Plates were spot inoculated with a suspension of pure culture and incubated at 30ºC. The endoglucanase activity of the isolated bacterial isolates was tested through the detection of hydrolyzing clear zones on agar plates containing CMC-Na and Iodine staining (Kasana *et al.,* 2008). After 4 days, plates were flooded with gram's iodine stain (2.0 g KI and 1.0 g iodine in 300 ml distilled water) for 3 to 5 minutes. The diameter of the zone of decolorization around each well was recorded.

Quantitative screening of cellulolytic activity of isolated bacteria

The pure cultures of bacterial isolates were inoculated with 3 ml (1 x 10^7 CFU/ml) in modified basal mineral medium (g/L): KH_2PO_4, 2g; $(NH_4)_2SO_4$, 1.4g; Urea, 0.3g; $CaCl_2 \cdot 2H_2O$, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; peptone , 1.0 g; Tween 80, 0.2%; $CoCl_2 \cdot 6H_2O$, 2.0 mg; $FeSO_4 \cdot 7H_2O$, 5.0 mg; $MnSO_4 \cdot 2H_2O$, 1.6 mg; $ZnSO_4 \cdot 7H_2O$, 1.4 mg; Distilled water , 1000 ml. One gram of treated sugar cane bagasse (Gutierrez-Correa and Tengerdy, 1997) was added into 100 ml of the production medium (pH 5.5) in 250 ml Erlenmeyer flask then incubated at 30ºC with an agitation speed of 180 rpm in a rotary shaking incubator. The cultures filtrate were collected after four days by centrifugation at 6000 rpm for 15 min and the supernatant was used as a source of crude cellulase enzyme (Vega *et al.*, 2012). filter-paper enzyme (FPase) and carboxymethyl cellulase (CMCase) activities were determined using the method of Ghose, (1987). The activity of the enzymes was expressed in U/ml. One unit (IU) of enzyme activity is defined as the amount of enzyme required to release 1µmol of glucose in the reaction mixture per minute under specified conditions.

Optimization of parameters for enzymatic hydrolysis

The cellulolytic activities of the bacterial isolates were tested at varying pH ranged from 4-10, varying temperatures ranged from 30ºC - 37ºC, and varying incubation periods ranged from 4-10 days, while all other parameters were kept constant. For carbon source induction experiments; sugar cane bagasse (SCB), as natural cellulose and carbon methylcellulose (CMC), as artificial cellulose, were tested individually as a sole carbon source in a basal mineral media at 30ºC and 170 rpm. The production media was inoculated by 3ml (1 x 10^7 CFU/ml) and all the flasks were incubated in a shaking incubator at 180 rpm (New Brunswick, USA). Enzyme assay of both FPase and CMCase was determined for each experiment as described before.

Identification of bacterial isolate using 16S rRNA sequencing

Genomic DNA of AGE-B8 isolate was purified using GeneJET Genomic DNA Purification Kit (ThermoFisher®, USA). The Primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) were used to amplify nearly full-length *16S rRNA* gene (Youseif *et al.*, 2021). The PCR product was purified, and the sequencing reaction of the PCR product was carried out using an ABI BigDye Terminator V3.1 cycle sequencing kit and ABI PRISM 3500 genetic analyzer (Applied Biosystem, USA).

Library amplification and genome sequencing of *AGE-B8* isolate.

Genomic DNA extraction was performed using the GeneJET Genomic DNA Purification Kit (ThermoFisher®, USA). DNA extraction quality was performed using DeNovix DS-11 (DENovix, USA). Sequencing library was prepared by random fragmentation of the DNA, followed by 5' and 3' adapter ligation using truseq nano DNA library kit (Illumina, USA), and the size of PCR enriched fragments was confirmed by electrophoresis on Agilent Technologies 2100 Bioanalyzer (Agilent, USA). For cluster generation, the library was loaded into a flow cell where fragments are captured on a lawn of surface-bound oligos complementary to the library adapters. Each fragment is then amplified into distinct, clonal clusters through bridge amplification. The sequencing was performed using NovaSeq 6000 (Illumina, USA). Library preparation and sequencing were performed at macrogen (Seoul, Korea).

Genome assembly and genome annotation of *AGE-8B*

FastQC (V0.11.8) was used to check the high throughput sequencing data. Adapter sequences and low quality reads quality filtering of the fastaq raw files was performed using Trimmomatic (Bolger *et al.,* 2014). Map with BWA-MEM (Galaxy Version 0.7.17.1) was used to map the filtered reads to the *Bacillus altitudinis* reference genome (Li and Durbin, 2009). SAMTools was used to manipulate the SAM/BAM files produced from the BWA-MEM software (Li *et al.,* 2009). Variants (SNPs and short indels) were identified by SnpEff (v4.3t) (Cingolani *et al.*, 2012). Genome annotation was performed using Prokka Prokaryotic genome annotation (Seemann, 2014), and RAST (Rapid Annotation using Subsystem Technology (Aziz *et al.*, 2008).

Phylogenetic analysis

The *16S rRNA* gene sequencing reads were edited and assembled using Sequencher (Gene Code Corporation, MI, USA). The relevant sequences were obtained using the nucleotide BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned using ClustalW version1.8 (Altschul *et al.*, 1997). The *gry*B, *rpo*B, *pyc*A housekeeping genes were identified from the draft genome assembly, BLASTed in GenBank

(www.ncbi.nlm.nih.gov) using the nucleotide BLAST program, and were aligned using ClustalW version1.8 (Altschul *et al.*, 1997). The maximum likelihood (ML) algorithm (Saitou and Nei, 1987) was selected for performing the phylogenetic analyses. Phylogenetic analyses were performed using MEGA 6.06 (Kumar *et al.*, 2016) using the Tamura-Nei model. Bootstrap support (BT) for each node was evaluated with1000 replicates.

RESULTS AND DISCUSSION

Isolation, screening, and identification of most efficient cellulase producing bacteria

Twenty bacterial isolates were acquired based on their ability to grow on basal mineral medium containing sugarcane bagasse pulp as the sole carbon source on solid media. The hydrolyzing zone diameter and colony diameter were recorded for all tested isolates, and only isolates hydrolyzing zone diameter larger than 10 mm, ten isolates, were selected for quantitative analysis. The ten bacterial isolates showing the highest clear zone were further quantitatively screened using DNS (3,5-dinitrosalicylic acid) method (Adney and Nrel, 1996). Bacterial isolates were inoculated in production media containing Sugarcane Bagasse (SCB) as the sole carbon source for seven days at 30ºC, and the induced exoglucanase (FPase) and endoglucanase (CMCase) were recorded (Table 1). The bacterial isolates showed FPase activity in a range from 0.022 to 0.092 U/ml, while the CMCase activity was ranged from 0.31 to 0.527 U/ml. The bacterial isolate AGE-B8 showed the highest CMCase activity with 0.527 U/ml in addition to 0. 092 U/ml for FPase, and was selected for further investigations. The results show a direct relationship between the enzyme concentration and the diameter of the cleared zone in the Congo-red staining method. The results are in accordance with other reports that confirmed this pattern (Bradner *et al.*, 1999; Florencio *et al.*, 2012; Teather and Wood, 1982).

Optimization of cellulase production

Cellulase production from AGE-B8 isolate was assessed after fermentation at the different parameters. The effect of pH on the induction of exoglucanase (FPase) and endoglucanase (CMCase) was assessed for the AGE-B8 bacterial isolate at 30ºC (Fig. 1 A). The results illustrated by Fig. (1. A) show that both Fbase and CMCase gradually increases as the pH values increased and reached maximum values at pH from 5.5 to 6.0 . At a pH of 5.5, the values were 0.532 and 0.094 U/ml for CMCase and FBase, respectively.

The results illustrated by Fig. (1) clearly show that CMCase production, expressed as enzyme activity, gradually increased as the incubation period increased reaching the maximum (0.72 U/ml ±0.004) at the tenth day, when incubated at 30ºC. The maximum activity for CMCase was 0.713 U/ml ± 0.02 on day 7 when the bacterial isolate incubated at 34ºC, and 0.80 U/ml ± 0.05 when incubated at 37ºC. The FPase production showed a similar pattern. It gradually increased as

the incubation period increased reaching the maximum (0.082 U/ml ±0.004) on the tenth day, when incubated at 30ºC. The maximum activity for FPase was 0.274 U/ml ± 0.04 on day 7 when the isolate incubated at 34ºC, and 0.191 U/ml ± 0.03 when incubated at 37ºC.

Sugarcane bagasse and carboxymethyl cellulose were tested for their effects on cellulase production. Data presented in Fig (2) showed that Bagasse is a better sole source of carbon for cellulase production as cellulase activity was 0.072U/ml for FPase and 0.54 U/ml for CMCase (Fig. 2).

Identification of *AGE-B8* using *16S rRNA*

Nearly full-length *16S rRNA* gene (1500 bp) was successfully amplified and sequenced. The *16S rRNA* sequence of AGE-B8 was submitted to the GenBank under accession numbers LC593153. Nucleotide BLAST of the *16S rRNA* showed the highest similarity to *B. altitudinis* strain GQYP101 (Accession number CP040514.1), *B. altitudinis* strain BIM (Accession number CP063360.1), *B. altitudinis* strain CHB19 (Accession number CP043559.1), *B. pumilus* strain TUAT1 (Accession number AP044928.1), *B. pumilus* strain SH B11 (Accession number C010997.1P), *B. cellulasensis* strain NJ-M2 (Accession number CP012329.1), *B. cellulasensis* strain GQYP101 (Accession number CP0405141.1), *B. cellulasensis* strain NJ-V2 (Accession number CP012482.1). Maximum likelihood (ML) phylogenetic tree based on *16S rRNA* was generated to determine the evolutionary relatedness of the bacterial isolates AGE-B8 (Fig. 3). According to the sequence similarity, the AGE-B8 isolate was grouped and could not be differentiated from the aforementioned strains (Fig. 3). *B. pumilus*, *B. cellulasensis* and *B. altitudinis*, form one group that can be differentiated from other closely related group. Some *Bacillus* isolates closely related to the *B. pumilus*, are not easily distinguished from *B. cellulasensis* and *B. altitudinis* by *16S rRNA* gene sequence alone, which are nearly identical in *16S rRNA* gene sequence, sharing similarity over 99.5% (Liu *et al.*, 2013).

Genome assembly and genome annotation of AGE-B8 bacterial isolate

For sequencing, genomic DNA was extracted, and one paired-ends library was performed. The genome was sequenced using the Novaseq 6000 (Illumina). In total, 15,882,070 reads with 2,398,192,570 bases were generated, achieving about 600X-fold coverage of the *B. pumilus*, *B. cellulasensis* and *B. altitudinis* reference genomes. Trimmomatic was used to remove adapter sequences and low quality reads to reduce biases in further analysis. After quality trimming at Q30, (~93.5%) of total length passed the filtering. Table (2) summarizes the resulting sequencing data and related quality metrics, before and after the quali-

ty trimming. The GC% of the reads was 41.57%.

The reads were submitted to NCBI under project number PRJNA675729, BioSample number SAMN16712403, and SRA accession number SRR13084424. To my knowledge, this is the first draft genome for *Bacillus altitudinis* with ability for lignocellulosic degrading. The reference genome strategy was applied for building the draft genome assembly. The NCBI reference genome of the *B. altitudinis* (accession number GCF_004563755.2) was selected as reference for aligning the reads sequences. More than 94% of the filtered reads could be aligned to it, compared to 85% match in case of *B. pumilus, B. cellulasensis.* The BWA-MEM (Galaxy Version 0.7.17.1) was used to map the filtered reads to the *Bacillus altitudinis* reference genome. The reads bases can be aligned to ninety-two percent of the 3,867,833 bases of the *B. altitudinis* reference genome (Table 3). The average alignment depth was 504.14.

Genome annotation was performed using Prokka Prokaryotic genome annotation (Seemann, 2014). The resulting assembly generated 208 contigs, with 3,575,132 bases, with N50 of 51,914. The assembly identified twenty-two rRNA, 74 tRNA, one tmRNA, and 3,540 coding regions. The functional annotation was carried out by RAST (Rapid Annotation using Subsystem Technology (Aziz *et al.*, 2008). The annotated genome revealed 232 genes potentially involved in carbohydrates including 76 Central carbohydrate metabolism genes (Fig. 4). These genes, with the mutations identified within it, can be characterized for the identification of new biomass-degrading enzymes, and testing its efficient conversion processes on bagasse that is characterized by the recalcitrant nature of its polymers.

Identification of *AGE-B8* using Multilocus Sequence Analysis

To confirm the taxonomy of the AGE-B8 isolate, phylogenetic analysis based upon the Multilocus Sequence Analysis (MLSA) of *gyr*B, *rpo*B, *pyc*A housekeeping genes was constructed. The sequence of *gyr*B, *rpo*B, *pyc*A housekeeping genes were extracted from the AGE-B8 assembly and aligned to those of bacterial strains that showed the highest similarity of 16S rRNA to AGE-B8 strain. The maximum likelihood (ML) phylogenetic tree based on *gyr*B, *rpo*B, *pyc*A housekeeping genes were generated and confirmed the identification of the AGE-B8 isolated as *B. altitudinis* isolate. The AGE-B8 can be grouped in a separate clade and could be differentiated from the *B. pumilus*, *B. cellulasensis* strains (Fig. 5). The bacteria of the *B. pumilus*, groups are repeatedly misnamed at the species level due to the high similarity in their *16S rRNA* gene sequence. The use of housekeeping genes can be used as a molecular marker to distinguish these closely related strains (Liu *et al.*, 2013). Inside the *B.* subtilis group, the gyrB gene has a better discrimination power than *16S rRNA* (Wang *et al.*, 2007). Also three concatenated housekeeping genes was used to

distinguish closely related rhizobial isolates (Youseif *et al.*, 2014). In this study, the *gyr*B, *rpo*B, *pyc*A housekeeping gene could successfully identify the *B. altitudinis* isolates from the *B. pumilus*, *B. cellulasensis* strains.

Genetic variation analysis of *AGE-8B* bacterial isolate

Comparing with the reference genome, *B. altitudinis* (accession number GCF_004563755.2), the produced genome assembly was tested for genetic variation using the SNPeff software. After removing duplicates and identifying variants, information on each variant was gathered and classified. Compared with the reference genome, 375 deletions, 398 insertions, and 60,009 SNP were identified. Supplementary file 1 lists all deletions, insertions, and SNPs along with their annotations (Supp. File 1).

Base changes (DNA substitution) are of two types. Interchanges of purines (A <-> G), or of pyrimidines (C <-> T) are transitions, while interchanges of purine for pyrimidine bases, and vice versa, are transversions. Generally, transversions are more likely to cause amino acid sequence changes. The number of transition (Ts) and transversion (Tv), and the Ts/Tv ratio were calculated using the base change count. Base changes (DNA substitution) are of two types. Interchanges of purines (A <-> G), or of pyrimidines (C <-> T) are transitions, while interchanges of purine for pyrimidine bases, and vice versa, are transversions. A total of 40,521 transitions and 19,488 transversions were identified (Table 4). Generally, transversions are more likely to cause amino acid sequence changes.

To find out the annotation information such as amino acid changes by variants, SnpEff was used. Table (5) shows the number of variants per type (based on the representative transcript). Seventy-eight percent of the identified 53,790 variations were synonymous. Twenty-one percent were missense_variant (Table 5). A list of 60,765 identified variants is listed in supp. file (2).

SUMMARY

Bagasse is a major source of lignocellulose that is produced in a huge amount in Egypt. It can be used as a second-generation biofuel starting material, fibers for paper, and substrate for high-value microbial products. The present study describes the isolation of cellulose-degrading bacterial strain, AGE-B8, isolated from rotted sugar cane bagasse. AGE-B8 bacterium was tested for the cellulases induction under various parameters such as pH, incubation temperature, incubation time, carbon sources. The best filter-paper enzyme (FPase) activity was obtained after incubation for 7 days at 34ºC, while best carboxymethyl cellulase (CMCase) activity was obtained after incubation for 7 days at 37ºC. The strain was identified using multilocus sequence analysis as *Bacillus altitudinis*. A draft assembly of the AGE-B8 genome was performed. The assembly was submitted to NCBI under project number

PRJNA675729, BioSample number SAMN16712403, and SRA accession number SRR13084424. A total of 3,540 genes were identified in the produced bacterial genome assembly. Gene annotation and genetic variation analysis was performed for the identified isolate. A detailed list of all mutations, in reference to the NCBI *Bacillus altitudinis* reference genome, was identified. The results will significantly contribute to a better understanding of the genetics of the lignocellulosic degrading bacteria.

REFERENCES

Adney B., and J. Nrel B., (1996). Measurement of cellulase activities. In: Labora-tory Analytical Procedure No. 006. Golden, CO: National RenewableEnergy Laboratory.

Altschul S. F., Madden T. L., Schäffer A. A., Zhang J., Zhanger W., and Lipman D. J., (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Research. 25:.3389-3402. https: //doi.org/10.1093/nar/25.17.3389

Aziz R. K., Bartels D., Best A., DeJongh M., Disz T., Edwards R. A., and Zagnitko O., (2008). The RAST Server: Rapid annotations using subsystems technology. BMC Genomics, 9:1-15. https://doi.org/ 10.1186/1471-2164-9-75

Bolger A. M., Lohse M., and Usadel B., (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics, 30: 2114-2120.https://doi.org/10.1093/bioinformatics/btu170

Bradner J. R., Gillings M., and Nevalainen K. M. H., (1999). Qualitative assessment of hydrolytic activities in antarctic microfungi grown at different temperatures on solid media. World Journal of Microbiology and Biotechnology, 15: 143-145. https://doi.org/ 10.1023/A:1008855406319

Cingolani P., Platts A., Wang L. L., Coon M., Nguyen T., Wang L., and Ruden D. M., (2012). A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly, 6: 80-92. https://doi.org/10.4161 /fly.19695

Davis J. R., Goodwin L. A., Woyke T., Teshima H., Bruce D., Detter C., and Sello J. K., (2012). Genome sequence of Amycolatopsis sp. strain ATCC 39116, a plant biomass-degrading actinomycete. Journal of Bacteriology, (2012): 2396-2397. https://doi.org/10.1128 /JB.00186-12

Florencio C., Couri S., and Farinas C. S., (2012). Correlation between agar plate screening and solid-state fermentation for the prediction of

cellulase production by trichoderma strains. Enzyme Research, 2012. https://doi.org/10.1155/2012/793708

García-Huante Y., Cayetano-Cruz M., Santiago-Hernández A., Cano-Ramírez C., Marsch-Moreno R., Campos J. E., and Hidalgo-Lara M. E., (2017). The thermophilic biomass-degrading fungus Thielavia terrestris Co3Bag1 produces a hyperthermophilic and thermostable β-1,4-xylanase with exo- and endo-activity. Extremophiles, 21: 175-186.https://doi.org/10.1007/s00792-016-0893-z

Gebbie L., Dam T. T., Ainscough R., Palfreyman R., Cao L., Harrison M., and Speight R., (2020). A snapshot of microbial diversity and function in an undisturbed sugarcane bagasse pile. BMC Biotechnology, 20: 1-16. https://doi.org/10.1186 /s12896-020-00609-y

Ghose T. K., (1987). Measurement of cellulase activities. Pure and Applied Chemistry, 59: 257-268. https:// doi.org/10.1351/pac198759020257

Gusakov A. V., (2011). Alternatives to Trichoderma reesei in biofuel production. Trends in Biotechnol-ogy,. (9):.419-425. https://doi.org/ 10.1016/ j.tibtech.2011.04.004

Gutierrez-Correa M., and Tengerdy R. P., (1997). Production of cellulase on sugar cane bagasse by fungal mixed culture solid substrate fermentation. Biotechnology Letters, 19: 665-667. https://doi.org/10.1023/A:1018342916095

Kasana R. C., Salwan R., Dhar H., Dutt S., and Gulati A., (2008). A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. Current Microbiology, 57: 503-507. https://doi.org/10.1007/s00284-008-9276-8

Kucharska K., Rybarczyk P., Hołowacz I., Łukajtis R., Glinka M. and Kamiński M., (2018). Pretreatment of lignocellulosic materials as substrates for fermentation processes. Molecules, 23: 2937.

Kumar S., Stecher G., and Tamura K., (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular Biology and Evolution, 33: 1870-1874. https://doi.org/ 10.1093/molbev/msw054

Li H., and Durbin R., (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics, 25: 1754-1760. https: //doi.org/10.1093 /bioinformatics/btp324

Li H., Handsaker B., Wysoker A., Fennell T., Ruan J., Homer N., and Durbin R., (2009). The Sequence Alignment/Map format and SAMtools. Bioinformatics, 25: 2078-2079. https://doi.org/ 10.1093 /bioinformatics/btp352

Liu Y., Lai Q., Dong C., Sun F., Wang L., Li G., and Shao Z., (2013). Phylogenetic diversity of the Bacillus pumilus group and the marine ecotype revealed by multilocus sequence analysis. PLoS ONE, 8: 1-11. https://doi.org/ 10.1371/ journal.pone.0080097

Luo X., Liu J., Zheng P., Li M., Zhou Y., Huang L., Chen L. and Shuai L., (2019). Promoting enzymatic hydrolysis of lignocellulosic biomass by inexpensive soy protein. Biotechnology for biofuels, 12: 1-13.

Maki M., Leung K. T., and Qin W., (2009). The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. International Journal of Biological Sciences, 5: 500-516. https://doi. org/10.7150 /ijbs.5.500

Mhuantong W., Charoensawan V., Kanokratana P., Tangphatsornruang S., and Champreda V., (2015). Comparative analysis of sugarcane bagasse metagenome reveals unique and conserved biomass-degrading enzymes among lignocellulolytic microbial communities. Biotechnology for Biofuels, 8(1). https://doi .org/10.1186/s13068-015-0200-8

Mosier N., Wyman C., Dale B., Elander R., Lee Y. Y., Holtzapple M., and Ladisc M., (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology, 96: 673-686. https://doi. org /10.1016/j.biortech.2004.06.025

Ohta Y., Nishi S., Kobayashi K., Tsubouchi T., Iida K., Tanizaki A., and Hatada Y., (2015). Draft genome sequence of Novosphingobium sp. strain MBES04, isolated from sunken wood from Suruga Bay, Japan. Genome Announcements, 3(1). https://doi.org/10.1128 / genomeA. 01373-14

Pandey A., Soccol C. R., Nigam P., and Soccol V. T., (2000). Biotechnological potential of agro-industrial residues. I: Sugarcane bagasse. Bioresource Technology, 74: 69-80. https://doi. org /10.1016/S0960-8524(99)00142-X

Pham J. V., Yilma M. A., Feliz A., Majid M.T., Maffetone N., Walker J. R., Kim E., Cho H. J., Reynolds J. M., Song M. C. and Park S. R., (2019). A review of the microbial production of bioactive natural products and biologics. Frontiers in microbiology, 10: 1404.

Prabhakaran M., Couger M. B., Jackson C. A., Weirick T., and Fathepure B. Z., (2016). Genome sequences of the lignin-degrading Pseudomonas sp. strain YS-1p and *Rhizobium* sp. strain YS-1r isolated from decaying wood. Genome Announcements, 3(2). https://doi.org/10.1128/genomeA.00019-15

Rattanachomsri U., Kanokratana P., Eurwilaichitr L., Igarashi Y., and Champreda V., (2011). Culture-independent phylogenetic analysis of the microbial community in industrial sugarcane bagasse feedstock piles. Bioscience, Biotechnology and Biochemistry, 75: 232-239. https: //doi.org/10.1271/bbb.100429

Rubin E. M., (2008). Genomics of cellulosic biofuels. Nature, 454(7206): 841-845. https://doi .org/10.1038 /nature07190

Saitou N., and Nei M., (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4: 406-425. https:// doi.org/10.1093/oxfordjournals.molbev.a040454

Seemann T., (2014). Prokka: Rapid prokaryotic genome annotation. Bioinformatics, 30: 2068-2069. https://doi.org/10.1093/bioinformatics/btu153

Shi Y., Chai L., Tang C., Yang Z., Zhang H., Chen R., and Zheng Y., (2013). Characterization and genomic analysis of kraft lignin biodegradation by the beta-proteobacterium Cupriavidus basilensis B-8. Biotechnology for Biofuels, 6(1). https:// doi.org/ 10.1186/1754-6834-6-1

Teather R. M. and P. Wood J., (1982). Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Applied and Environmental Microbiology, 43: 777-780. https:// doi.org/10.1128/aem.43.4.777-780.1982

Vega K., Villena G. K., Sarmiento V. H., Ludeña Y., Vera N., and Gutiérrez-Correa M., (2012). Production of Alkaline Cellulase by Fungi Isolated from an Undisturbed Rain Forest of Peru. Biotechnology Research International, 2012: 1-7. https://doi.org/ 10.1155/2012/934325

Wang L. T., Lee F. L., Tai C. J., and Kasai H., (2007). Comparison of gyrB gene sequences, *16S rRNA* gene sequences and DNA-DNA hybridization in the *Bacillus subtilis* group. International Journal of Systematic and Evolutionary Microbiology, 57: 1846-1850.https: //doi.org/10.1099 /ijs.0.64685-0

Woo H. L., Ballor N. R., Hazen T. C., Fortney J. L., Simmons B., Davenport K. W., and DeAngelis K. M., (2014a). Complete genome sequence of the lignin-degrading bacterium *Klebsiella* sp. strain BRL6-2. Standards in Genomic Sciences, 9(1): 19. https://doi .org/10.1186 /1944-3277-9-19

Woo H. L., Utturkar S., Klingeman D., Simmons B. A., DeAngelis K. M., Brown S. D., and Hazen T. C., (2014b). Draft genome sequence of the lignin-degrading Burkholderia sp. strain LIG30, isolated from wet tropical forest soil. Genome Announcements, 2(3). https://doi. org/ 10.1128/ genomeA.00637-14

Youseif S. H., Abd El-Megeed F. H., Ageez A., Cocking E. C., and Sale S. A., (2014). Phylogenetic multilocus sequence analysis of native rhizobia nodulating faba bean (*Vicia faba* L.) in Egypt. Systematic and Applied Microbiology, 37: 560-569. https:// doi.org/10.1016/j.syapm.2014.10.0 01

Youseif Sameh H, El-megeed F. H. A., Mohamed A. H., Ageez A., Veliz E., and Martínez-romero E., (2021). Diverse Rhizobium strains isolated from root nodules of Trifolium alexandrinum in Egypt and symbiovars. Systematic and Applied Microbiology, 44(1): 126156. https: //doi.org/10.1016/j.syapm.2020.12 6156.

Supporting Files

- **S1 File**: list of all deletions, insertions, and SNPs along with their annotations in the *Bacillus altitudinis* AGE-B8 genome. https://drive.google.com/file/d/1CfiYq1rrvax0AErYguNcy3MWqbqoJUjd/view?usp=sharing

- **S2 File:** A list of 60,765 identified variants in the *B. altitudinis* AGE-B8 genome. https://drive.google.com/file/d/1BfZJIrZ5g7kHbule5Dg044NgtAn-5aG_/view?usp=sharing

Table (1): Evaluation of Exoglucanase (FPase) activity and Endoglucanase (CMCase) activity of the cellulose-degrading bacterial isolates.

Isolate code	D/d	FPase (IU/ml)	CMCase (IU/ml)
AGE-B1	27/2.1	0.037 ± 0.002	0.352 ± 0.002
AGE-B2	28/2.2	0.032 ± 0.001	0.425 ± 0.004
AGE-B3	18/2.0	0.022 ± 0.001	0.310 ± 0.003
AGE-B4	29/2.1	0.075 ± 0.01	0.499 ± 0.002
AGE-B5	24/2.3	0.042 ± 0.004	0.400 ± 0.003
AGE-B6	23/1.8	0.056 ± 0.002	0.325 ± 0.001
AGE-B7	24/2.2	0.053 ± 0.003	0.325 ± 0.004
AGE-B8	31/1.9	0.092 ± 0.001	0.527 ± 0.008
AGE-B9	17/2.0	0.025 ± 0.002	0.410 ± 0.005
AGE-B10	29/2.1	0.086 ± 0.001	0.458 ± 0.002

"*D*/*d*": hydrolyzed zone diameter/colony diameter, in millimeter, on agar media containing CMC as a sole carbon source.

Table (2): Raw data Stats before and after trimming using the trimmomatic software.

	Total read bases (bp)	Total number of reads	Total reads GC(%)	Q20(%)	Q30(%)
Before trimming	2,398,192,570	15,882,070	41.58	96.20	90.73
After trimming	2,170,161,529	14,675,394	41.57	98.29	93.54

Table (3): Mapped data Stats.

Ref. Length (GCF_004563755.2)	Mapped Sites (>=1x)	Total Reads	Mapped Reads	Mapped Bases Mean Depth
3,867,833	3,586,315 (92.73%)	14,675,394	13,813,626 (94.13%)	504.14

Table (4): Transition, Transversion information table.

Ref. Length	Number of SNPs	Transition	Transversion	Ts/Tv
3,867,833	60,009	40,521	19,488	2.08

Table (5): Annotation type count.

Type of annotation	Count	Ratio
synonymous_variant	42,117	78.3%
missense_variant	11,398	21.19%
stop_gained	53	0.1%
frameshift_variant	50	0.09%
downstream_gene_variant splice_region_variant	40	0.07%
and stop_retained_variant	31	0.06%
conservative_inframe_insertion	18	0.03%
start_lost	14	0.03%
conservative_inframe_deletion	14	0.03%
upstream_gene_variant	10	0.02%

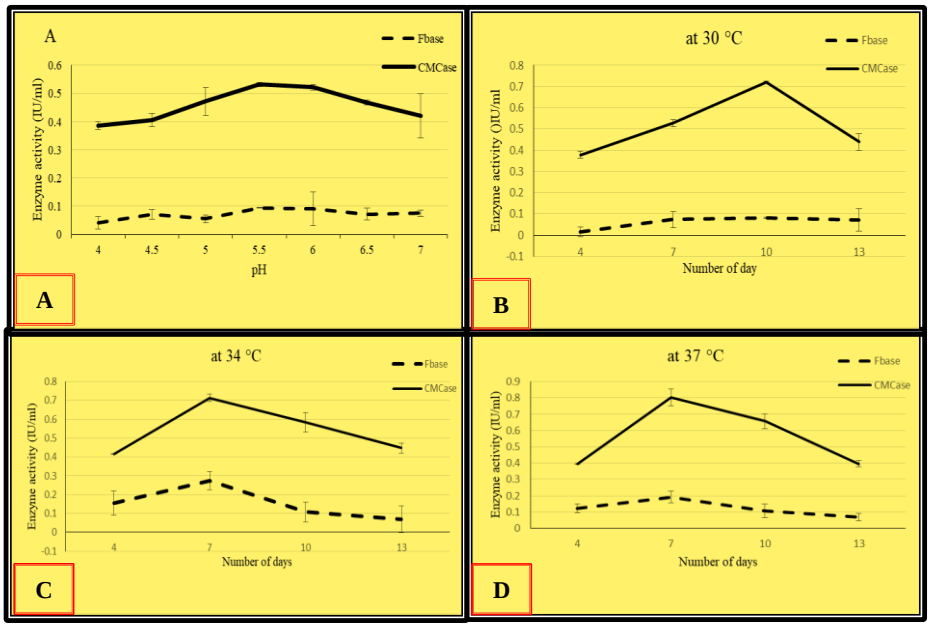

Fig. (1): Effect of pH and incubation time at different incubation temperatures on the activity of FPase and CMCase for *B. altitudinis* AGE-B8. (A): Effect of different pH, at 30ºC, on the production of cellulases by *B. altitudinis* AGE-B8. Effect of incubation period on the production of cellulases at incubation temperature 30ºC (B), 34ºC (C) and 37ºC (D).

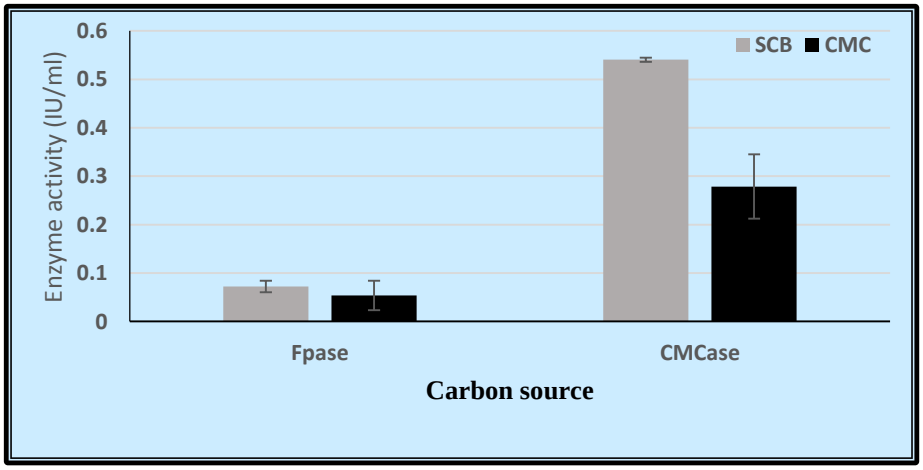

Fig. (2): Effect of SCB and CMC as sole carbon sources for the induction of FPase and CMCase.

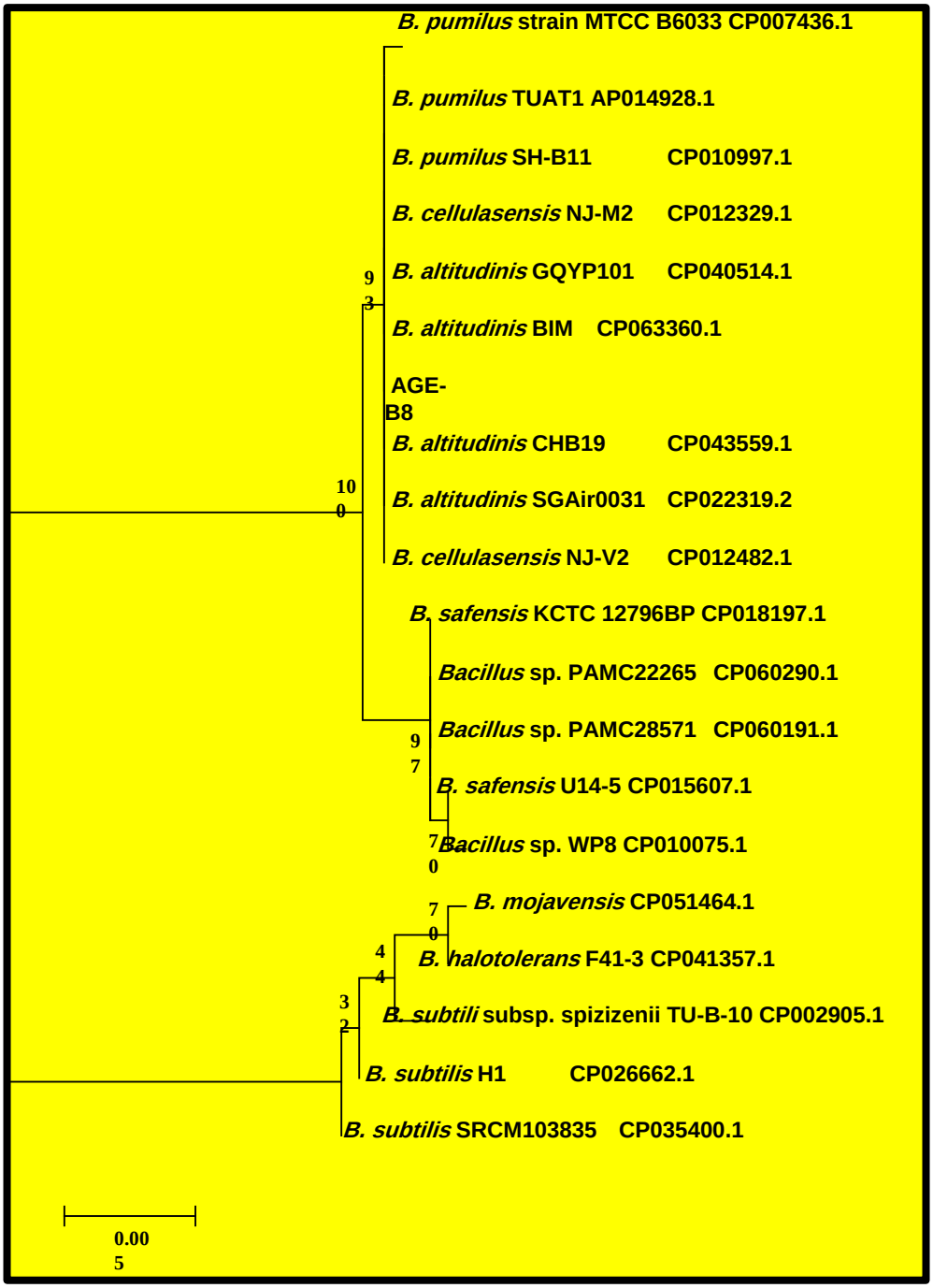

Fig. (3): Phylogenetic tree based upon the *16S rRNA* sequences obtained by maximum likelihood (ML) method. GenBank accession numbers are indicated next to the strain name. Bootstrap values were calculated from 1000 trees compared with the sequence of standard strains. Bootstraps are shown at the branching points.

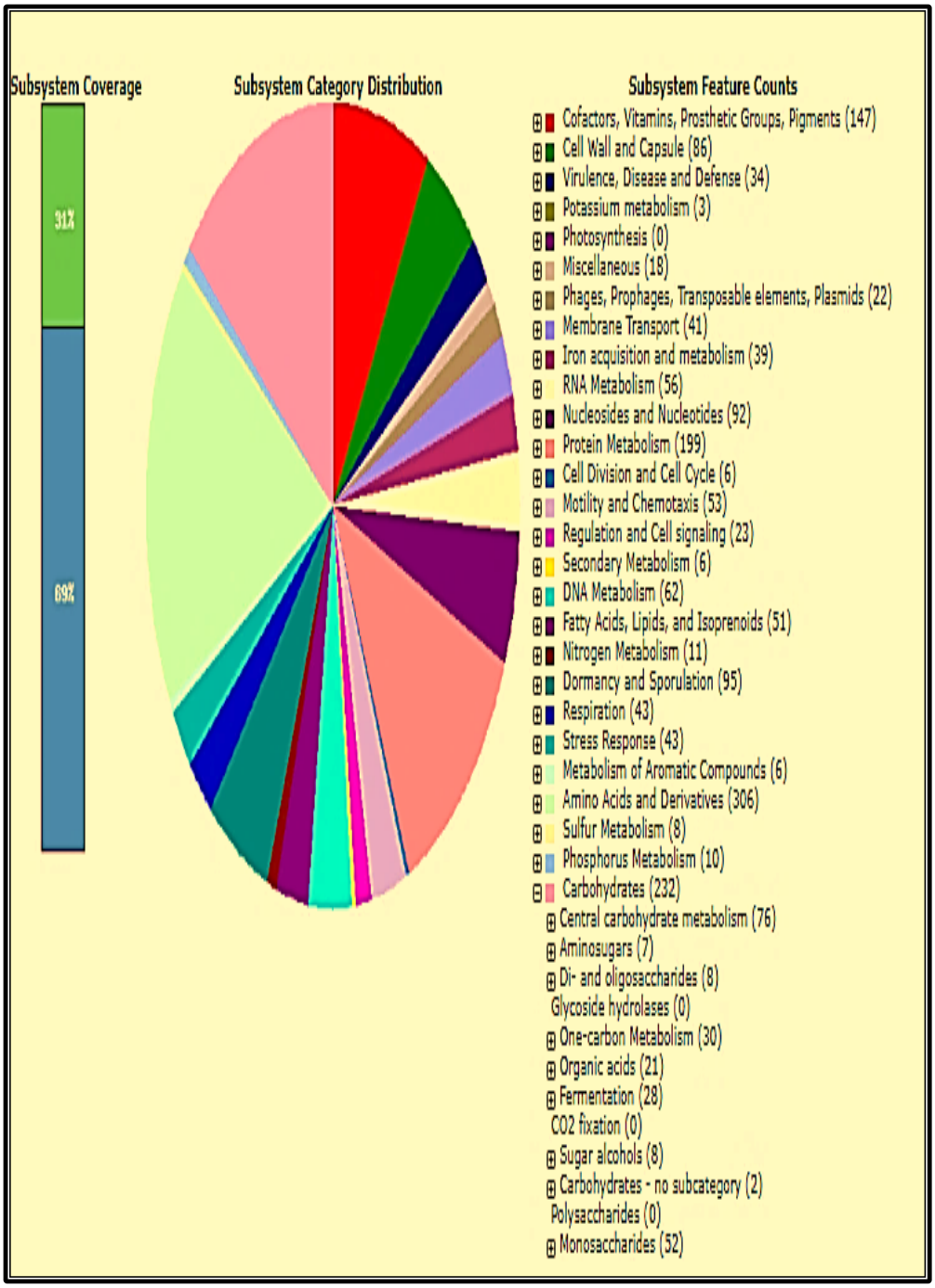

Fig. (4): Subsystem distribution of AGE-8B genes based on RAST annotation server.

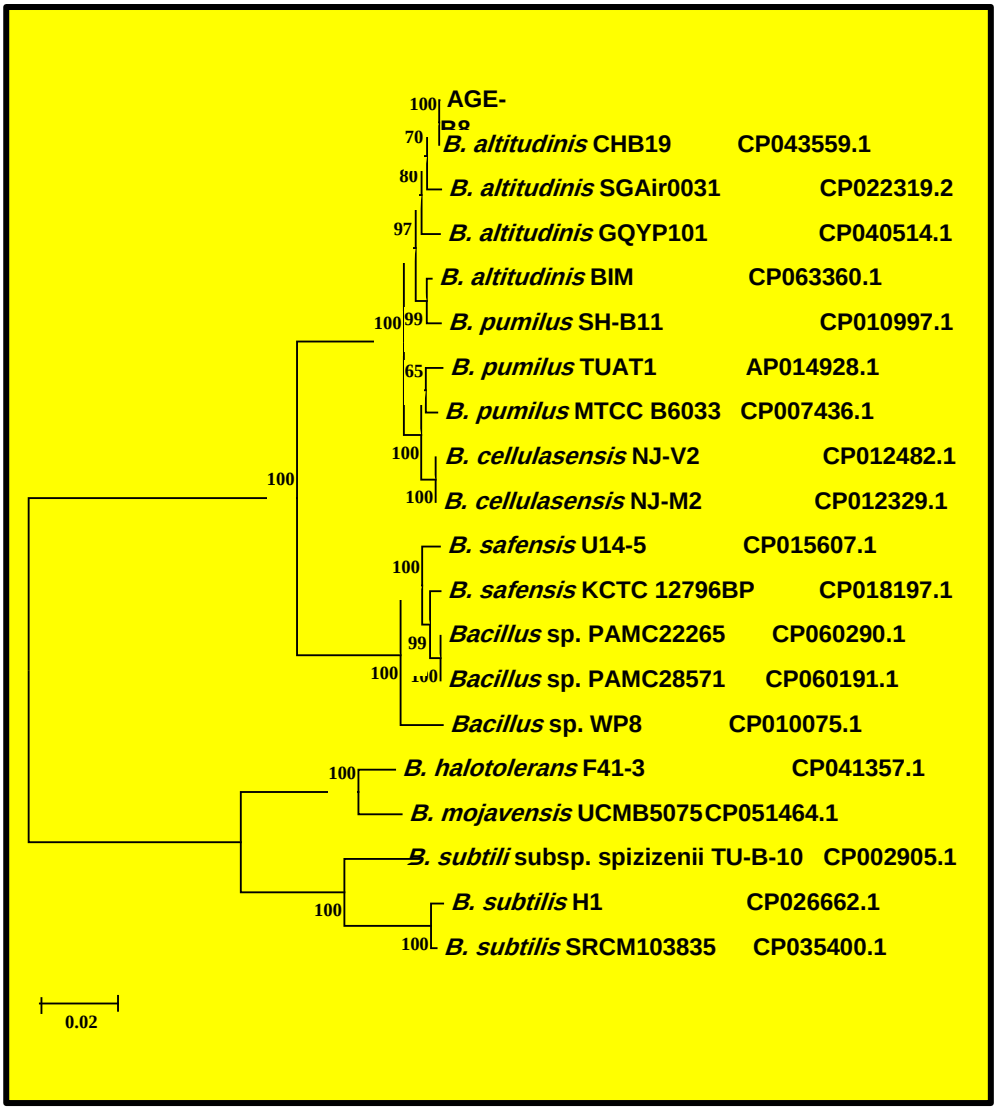

Fig. (5): ML tree based on three concatenated *gyr*B, *rpo*B, *pyc*A housekeeping genes. GenBank accession numbers are indicated next to the strain name. Bootstrap values were calculated from 1000 trees compared with the sequence of standard strains. Bootstraps are shown at the branching points.

PHYLOGENETIC RELATIONSHIPS OF SOME BARLEY (*Hordeum vulgare* L.) ACCESSIONS IN EGYPT

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Barley (*Hordeum vulgare* L.) is one of the founder crops of Old-World agriculture, it was one of the first domesticated cereals. It is also a model experimental system because of its short life cycle and morphological, physiological, and genetic characteristics. Barley is used for, in order of importance, animal feed, brewing malts and human food. Barley is found in widely varying environments globally. It has wide adaptation ability to different climatic conditions and various environments comprising drought and irrigated environments (Nevo, 1992; Gomez-Macpherson, 2001).

Kianoosh *et al.*, (2017) stated that barley, is one of the oldest cultivated crops all over the world. The study of origin, genetic variation and evolutionary relationships in barley is important in the conservation and restoration of biodiversity of wild germplasm. Genetic diversity in domesticated crop species is crucial to fight new pests and diseases and to produce better-adapted varieties for the changing environments.

Besides morphological markers, DNA markers are ordinarily used to allow cultivar identification and fingerprint of genomes in crops; SSR and SCoT are rapid and efficient applications in evaluation, characterization of genetic material (Olgun *et al.*, 2015).

Microsatellites, or simple sequence repeats (SSRs), are stretches of DNA consisting of tandemly repeated short units of 1–6 base pairs in length. The value and uniqueness of microsatellites arise from their codominant inheritance, multiallelic nature, extensive genome coverage, relative abundance, and simple detection by PCR using two unique primers, that flank the microsatellite and hence define the microsatellite locus (Thiel *et al.*, 2003).

Start codon targeted (SCoT) is a novel method for generating plant DNA

markers. Developed based on the short conserved region flanking the ATG start codon in plant genes. This method uses single primers 18-mer in single primer polymerase chain reaction (PCR) and an annealing temperature of 50ºC. PCR amplicons are resolved using standard agarose gel electrophoresis (Collard and Mackill, 2009).

The present study aims to derive data from morphological and molecular attributes to assess the phylogenetic relationships of a collection of barley (*Hordeum vulgare* L.) taxa, based on some morphological, SSR and SCoT analysis

MATERIALS AND METHODS

This study includes eleven accessions of barley *Hordeum vulgare* L., Morphological study was carried out using quantitative traits and molecular study was carried out using SSR and SCoT techniques and further for developing new crop accessions.

1. Plant materials

Eleven Egyptian barley accessions *Hordeum vulgare* L. seeds were kindly provided by Gene Bank of Egyptian National Gene Bank (NGB) Giza, Egypt. Accessions were collected from different regions of Egypt, Their code numbers and regions are recorded in Table (1)

2. Field experiment

This experiment was carried out at the research farm of Faculty of Agricultural, Ain Shams University, Shubra El-Khima, Egypt, during the growing season of 2016. Based on randomized complete block design (RCD) with three replications, to reduce the variance introduced into the data arising from location during the growth seeds were planted by hand in 1 x 3 m plots spacing 20 cm between rows. Ten seeds of each accession were planted in 5 hills and maintained until the end of the experiment. Plots were kept free from weeds. Irrigation was conducted at weekly intervals.

3. Morphological Characterization

Fifteen quantitative morphological traits of *Hordeum* accessions were recorded (Table 2). Ten randomly selected individual plants were used for recording charactrers.

4. Molecular analysis

4.1 DNA extraction and purification

DNA extraction was performed using DNeasy plant Mini Kit (QIAGEN).

PCR was performed in 25µl volume tubes according to Williams *et al.*, (1990).

4.1.1 Polymerase Chain Reaction (PCR) conditions for Simple Sequence Repeats (SSRs) and Start Codon Targeted (SCoT)

The DNA amplifications were performed in an automated thermal cycle (model Techno 512) programmed for one cycle at 94ºC for 4 min followed by 45 cycles of 1 min at 94ºC, 1 min at 37ºC, and 2 min at 72ºC, reaction was finally stored at 72ºC for 10 min. 10 used primers and their sequences were recorded in Tables (3 & 4)

4.2 Molecular data Analysis

The banding patterns generated by PCR marker analyses were compared to determine the genetic relatedness of the samples under study. Clear and distinct amplification products were scored as '1' for presence and '0' for absence of bands. Bands of the same mobility were scored as identical. The genetic similarity coefficient (GS) between two genotypes was estimated according to Dice coefficient (Sneath and Sokal, 1973).

Dice formula: GSij = 2a/(2a+b+c)

Where: GSij is the measure of genetic similarity between individuals i and j, a is the number of bands shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i.

The similarity matrix was used in the cluster analysis. The cluster analysis was employed to organize the observed data into meaningful structures. At the first step, when each accession represents its own cluster, the distances between these accessions are defined by the chosen distance measure (Dice coefficient). However, once several accessions have been linked together, the distance between two clusters is calculated as the average distance between all pairs of accessions in the two different clusters. This method is called Unweighted Pair Group Method using Arithmetic Average (UPGMA) (Sneath and Sokal, 1973). The polymorphism information content (PIC) was calculated for each marker according to a simplified version of *Botstein et al.*, (1980) as follows:

PIC=1-Σ(Pij)2

Where: Pij is the frequency of the i alelle of the locus in 11 accessions revealed by the j primer.

RESULTS AND DISCUSSIONS

1. Quantitative morphological traits of the 11 *Hordeum vulgare* L. accessions

Fifteen quantitative morphological traits of 11 *Hordeum vulgare* L. accessions were recorded in Table (5). The results indicate the existence of high morphological variations in barley accessions. Mean of root length clearly indicated that the Egyptian barley B1 recorded the highest value (13 cm), while the lowest value was 9.8cm recorded by accession B 11. The tallest plants were observed in accession B4 with mean of 106cm from Marsa Matrouh, while the shortest was recorded

in accession B1 (85 cm) from Sharkia. Number of tillers also varied from 10 in accession B1 to 5.5 in accession B11 from Red sea. The highest of stem length was 79 cm recorded in accession B11, on the other hand, the lowest value of stem length was 63.3cm recorded by accession B2 from Dakahlia. Number of nodes was highest in accession B1 and B4 (6.3) and lowest in accession B10 and B11 from Alexandria and Red sea respectively. Data showed that sheathing leaf base length in accession B1, B4 and B5 recorded the highest value (13.5 cm), while the lowest values were recorded in accession B2 (9.3 cm). The highest values of flag leaf length (22.2 cm) in accession B4, while the lowest values were (16.8 cm) in accession B1. Flag leaf width ranges from 1.27 cm in accession B5 and B6 to 1.3 cm in accession B2, B4 and B9. For spike length, the tallest was recorded in accession B6 (19 cm), while the shortest recorded in accession B9 (16cm). Spike axis length was the longest in accession B11 (10.2 cm) and shortest in accession B3 (5.2 cm) . Highest values of central awn length in accession B3 and B4 (14.3cm), while the lowest values were recorded in accession B8 (11.7 cm). Number of spikelets was highest (72) in accession B11 and lowest (52.8) in accession B8. Days of heading were highest (72 days) in accession B2 and B11 and lowest in accession B3 (66 days). Leaf area was highest (25 cm^2) in accession B5 and lowest (16.2 cm^2) in accession B1. Weight of 1000 grain was highest (54.2 g) in accession B4 and lowest (28.9 g) in accession B2.

Analysis of variance (ANOVA) indicate the presence of significant differences in several traits such as awn of central lemma length, number of spikelets per spike, days of heading and leaf area, and some high significant traits such as leaf base length, leaf width, weight of 1000 grain and spike axis length.

1.1. Cluster analysis of 15 Quantitative morphological traits of 11 *Hordeum valgare* L. accessions

The similarity coefficient of 15 quantitative morphological traits shown in Table (6), maximum ratio of similarity coefficient was observed between accessions (B7 & B8) 98% both from North Sinai and (B9 & B10) 98 % both six-rowed from Sharkia and Alexandria, respectively, whereas the lowest similarity ratio was 0.91% between accessions B2 and B11 both tow- rowed from Dakahlia and Red sea, respectively.

The dendrogram obtained from UPGMA cluster analysis of 15 quantitative morphological characteristics of 11 *Hordeum* accessions is illustrated in Fig. (1). It showed that the accessions were divided into two distinct clusters with genetic similarity rang from 0.936 to 1.00.

Cluster I contain three accessions, accession B4 was separated at taxonomic distance of 0.952 from accessions B5 and B11 both were two-rowed and grouped together at taxonomic distance of 0.965.

Cluster II contain 8 accessions; all of them were six-rowed except B2 with two rowed but it was integrated with the second cluster. This cluster was further subdivided into two subclusters at taxonomic distance of 0.944, subcluster A included six accessions, accessions B3 separate at taxonomic level 0.956, while accession B6 was separated at taxonomic distance of 0.965, on the other hand, accessions B7 and B8 were closely related both from North Sinai grouped together at 0.978. As for accessions B9 and B10 they were grouped together at a taxonomic distance of 0.971. Subcluster B contain accession B2 and B1 at taxonomic level 0.949.

Variation in morphological traits based on geographic origin has been established before, where differences are probably related to agronomic adaptation to environments (Bothmer *et al*., 1992). *H. vulgare* cultivated though many phytogeographical regions in Egypt (Boulos, 1995; 2005; 2009)

The most important characters for determination and recognizing of different species number of spikes and spikelets but should always use combination of different characters (von Bothmer and Jacobson, 1985).

Phenotypic heterogeneity in different species is reported because of phenotypic plasticity, which is defined as the flexibility of individual genotypes to grow and develop alternation in response to changing the environmental factors, also somatic polymorphism related to several important characteristics, particularly during their reproductive period (Booy *et al*., 2000; Dekker, 2003).

Hagenblad *et al.,* (2019) studied morphological and genetic characterization of barley accessions in the Canary Islands using 57 accessions collected from Gene-banks. they found that although accessions from the same island tended to be similar, the results showed morphological and genetic diversity both within and among islands

2. Molecular analysis

DNA markers have numerous applications in plant molecular genetic research. One of the most common uses of DNA markers have been the assessment of genetic diversity within crop germplasm (Collard and Mackill, 2009)

2.1. SSR analysis

PCR amplification of the genomic DNA isolated from the 11 barley accessions yielded a total of 50 bands, of which 26 were polymorphic (64 %) and 24 were monomorphic, overall size of the PCR amplified fragments ranged from 50 to 722 bp. (Table 7) shows list of SSR primers, number of total bands (TB), polymorphic band (PB), monomorphic band (MB), the percentage of polymorphism (PP), specific band (SB) and Polymorphic Information Content (PIC). The banding patterns of barley genotypes are shown in Fig. (2).

2.1.1. Cluster analysis as revealed by 10 SSR primers for 11 *Hordeum* accessions

Table (8) shows the genetic similarity indexes among 11 accessions of barley based on banding patterns of 10 SSR markers, detected the maximum value of genetic similarity was 94% between B3 and B4, whereas the minimum value was 70 % between accessions B1 and B5.

The dendrogram obtained from UPGMA cluster analysis of the ten SSR markers of 11 *Hordeum* accessions is illustrated in Fig. (3). It was divided into two distinct clusters with genetic similarity ranged from 0.75 to 0.99.

Cluster I contain the 10 accessions in four subclusters, subcluster A included three accessions, accessions B2 and B9 were grouped together at genetic level of 0.95 and were closely related, while accession B6 was separated from them at genetic distance of 0.91. Subcluster B also include three accessions, accessions B3 and B4 were grouped together at genetic similarity of 0.93 separated from accession B10 at genetic distance of 0.92. As for subcluster C, it included three accessions; accession B5 was separated at single distance from the closely related B7, B8. On the other hand, subcluster D included single accession, accession 11 separated at genetic distance 0.84. Cluster II contained only one accession B1 separated from the rest at genetic distance 0.75.

SSR have become important molecular markers for a wide range of applications, such as phenotype mapping, marker assisted selection of crop plants, genome mapping and characterization and a range of molecular ecology and diversity studies (Robinson *et al.*, 2004).

SSR amplification products (alleles) are amplified using PCR so that only a small amount of starting DNA is required. Alleles vary according to the number of repeat units present, but other mutations have also been shown to be responsible for allele length variation (Kumar *et al*., 2009).

Naceur *et al.*, (2012) studied the phylogenetic relationships of 31 barley accessions from North Africa (Algeria, Tunisia, and Egypt) using 11 SSR primers. A total of 478 reproducible bands were scored, Nandha and Singh, (2014) stated that species specific markers have a great potential for introgression of important traits from wild to cultivated barley, tomato, potato, rice, maize and barley. An overview of the reported results for barley indicated that diversity parameters varied significantly among studies (Matus and Hayes, 2002).

It is noteworthy that cluster analysis is a valuable tool for subdividing genotypes into groups including similar and dissimilar accessions and has a great value from the breeder's point of view for initiating barley hybrid programs.

2.2. SCoT analysis

PCR amplification of the genomic DNA isolated from the 11 barley accessions yielded a total of 143 bands, of which 108 were polymorphic (72%) and 35 were monomorphic (28 %). Overall size of the PCR amplified fragments ranged from 125 to 2060 bp . List of SCoT primers, number of total bands (TB), polymorphic band (PB), monomorphic band (MB), the percentage of polymorphism (PP), specific band (SB) and Polymorphic information content (PIC) are shown in Table (9). The Scot banding patterns of barley genotypes are shown in Fig. (4).

2.2.1. Cluster analysis as revealed by 10 SCoT primers for 11 *Hordeum* accessions

Table (10) show the genetic similarity indexes among the 11 accessions of barley based on banding patterns of 10 SCoT markers and detected the maximum value of genetic similarity was 80 % between B9 and B11, on the other hand the minimum value was 50 % detected between B3 and B10, and also between B4 and B8.

The dendrogram obtained from UPGMA cluster analysis of ten SCoT markers of 11 *Hordeum* accessions is illustrated in Fig. (5). The results showed that the dendrogram was divided into two distinct clusters with genetic similarity range from 0.55 to 0.95.

Cluster I was divided into two subclusters at genetic distance 0.95; subcluster A contains four accessions, accession B7 separated at 0.71, accessions B9 and B11 were closely related grouped in a single sub-cluster at genetic distance of 0.80, while accession B8 was separated at genetic distance of 0.67. Sub-cluster B contained only accession B10 in a separate sub-cluster at 0.65.

On the other hand, cluster II was divided into sub-cluster C which included three accessions; accession B6 was separated at genetic distance of 0.66, while accessions B1 and B2 were grouped together at 0.71. Subcluster D also included three accessions, accession B3 was separated at genetic distance 0.69, while accessions B4 and B5 were grouped together at genetic distance of 0.76.

SCoT is based on PCR technology, as a new molecular marker method, it has several advantages: simple, low-cost, high polymorphic, extensive genetic information and its primers are universal in plants. These advantages have been validated through studies on genetic diversity in rice (Collard and Mackill, 2009), peanut (Xiong *et al.*, 2011) and potato (Gorji *et al*., 2011).

SCoT markers are multilocus which are helpful in obtaining high genetic polymorphism. Besides, SCoT markers are expected to be linked to functional genes and corresponding traits, thus the amplicons can be converted to

gene targeted marker systems (Xiong *et al*., 2011).

Guo *et al*., (2012) used SCoT technique to assess genetic relationships among 64 grape varieties. Seventeen informative primers were selected from 36 SCoT primers based on their ability to produce repeatable and clear polymorphic and unambiguous bands among the varieties.

3. Combined data of quantitative morphological traits with 10 SSR and 10 SCoT primers for 11 *Hordeum* accessions

Combination of the data obtained from the two molecular marker systems along with quantitative morphological traits could give more accurate estimation to the genetic relations among the studied accessions, as illusterated in Table (11) and (Fig. 6). This agreed with the results reported by Abdel-Tawab *et al*., (2008).

SUMMARY

phylogenetic relationships of eleven accessions of *Hordeum vulgare* L. collected from different region of Egypt were assessed. Fifteen quantitative morphologcal traits were used, the measured data were evaleuated statistically using ANOVA, phylogenetic tree were constructed using UPGMA. Also simple sequance repeats (SSRs) and start codon targeted (SCoT) mulecular marker techniques were used for DNA fingerprinting and assessing genetic divesity and phylogenetic relationships in barley germplasm. The results showed that SSR primers produced 50 bands ranged in size between 50-722 bp with 64% polymorphism percentage and SCoT primers produced 143 bands, ranged in size between 165-2060 bp with 72% polymorphism percentage. Polymorphic information content PIC was 0.44 and 0.82 for SSRs and SCoT, respectively. UPGMA dendrogram was divided into two clusters by each of morphological traits, SSRs and SCoT analysis. Genetic similarity matrix was examined with Jacard's coefficient, maximum similarity was found between B7 and B8 (0.98%) with quantitative morphological analysis, between B3 and B4 (94%) with SSRs analysis and between B9 and B11 (80%) with SCoT analysis. However, combined cluster analysis between the three components together revealed better resolution for distinction between these taxa.

REFERENCES

Abdel-Tawab F. M., Rashed M. A., Azer S. A., El-Metabteb G. M. and khafaga F., (2008). Molecular fingerprinting and marker-assisted selection in citrus species. Egypt. J. Genet. & Cytol., 37: 335-358.

Booy G., Hendriks R. J. J., Sulderns M.J.M., Van Groenendeal J.M. and Vosman B., (2000). Genetic diversity and the survival of populations. Plan Biol., 2: 379-395.

Bothmer R. V., (1992). The wild species of Hordeum: relationships and potential use for improvement of cultivated barley. Barley: genetics, biochemistry, molecular biology and biotechnology., 3-18.

Botstein D., White R. L., Skolnick M., Davis R. W., (1980) Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet., 32: 314-331.

Boulos L., (1995). Flora of Egypt checklist. Al Hadara Publishing Cairo Egypt, 1-283.

Boulos L., (2005). Flora of Egypt Vol.VI. Al Hadara Publishing Cairo Egypt, 1-617.

Boulos L., (2009). Flora of Egypt checklist. Revised annotated edition. Al Hadara Publishing Cairo Egypt, 292.

Collard B. C. , and Mackill D. J., (2009). Start codon targeted (SCoT) polymorphism: a simple, novel DNA marker technique for generating gene-targeted markers in plants. Plant Molecular Biology Reporter, 27: 86.

Dekker J., (2003). The foxtail (Setaria) species-group. Weed Science, 51: 641-656.

Gomez-Macpherson H., (2001). *Hordeum vulgare*. http://ecoport.org/ep?Plant=1232&entityType=PL****&entityDisplayCategory=full

Gorji A. M., Poczai P., Polgar Z. and Taller J., (2011). Efficiency of arbitrarily amplified dominant markers (SCoT, ISSR and RAPD) for diagnostic fingerprinting in tetraploid potato. Am. J. Potato Res., 88: 226-237.

Guo D. L., Zhang J. Y., and Liu C. H., (2012). Genetic diversity in some grape varieties revealed by SCoT analyses. Molecular Biology Reports, 39: 5307-5313.

Hagenblad J., Leino M. W., Afonso G. H., and Morales D. A., (2019). Morphological and genetic characterization of barley (*Hordeum vulgare* L.) landraces in the Canary Islands. Genetic Resources and Crop Evolution, 66: 465-480.

Kianoosh C., Leila Z., Reza Z. A. and Saeid J. H., (2017). Study of genetic variation among diverse barley (*Hordeum vulgare* L.) genotypes using agro-morphological traits and RAPD, ISSR, AFLP markers. Research Journal of Biotechnology, 12: 6.

Kumar P., Gupta V. K., Misra A. K., Modi D. R., and Pandey B. K., (2009). Potential of molecular

markers in plant biotechnology. Plant omics, 2: 141.

Matus I. A. and P. Hayes M., (2002). Genetic diversity in three groups of barley germplasm assessed by simple sequence repeats. Genome, 45: 1095-1106.

Naceur A. B., Chaabane R., El-Faleh M., Abdelly C., Ramla D., Nada A. and Sakr M., (2012). Genetic diversity analysis of North Africa's barley using SSR markers. Journal of Genetic Engineering and Biotechnology, 10: 13-21.

Nandha P. S. and Singh J., (2014). Comparative assessment of genetic diversity between wild and cultivated barley using g SSR and EST-SSR markers. Plant breeding, 133: 28-35.

Nevo E., (1992). Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology. University of Haifa: 19-43.

Olgun M., Budak Başçiftçi Z., Ayter N. G., Turan M., Koyuncu O., Ardıç M., Ağar G., and Takıl E., (2015). Genetik divergence in some barley (*Hordeum vulgare* L.) genotypes by RAPD and ISSR Analyses. Süleyman Demirel Üniversitesi Ziraat Fakültesi Dergisi, 10:102-109.

Robinson A. J., Love C. G., Batley J., Barker G. and Edwards D., (2004). Simple sequence repeats marker loci discovery using SSR primer. Bioinformatics, 20: 1475-1476.

Sneath P. H. A. and Sokal R. R., (1973). Numerical Taxonomy. Freeman, San Francisco, California.

Thiel T., Michalek W., Varshney R., and Graner A., (2003). Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). Theoretical and Applied Genetics, 106: 411-422.

Von Bothmer R. and Jacobson N., (1985). Barley origin, taxonomy, and related species. Barley Agronomy, 26: 19-51.

Williams J. G. K., Kubelike A. R., Livake K. T., Rafalski J. A. and Tingoy S., V. (1990). DNA polymorphism amplified by random primers are useful as genetic markers. Nucleic Acid Research, 18: 6531-6539.

√Xiong F., Zhong R., Han Z., Jiang J., He L., Zhuang W. and Tang R., (2011). Start codon targeted polymorphism for evaluation of functional genetic variation and relationships in cultivated peanut (*Arachis hypogaea* L.) varieties. Mol. Biol. Rep., 38: 3487-3494.

Table (1): Accession of barley *Hordeum vulgare L*. with their Gene-Bank bar code number and regions of collection.

no	Accession	Gene bank bar code	Regions
1-	B1	11418	Sharkia
2-	B2	11514	Dakahlia
3-	B3	11717	Marsa Matruh
4-	B4	11721	Marsa Matruh
5-	B5	11726	Minya
6-	B6	11727	Red sea
7-	B7	11773	North Sinai
8-	B8	11782	North Sinai
9-	B9	11820	Sharkia
10-	B10	11917	Alexandria
11-	B11	11937	Red sea

Table (2): Quantitative morphological traits of *Hordeum vulgare* L. accessions.

Trait	Trait definition
1-Root length	Measured at maturity from the ground to the tip of the longest fibrous root (pulling carefully to avoid rupture).
2-Plant height	Length of randomly selected plants measured from the ground to the tip of the spike excluding awns at maturity.
3-No. of tillers	Number of fertile tillers (spike bearing) of randomly selected plants counted at maturity.
4-Stem length	Length of the main stem by cm, measured from the cotyledonary node up to the shoot apex on the base of terminal spike.
5-No. of nodes	Number of internodes of the main stem.
6-Leaf base length	Sheathing base of flag leaf.
7-Leaf length	Length of flag leaf by cm.
8-Leaf width	At the widest part of flag leaf by cm.
9-Spike length	From the base of the spike to the top of the awn.
10-Spike axis length	From the base of the spike to the top of the awn excluding awns.
11-Awn of central lemma length	Distance from tip of the spike to the end of the awn (cm).
12-No. of spikelets	Number of all spikelets per spike on randomly selected plants, counted at maturity.
13- Days of heading	Number of days from planting to the day when 50% of the heads fully flower (heading) emerge of flag leaf.
14- Leaf area	Leaf length X leaf width X 0.905 (Kemp, C. D. 1959).
15- Wt 1000 grain	As the weight of 200 kernels taken from the bulk seed of the plot and multiplied by 5.

Table (3): SSR primers with their sequence and chromosome number.

No	Primer name	Sequence 5'-3' forward	Sequence 5'-3' revers	Chromosome no.
1	MGB391	AGCTCCTTTCCTCCCTTCC	CCAACATCTCCTCCTCCTGA	2H
2	HVITR1	CCACTTGCCAAACACTAGACCC	ATTCATGCAGATCGGGCCAC	3H
3	HV13GEIII	AGGAACCCTACGCCTTACGAG	AGGACCGAGAGTGGTGGTGG	3H
4	MGB396	CGCTAGCTTGTTTCTCGTTTG	TCGCATGGCATCAACTACAG	4H
5	MGB402	CAAGCAAGCAAGCAGAGAGA	AACTTGTGGCTCTGCGACTC	1H
6	Bmag149	CAAGCCAACAGGGTAGTC	ATTCGGTTTCTAGAGGAAGAA	1H
7	HVGLUEND	TTCGCCTCCATCCCACAAAG	GCAGAACGAAAGCGACATGC	1H
8	MGB371	CACCAAGTTCACCTCGTCCT	TTATTCAGGCAGCACCATTG	6H
9	MGB356	TGGTCTGGAGCTCTCAACAG	AAGCCACATTGAAGGAGCAC	6H
10	EBmac624	AAAAGCATTCAACTTCATAAGA	CAACGCCATCACGTAATA	6H

Table (4): SCoT primers with their sequence.

No	Primer	Primer sequence 5´→ 3´
1	**SCoT 1**	5'- ACG ACA TGG CGA CCA CGC -3'
2	**SCoT 2**	5'- ACC ATG GCT ACC ACC GGC -3'
3	**SCoT 3**	5'- ACG ACA TGG CGA CCC ACA -3'
4	**SCoT 4**	5'- ACC ATG GCT ACC ACC GCA -3'
5	**SCoT 9**	5'- ACA ATG GCT ACC ACT GCC -3'
6	**SCoT 10**	5'- ACA ATG GCT ACC ACC AGC -3'
7	**SCoT 12**	5'- CAA CAA TGG CTA CCA CCG -3'
8	**SCoT 13**	5'- ACC ATG GCT ACC ACG GCA -3'
9	**SCoT 14**	5'- ACC ATG GCT ACC AGC GCG -3'
10	**SCoT 15**	5'- CCA TGG CTA CCA CCG GCT -3'

Table (5): Quantitative morphological traits of 11 *Hordeum vulgare* L. accessions.

Trait	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	F value
1-Root length	13^{a} ±1	10^{b} ±2	11.7^{ab} ±1.5	11.7^{ab} ±1.5	12^{ab} ±1	10^{b} ±1	10^{b} ±2	10^{b} ±1	10.3^{ab} ±2.1	10^{b} ±1	9.8^{b} ±1.3	1.69
2-Plant height	85^{c} ±5	98.3^{abc} ±10.4	105^{ab} ±13.2	106^{a} ±15.3	95.3^{abc} ±4.5	95^{abc} ±5	96^{abc} ±5.8	96^{abc} ±5.8	91^{abc} ±10.4	96^{abc} ±11.5	86^{bc} ±11.5	1.37
3-No. of tillers	9.3^{a} ±1.5	9^{a} ±1	9.3^{a} ±0.6	8.7^{a} ±1.2	9^{a} ±1	7.3 ^{a} ±0.6	8^{a} ±1	7.3^{a} ±2.1	9^{a} ±1	9^{a} ±1	8^{a} ±1	1.26
4-Stem length	64.7^{b} ±11.2	63.3^{b} ±1.2	70.7^{a} ±1.2	77.3^{a} ±14.2	73.7^{a} ±11.7	79^{a} ±7.9	76^{a} ±16.8	72.3^{a} ±6.4	67.3^{a} ±4	68.3^{a} ±7.6	79^{a} ±5.3	1.07
5-No. of nodes	6.3^{a} ±1.2	5.7^{a} ±0.6	5.7^{a} ±1.2	6.3^{a} ±1.2	5.7^{a} ±0.6	5.7^{a} ±0.6	5.3^{a} ±0.6	5.3^{a} ±0.6	5.3^{a} ±0.6	5.7^{a} ±0.6	5^{a} ±0	0.90
6-Leaf base length	13.5^{a} ±0.5	9.3^{de} ±0.6	12.7^{abc} ±0.6	13.5^{a} ±0.5	13.2^{ab} ±0.8	10.8^{ab} ±1	12.7^{cde} ±1.5	11.3^{abc} ±0.6	10.3^{bcd} ±0.6	12.3^{de} ±2.1	11.7^{abc} ±0.6	5.85**
7-Leaf length	16.8^{b} ±0.8	18.3^{ab} ±2.3	19.3^{ab} ±1.2	22.2^{a} ±1.9	21.7^{a} ±5.1	21.3^{a} ±1.5	20.3^{ab} ±2.5	20.7^{ab} ±1.5	20.7^{ab} ±1.5	20.3^{ab} ±0.6	22^{a} ±1	1.68
8-Leaf width	1.1^{ab} ±0.1	1^{c} ±0.1	1.1^{bc} ±0.1	1^{c} ±0.1	1.3^{a} ±0.1	1.3^{a} ±0.1	1.1^{bc} ±0.1	1.1^{bc} ±0.1	1^{c} ±0.1	1.2^{ab} ±0.1	1.1^{bc} ±0.1	5.60**
9-Spike length	16.8^{ab} ±2.5	17^{ab} ±1	17.7^{ab} ±0.6	17.7^{ab} ±0.6	19^{ab} ±2.6	16.2^{b} ±0.8	19.3^{a} ±2.1	16^{b} ±1	16.5^{ab} ±0.9	16.7^{ab} ±1.5	19.3^{a} ±2.1	1.80
10-Spike axis length	6.7^{bcd} ±0.6	6.3^{cd} ±0.3	5.2^{d} ±0.3	5.5^{cd} 0.5±	9.8 ^{a} ±1.6	5.2^{d} ±0.3	7.9^{b} ±1.0	6.8^{bc} ±0.6	6.5^{bcd} ±0.5	7.0^{bc} ±1.0	10.2^{a} ±1.0	13.3**
11-Awn of central lemma length	14.0^{a} ±0.9	14.2^{a} ±0.8	14.3^{a} ±0.6	14.3^{a} ±1.0	13.2^{ab} ±1.0	11.8^{b} ±0.3	14.2^{a} ±0.8	11.7^{b} ±1.5	13.2^{ab} ±1.0	12.8^{ab} ±0.8	12.7^{ab} ±0.8	3.4*
12-No. of spikelets	54.9^{bcd} ±3.5	49.8^{d} ± 0.6	54^{cd} ±1.5	68.1^{abc} ±3.5	69^{ab} ±3.0	57^{bcd} ± 1.0	56.1^{bcd} ± 0.6	52.8^{d} ± 2.3	57.9^{abcd} ±2.5	63^{abcd} ±4.4	72 ^{a} ±1.7	2.8*
13- Days of heading	70.3^{ab} ±1.5	72.0^{a} ±2	66.0^{b} ±1	70.7^{ab} ±2.5	71.0^{ab} ±2	68.0^{b} ±1	67.3^{b} ±2.1	67.0^{b} ±2	70.3^{ab} ±1.5	67.0^{b} ±2	72.0^{a} ±2	4.9*
14- Leaf area	16.2^{b} ±1.1	16.6^{b} ±2.1	19.2^{ab} ±1	20.8^{ab} ±2.4	25.0^{a} ±6.8	24.5^{a} ±2.3	19.5^{ab} ±1.5	21.2^{ab} ±2.5	19.4^{ab} ±2.4	21.5^{ab} ±1.5	22.6^{ab} ±1.5	3.1*
15- 1000 grain wt	38.6^{b} ±1.2	28.9^{c} ±1.2	38.4^{b} ±2	54.2^{a} ±1.7	46.0^{a} ±1.3	33.5^{b} ±1.6	40.8^{a} ±1.6	41.5^{a} ±1.1	33.5^{b} ±1.1	34.4^{ab} ±1	45.3^{a} ±1	11.5**

Mean ± Standard deviation, means with the same letters was not significant differe. * P<0.01, **P<0.001

Table (6): Similarity indexes among 11 accessions of *Hordeum vulgare* L. based on 15 quantitative morphological traits.

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11
B1	**1**										
B2	**0.95**	**1**									
B3	**0.95**	**0.95**	**1**								
B4	**0.92**	**0.92**	**0.96**	**1**							
B5	**0.94**	**0.93**	**0.95**	**0.96**	**1**						
B6	**0.93**	**0.94**	**0.95**	**0.94**	**0.96**	**1**					
B7	**0.94**	**0.95**	**0.96**	**0.95**	**0.96**	**0.96**	**1**				
B8	**0.94**	**0.94**	**0.96**	**0.94**	**0.96**	**0.97**	**0.98**	**1**			
B9	**0.96**	**0.96**	**0.95**	**0.93**	**0.95**	**0.96**	**0.96**	**0.96**	**1**		
B10	**0.94**	**0.95**	**0.95**	**0.93**	**0.95**	**0.96**	**0.97**	**0.97**	**0.98**	**1**	
B11	**0.93**	**0.91**	**0.92**	**0.94**	**0.97**	**0.95**	**0.95**	**0.94**	**0.94**	**0.94**	**1**

Table (7): List of SSR primers, number of total bands (TB), polymorphic band (PB), monomorphic band (MB), the percentage of polymorphism (PP), specific band (SB) and polymorphic information content (PIC).

Primer	MS	TB	PB	MP	PP	SB	PIC
MGB391	90-450	8	4	4	50%	+(2), -(2)	0.29
HVITR1	90-110	3	2	1	67%	+(1), -(1)	0.15
HV13GEIII	55-390	7	0	7	0%	0	0.00
MGB396	50-170	4	3	1	75%	-(1)	0.55
MGB402	175-510	6	1	5	83%	+(1)	0.80
Bmag149	60-300	4	2	2	50%	0	0.32
HVGLUEND	400-100	4	3	1	75%	+(1)	0.66
MGB371	65-320	7	7	0	100%	+(3), -(1)	0.83
MGB356	60-722	5	2	3	40%	0	0.25
EBmac624	52-57	2	2	0	100%	0	0.57
Total	----	50	26	24	----	13	----
Mean	----	5	2.6	2.4	64%	----	0.44

Table (8): Similarity indices among 11 accessions of barley based on banding patterns of 10 SSR markers.

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11
B1	**100**										
B2	**72**	**100**									
B3	**84**	**86**	**100**								
B4	**84**	**86**	**94**	**100**							
B5	**70**	**83**	**82**	**82**	**100**						
B6	**74**	**91**	**87**	**87**	**84**	**100**					
B7	**74**	**84**	**90**	**87**	**93**	**82**	**100**				
B8	**78**	**86**	**89**	**89**	**91**	**84**	**93**	**100**			
B9	**74**	**85**	**88**	**88**	**84**	**89**	**86**	**85**	**100**		
B10	**79**	**91**	**93**	**93**	**87**	**88**	**91**	**93**	**92**	**100**	
B11	**75**	**86**	**82**	**85**	**79**	**84**	**81**	**85**	**91**	**87**	**100**

Table (9): List of SCoT primers, number of total bands (TB), polymorphic band (PB), monomorphic band (MB), the percentage of polymorphism (PP), specific band (SB) and polymorphic information content (PIC).

Primer	MS	TB	PB	MP	PP	SB	PIC
SCoT 1	240 - 745	9	6	3	66%	+(2), -(1)	0.88
SCoT 2	165 - 1160	14	8	6	57%	+(3)	0.70
SCoT 3	125 - 1245	14	7	**7**	50%	+(1)	0.80
SCoT 4	310 - 1870	15	12	3	80%	+(6)	0.83
SCoT 9	270 - 1920	15	13	2	86%	+(4)	0.88
SCoT 10	350 - 1790	16	15	1	93%	+(1), -(3)	0.90
SCoT 12	275 – 1935	**18**	**18**	**0**	**100%**	+(3), -(1)	0.90
SCoT 13	385 – 2060	16	11	5	68%	-(4)	0.90
SCoT 14	335 – 1050	**8**	**3**	5	**37%**	+(1)	0.88
SCoT 15	220 - 1335	**18**	15	3	83%	+(4)	0.48
Total	----	143	108	35	----	34	
Mean	----	14.3	10.8	3.5	72%	3.4	0.82

Table (10): Similarity index among 11 accessions of barley based on banding patterns of 10 SCoT markers.

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11
B1	**1.00**										
B2	**0.71**	**1.00**									
B3	**0.65**	**0.66**	**1.00**								
B4	**0.63**	**0.61**	**0.70**	**1.00**							
B5	**0.61**	**0.63**	**0.68**	**0.78**	**1.00**						
B6	**0.63**	**0.69**	**0.59**	**0.62**	**0.62**	**1.00**					
B7	**0.63**	**0.66**	**0.57**	**0.63**	**0.59**	**0.69**	**1.00**				
B8	**0.59**	**0.58**	**0.54**	**0.50**	**0.55**	**0.60**	**0.67**	**1.00**			
B9	**0.64**	**0.61**	**0.52**	**0.59**	**0.59**	**0.63**	**0.74**	**0.67**	**1.00**		
B10	**0.59**	**0.54**	**0.50**	**0.56**	**0.54**	**0.55**	**0.61**	**0.61**	**0.69**	**1.00**	
B11	**0.63**	**0.59**	**0.53**	**0.58**	**0.59**	**0.59**	**0.70**	**0.68**	**0.80**	**0.69**	**1.00**

Table (11): Similarity index among 11 accessions of barley based on combined quantitative morphological traits, 10 SSR markers and 10 SCoT markers.

	B_1	B_2	B_3	B_4	B_5	B_6	B_7	B_8	B_9	B_10	B_11
B_1	1										
B_2	0.70	1									
B_3	0.70	0.72	1								
B_4	0.69	0.69	0.79	1							
B_5	0.62	0.68	0.72	0.78	1						
B_6	0.65	0.75	0.68	0.70	0.68	1					
B_7	0.65	0.71	0.68	0.70	0.70	0.72	1				
B_8	0.64	0.66	0.65	0.62	0.67	0.67	0.75	1			
B_9	0.66	0.71	0.63	0.68	0.66	0.71	0.77	0.72	1		
B_10	0.64	0.65	0.64	0.68	0.64	0.65	0.70	0.71	0.76	1	
B_11	0.65	0.67	**0.61**	0.66	0.64	0.66	0.72	0.73	**0.83**	0.73	1

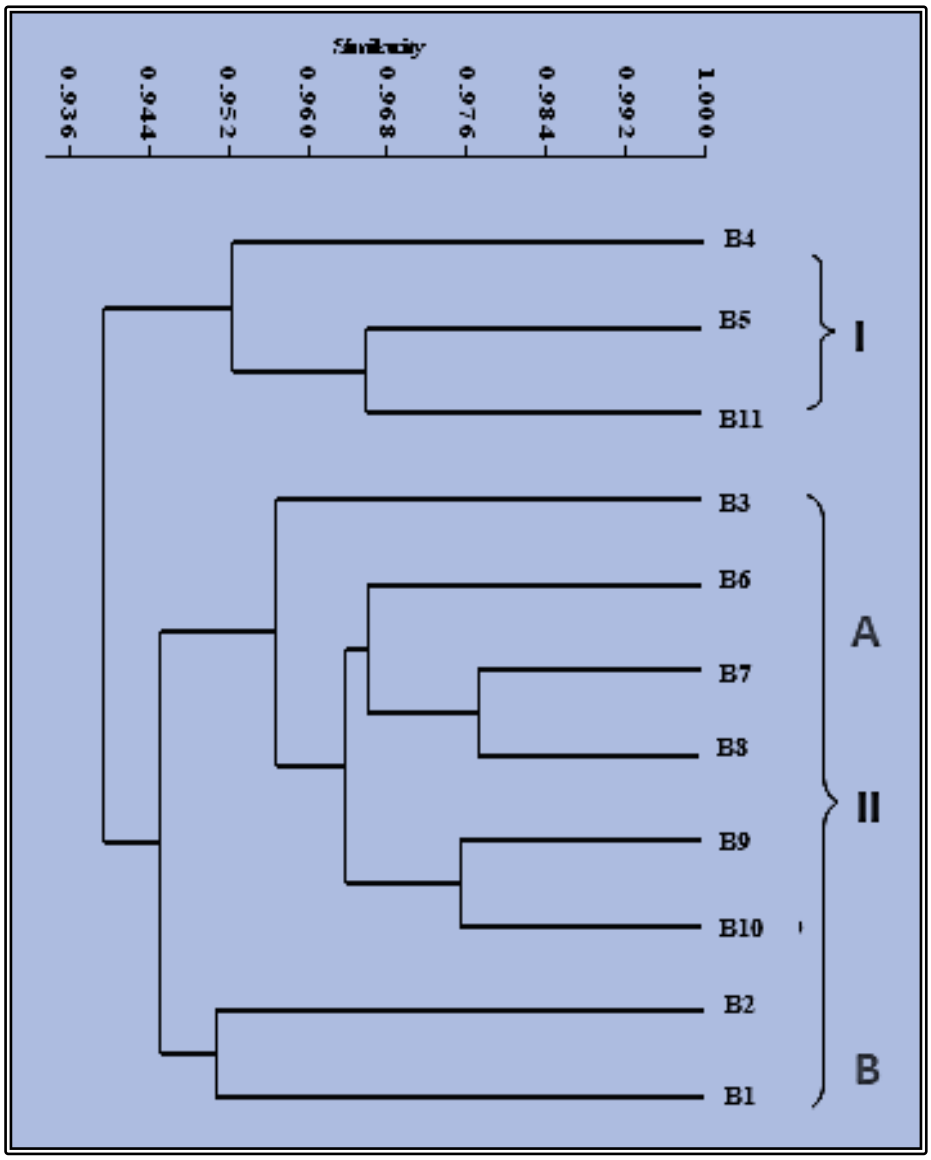

Fig. (1): Dendrogram representing the quantitative morphological traits of 11 *Hordeum vulgare* L. accessions using UPGMA cluster analysis generated from 15 quantitative morphological traits.

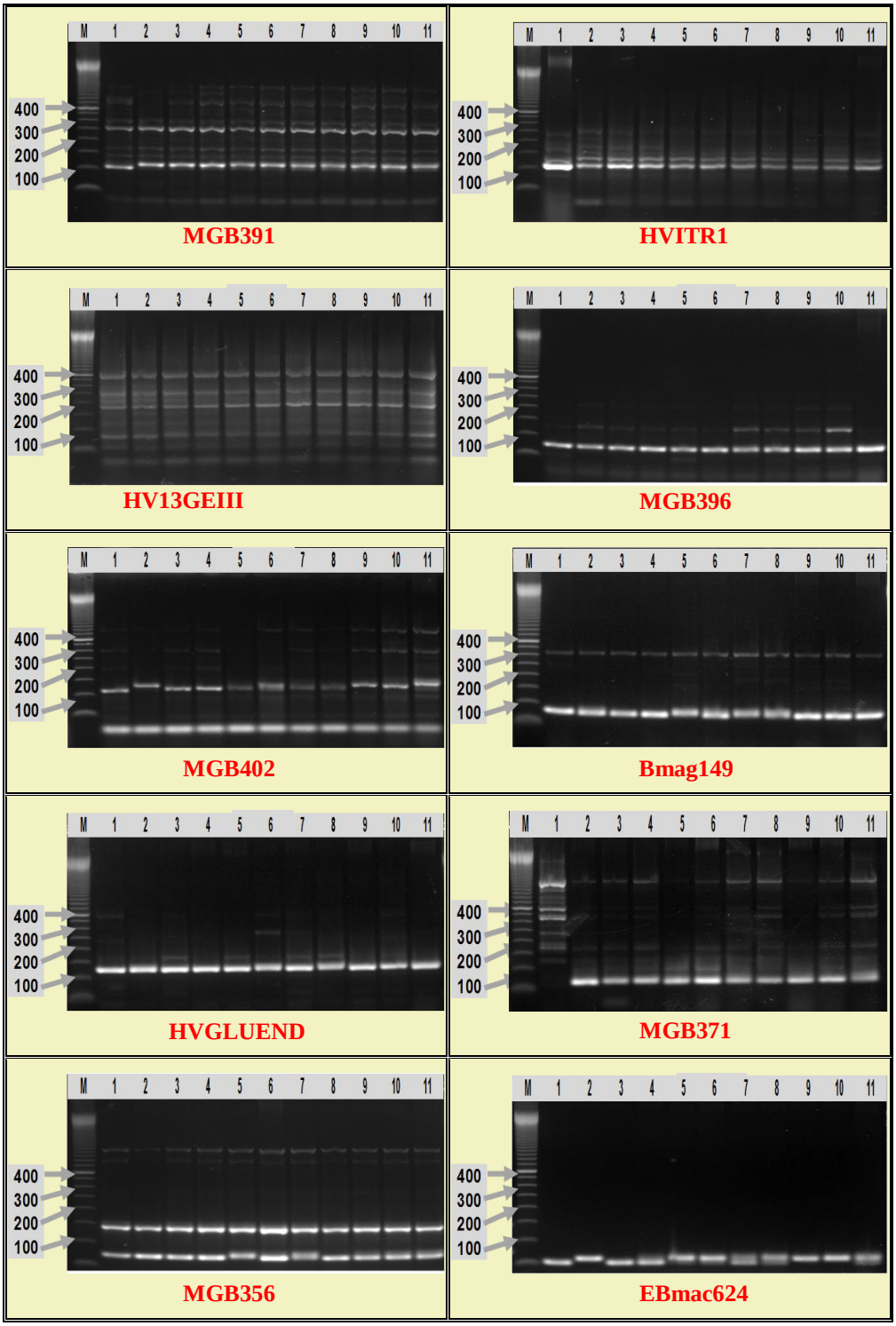

Fig. (2): Banding patterns of 11 *Hordeum vulgare* L. accessions using 10 SSR primers.

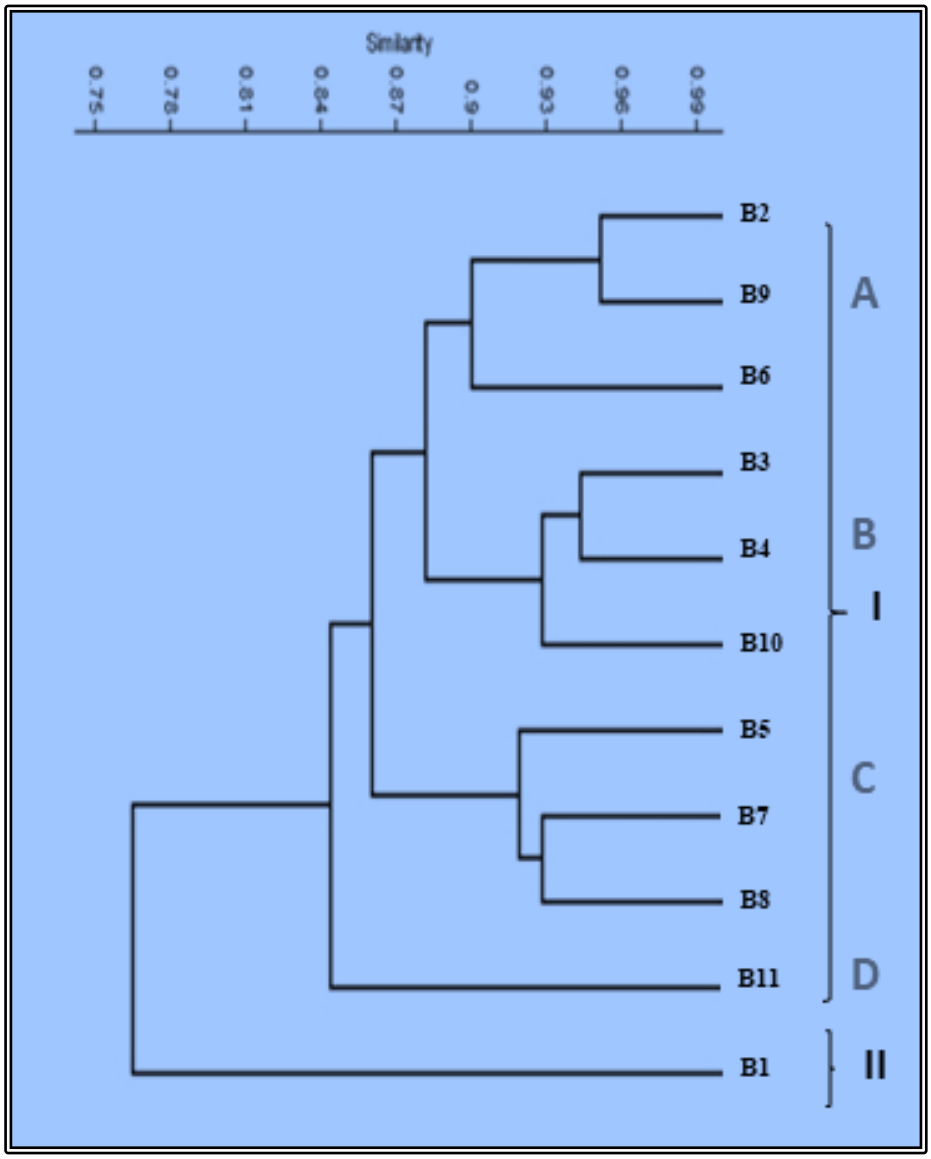

Fig. (3): Dendrogram using UPGMA cluster analysis of ten SSR markers.

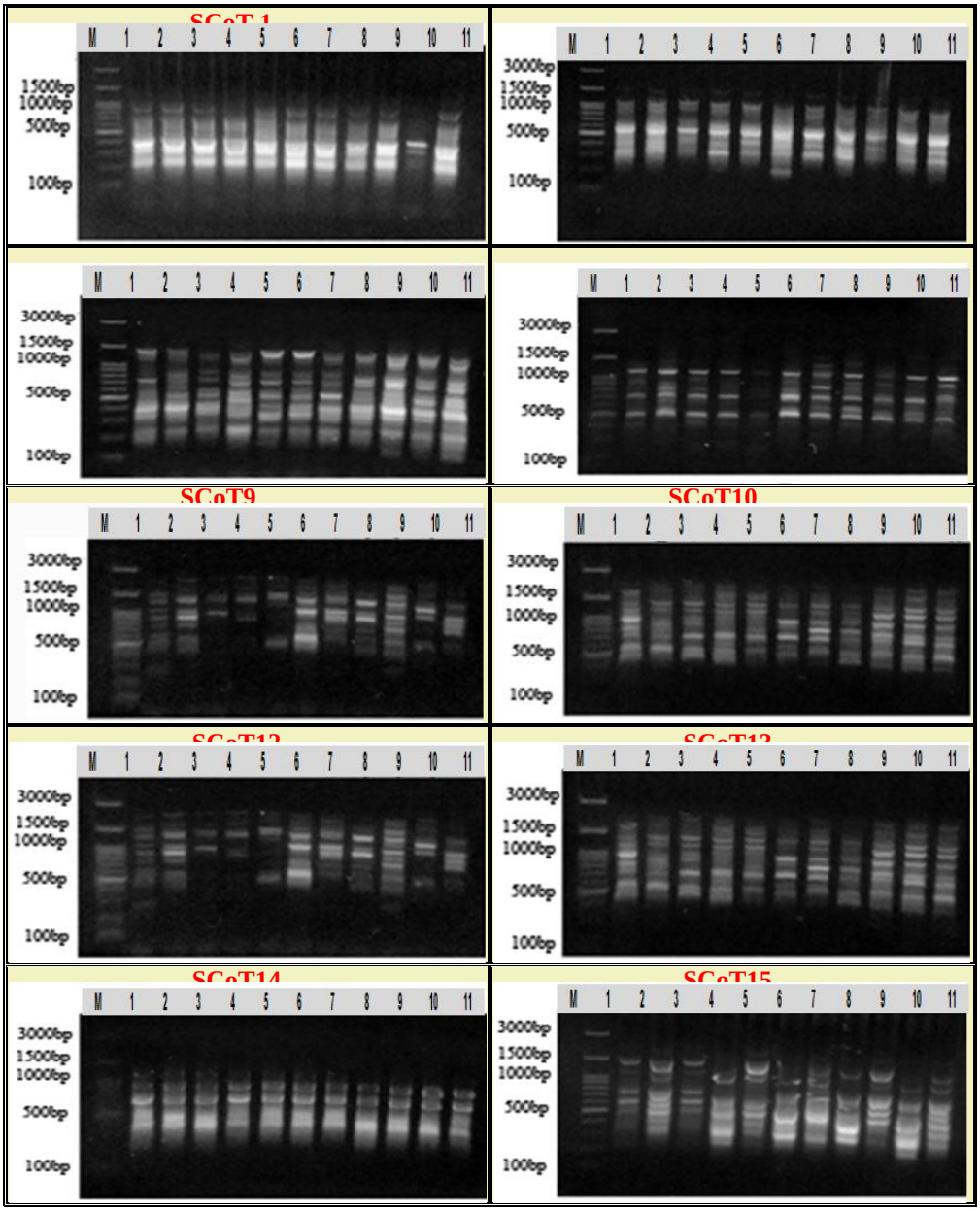

Fig. (4): Banding patterns of 11 *Hordeum vulgare* L. Accessions using 10 SCoT primers.

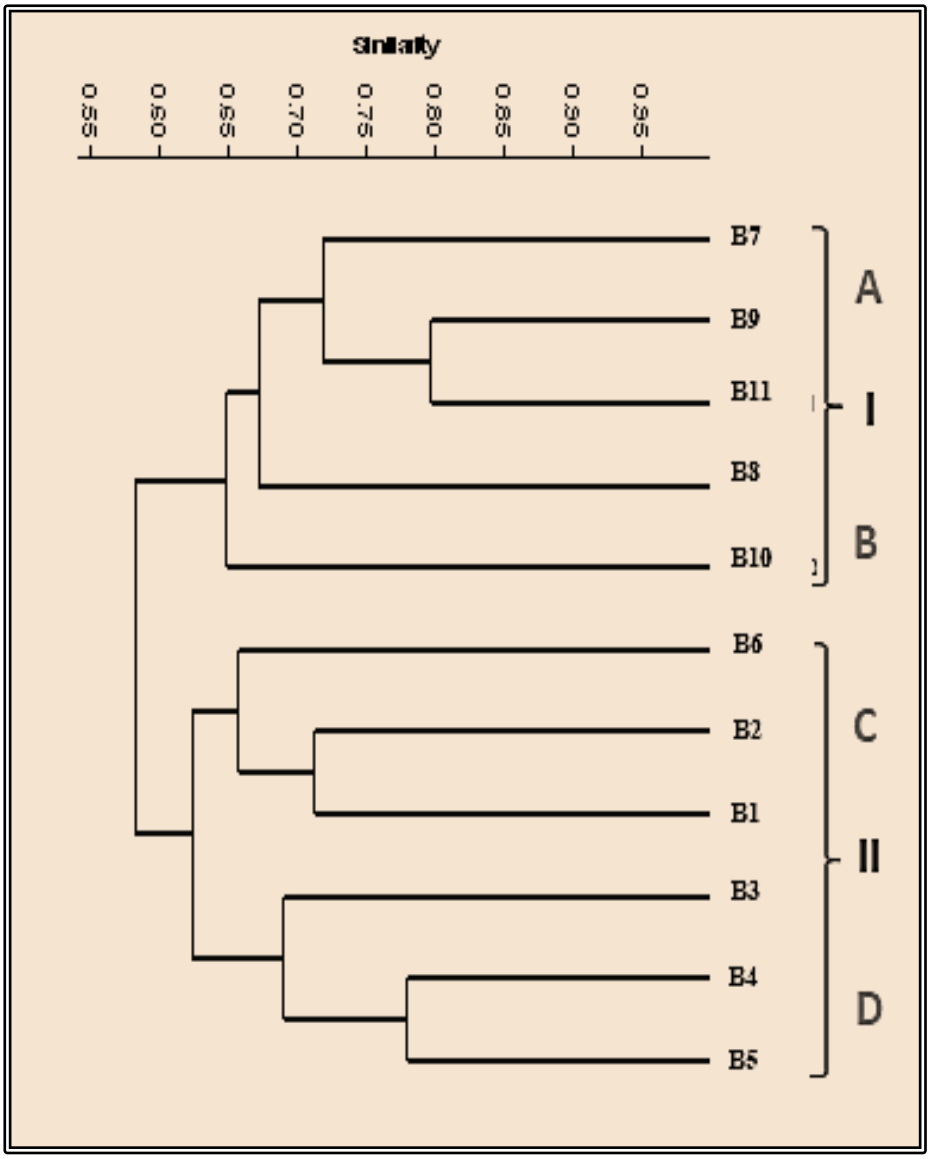

Fig. (5): Dendrogram using UPGMA cluster analysis of ten SCoT markers.

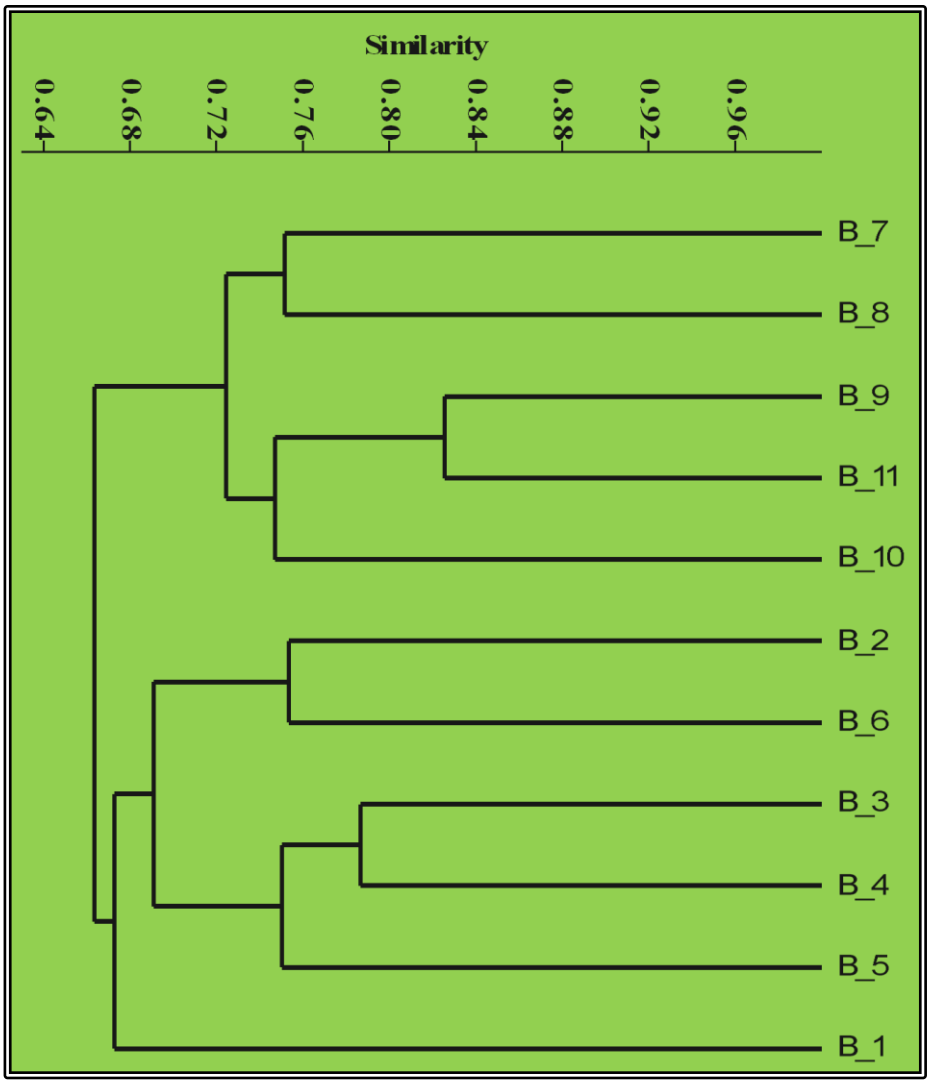

Fig. (6): Dendrogram using UPGMA cluster analysis of combined qualitative morphological traits with 10 SSR markers and 10 SCoT markers.

MtNOOT HETEROLOGOUS EXPRESSION WITH *FaWRKY1* OVER-EXPRESSION *CONFER STRWBERRY* RESISTANCE AGAINST *Macrophomina phasiolena*

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Strawberry (*Fragaria x ananassa*) is a cultivated member of the family *Rosaceae*, and globally, it is one of the most important fruits (Parikka, 2004). Wild strawberries were consumed by the native people since the Stone Age and they are native to Asia and Europe. Strawberry plants have distinctive variety regarding their genetic makeup and most Strawberry species are diploid species and the hybrids can be varied from tetraploid to decaploid. It has a high nutritional value (Giampieri *et al*., 2017) as they are favorable for people suffering from dysentery, arthritis, gout, high cholesterol, premature aging, high blood pressure, diabetes, a weak immune system, liver damage, respiratory infections, high toxicity, cancer risk, and constipation. Environmental condition including abiotic and biotic stresses is affecting such economically important fruits. Some verities display high tolerance against various stresses (Phukan *et al*., 2015; Garrido-Bigotes *et al*., 2018). Worldwide Strawberry production threat mainly is due to numerous black root rot (charcoal rot) diseases caused by *Macrophomina phaseolina* (Browne *et al*., 2002; Millner 2006; Chamorro *et al*., 2015; and Burkhardt *et al*., 2019) and *Rhizoctonia solani* (Sharma and Bhardwaj, 2001; Timudo-Torrevilla *et al*., 2005). *Macrophomina phaseolina* is highest abundance and wide distribution in strawberry plants in Egypt (Hussein *et al*., 2012). This article, aimed to produce some valuable resistant traits to the cultivated strawberry to enhance its ability to tolerate some destructive fungi and hence promote its high productivity.

Plants normally respond to the perceived signal molecules of any external hazardous by stimulation specific genes that are associated with the biotic and abiotic stress as well. The resistance to pathogens attacking is regularly harmonized by plants *via* a complex molecular defense network (Robert-Seilaniantz *et al*., 2011). The particular defense genes include transcription factors (TFs) like; WRKY, NAC, ERF, and MADS. WRKYs are involved in both biotic and abiotic stress response as well as in physiological and developmental processes (Robatzek and Somssich, 2002; Desveaux *et al*., 2005; Jiang *et al*., 2015; Seo *et al*., 2015; Liu *et al*., 2016). WRKYs is achieving cellular homeostasis by recognition of W-box existing in target genes' promoters and induce their expression. Diqiu *et al*. (2001) presented that W-box found in the promoter sequences of the NPR1 (Nonexpresser of PR genes 1) are recognized particularly by SA-induced WRKY DNA binding protein in Arabidopsis and thus, WRKY genes act upstream of the NPR1 genes and positively control their expression during plant defense actions. Previous data proved that overexpression of NPR1 genes leads to enhance disease resistance in various plant species like; rice, *Arabidopsis*, wheat, tobacco, and apple (Spoel and Dong, 2008; Chern *et al*., 2005, Makandar *et al*., 2006).

Cauliflower mosaic virus (CaMV) promoter P35S, the most efficient plant promoter, was used (Odell *et al*., 1985; and Benfey *et al*., 1989) to drive the expression of *FaWRKY* gene in *Escherichia coli*. Assaad and Signer (1990) presented evidence that *P35S* successfully directed the expression of neomycin phosphotransferase II (NPTII) gene in *E. coli* as the translation nucleotide sequence start site is strongly homologous to that of the consensus sequence of a prokaryotic promoter. In this article, *Macrophomina phaseolina* and *Rhizoctonia solani* were subjected to the effect of both *FaWRKY* transformed *E. coli* to assess the antagonistic ability of *E. coli* ecologically expressed the two chimeric gene in enhancing defense activity.

For additional insight into the biotic function of *FaWRKY1* as well as *MtNOOT* genes within the pathogen resistance mechanism, we have transiently overexpressed both genes in strawberry tissue. The two genes were introduced separately and in combination with each other into strawberry leaves *via Agrobacterium*-mediated transient transformation approach (Mangano *et al*., 2014). *FaWRKY* gene was previously isolated from the stressed strawberry tissues by applying jasmonic acid, salicylic acid, and *Macrophomina phasiolena* fungal homogenate (Hussein *et al*., 2016). *MtNOOT* encodes BTB/POZ-ankyrin repeat protein specific for NPR1 family. NPR1 family

are triggered during plant's attack *via* Systemic Acquired Resistance (SAR) (Klessig and Malamy 1994; Fu and Dong, 2013) and has a fundamental role in signaling activation of PR-mediated protection against various pathogens through salicylic acid SA signaling pathway (Yan and Dong, 2014). *MtNOOT* was isolated through investigating *noot*, the symbiotic mutant of *Medicago truncatula* (Couzigou *et al*., 2012).

This investigation proposed that susceptibility to *Macrophomina phasiolena*, is significantly decreased in the strawberry plant where *FaWRKY1* & *MtNOOT* were transiently overexpressed separately or in combination. This study highlights the positive regulation of resistance establishes after overexpression of *FaWRKY* with the ectopic expression of *MtNOOT* in strawberry due to the defense response network between the two genes.

MATERIALS AND METHODS

Plant Materials and Fungal Strains

Strawberry (*Fragaria* × *ananassa* cv. Camarosa) plants were kindly provided from the modern company (PICO). *Macrophomina phasiolena* and *Rhizoctonia solani* were preserved on potato dextrose agar (PDA) (Altindag *et al*., 2006) at 20ºC with 16/8 light/dark photoperiod. To rise the fungal infectivity prior to inoculation with the pathogen, the conidia stock suspensions were prepared by rubbing the four week old fungal mycelia surface in dH_2O supplied by 0.03% Tween80, then they were filtrated with glass wool. The conidia suspensions concentrations were quantified with a Neubauer Chamber Cell Counting and adjusted with dH_2O to be 10^6 conidia/ml (Higuera *et al*., 2019). The fungal spores' suspensions with 10^6 spore/ml concentration were autoclaved.

Binary Vectors

Modified pCambia 1390 binary vector was used for *FaWRKY1* construction under the control of S35 promoter and nos terminator, while pCP 42 was used for cloning of *MtNOOT* under the control of its promoter region and nos terminator. Modified pCambia 1390 and pCP 42 binary vectors were kindly provided from Dr. Pascal Ratet, Institut des Sciences des Plantes de Paris Saclay, IPS2BATIMENT 630 PLATEAU DU MOULON RUE NOETZLIN.

Plasmid Construction for Fungal Application and Strawberry Transient Transformation

For transient overexpression of *MtNOOT* gene in strawberry leaves, a fragment of 4700 bp including; 2811 bp of *NOOT* gene plus 1889 bp of its promoter fragment was amplified using the two previously designed primers (Couzigou *et al*., 2012); Pnoot 5'-GGACCGACGAATGTATTAA-GCCTTAAA-3' & nootR 5'-TTAGTAGTCATGACCATGAGAGT-3' was cloned in pGEM-Teasy and double digested by *Eco*RI/*Sal*I after destruction *of*

*Eco*RI site toward the SP6 promoter of pGEM-Teasy and sub-cloned in pCP42 binary vector to generate pCP42::*MtNOOT* under the control of *MtNOOT* gene promoter. For preparing pCambia1390::FaWRKY1 (35S::FaWRKY1) expression cassette, *Bam*H1/*Eco*R1 restriction sites were added to the start and the end of *FaWRKY1*, respectively *via* the two oligonucleotide primers*;* WR-F 5' C*GGATCC*ATGGATACCTACCCAGCATTC '3 & WR-R 5' G*GAATTC*TCACAAAGAAGTGTAGATTTGCAT '3 using pGEM-Teasy-FaWRKY1 clone (Hussein *et al*., 2016) as a DNA template to amplify a fragment of 575bp. The amplified FaWRKY1 fragment and the modified pCambia 1390 binary vector were double digested by *Bam*H1/*Eco*R1 and ligated together. pCP42::MtNOOT and pCambia1390::*FaWRKY1* were transformed into *E. coli* competent cells and the transformed colonies were selected on 100 mg/ml ampicillin LB plates and confirmed by the proper colony PCR protocol.

FaWRKY1 Antagonistic Activity

Antifungal activity of *FaWRKY1* was assayed by estimating the inhibition initiated by two parasitic fungi on potato dextrose agar plates. *E. coli*-containing *FaWRKY1* gene cloned in pGEM-Teasy was inoculated on the edges of both *Macrophomina phasiolena, Rhizoctonia solani* PDA plates. *Macrophomina phasiolena, Rhizoctonia solani* grown separately on PDA plates were used as a positive control and were inoculated at the edge with non-transformed *E. coli* cells to eliminate the effect of *E. coli* on the fungal growth. The plates were incubated at 28 ºC for seven days and fungi inhibition were determined.

FaWRKY and *MtNOOT* Construction and Agrobacterium Transformation

pCP42::MtNOOT and pCambia1390::FaWRKY1 constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 (Koncz *et al.*, 1989) by electroporation protocol (Sukharev *et al*., 1992) at 25 mF, 2.5 kV and 400 Ω. The transformed cells were selected on LB- agar media containing 50 mg/ml and streptomycin 50mg/ml kanamycin. Agro-infiltration experiment was carried out using the two constructs separately and together.

Agro-infiltration in Strawberry Leaves

Strawberry plant leaves were used for transient expression by Agro-infiltration (Kapila *et al*., 1997; Yang *et al*., 2000). One hundred microliters of transformed frozen cells stock was inoculated in 5 ml LB broth supplemented with 50 mg/ml streptomycin and 50 mg/ml kanamycin. The culture was incubated at 28ºC at 210 rmp shaking overnight. 500 µl of transformed *Agrobacterium* was used to inoculate 50 ml of LB medium and the cells were shaking at 28ºC and 210 rpm until the culture reached the O.D.600=0.6. The cells were harvested at 6000 rpm by centrifugation and re-suspended in 50 ml MES buffer (10 mM MES; pH 5.5, 10

mM $MgCl_2$). MES buffer was incubated at room temperature for two hours with 100 µM acetosyringone. The leaves of two months old strawberry plants were pressure injected into the lower epidermis of the leaf through a 3 ml disposable syringe. Post-infiltrated plants were kept in the growth chamber with a 16/8 light/dark photoperiod at 25ºC.

Mechanical Injury and Strawberry Leaves Infection

Two days post-agro-infiltration process, strawberry leaves were infected mechanically by *Macrophomina phasiolena* parasitic fungus, a wound was formed by a red hot pinhead touched the epidermis of the infiltrated strawberries leaves gently (De Meyer and Hofte, 1997). After wounding the leaves, the spores' suspensions were sprayed exogenously over the strawberries plants. Then the pots with the infected and control plants were incubated at closed chamber at 25ºC degree separately. The data were taken three, five, and seven days post fungal inoculation.

RNA Extraction and RT-PCR Conditions

RNA extracted was carried out from *MtNOOT* and *FaWRKY1* transformed leaves using RNeasy Mini Kit according to the manufacturer's instructions. All DNA contaminates were removed *via* Thermo Scientific, DNase1 RNase free kit. Recovered RNAs were measured by spectrophotometry at A260 and A280 nm. SuperScript-III, the one-step RT-PCR was carried out according to the manufacturer's instruction. NanoDrop Spectrophotometer (Thermo scientific) was used for quantifying the Purified RNA. Oligonucleotide pair; nootF 5'-ATGTCCCTTGAAGACTCACTAA-GATCT-3' & nootR and oligonucleotide pair WR-F & WR-R were used for *NOOT* and *WRKY* expression detection respectively in strawberry leaves.

RESULTS AND DISCUSSIONS

Strawberry is the major popular fruit and its health benefits were intensively dedicated by many researchers worldwide, but its yield and quality are extremely limited due to various pathogen attacks. *Macrophomina phasiolena*, *Rhizoctonia solani* are seriously limiting parasites that are thriving strawberry productivity. Thus, the strategies to expand resistance in such important crop will be the most relevant economically.

Expression Performance of *FaWRKY1* as an Antagonistic to Fungal Growth

Promoter Elements of prokaryotes and eukaryotes developed differently through evolution with regard to their structure and sequences. Previously many researcher demonstrated that the eukaryotic promoter sequences can't drive an efficient gene expression in prokaryotes upon its translocation. However, Assaad and Signer (1990) used cauliflower mosaic virus constitutive promoter *P35S* to direct the expression of neomycin phosphotransferase II gene in *E. coli.*

This study was designed an experiment to evaluate the effect of *FaWRKY1* transgene that is produced into *E. coli* transformed cells on limiting the growth of the harmful parasitic fungi. pCambia1390::FaWRKY1 (p35S::FaWRKY) transformed colonies were selected and confirmed by PCR. *Macrophomina phasiolena*, and *Rhizoctonia solani* growth plates were subjected to the effect of *FaWRKY1* as described in materials and methods section. An effectively inhibition zones were obviously detected on both *Macrophomina phasiolena*, and *Rhizoctonia solani* PDA plates treated with p35S::FaWRKY transformed *E. coli*. The detected Inhibition zones were almost similar in case of the two parasitic fungal growth. *Macrophomina phasiolena* and *Rhizoctonia solani* growing on PDA plates and inoculated with un-transformed *E. coli* were used as positive control as it hasn't any effect on fungal growth performance (Fig. 1). This result is a preliminary evidence that *FaWRKY1* can positively limits harmful fungal growth and inhibits its activity.

Previously, Jacob *et al*., (2002) succeeded to prove that constructs designed for transformed into plants is able to be expressed in *E. coli* as well. They demonstrated that Plants promoter contains elements that are recognized by eubacteria transcription machinery even though the promoter sequences are considerably less specific than of commonly expected. To prove their findings, they evaluated lux gene expression driven by ten diverse plant-specific promoters, mainly the *P35S* promoter, in five diverse bacteria species, including *E. coli*. Our finding presented that the designed constructs, P35S-*FaWRKY-3'nos* transformed into *E. coli* were thrived to generate an inhibition zone in *Macrophomina phasiolena*, and *Rhizoctonia solani* fungal growth plates. This result is great standing for our knowledge around the organization of gene expression evolution and on construction of various expression vectors. In a similar manner, many studies used *P35S* to drive gene expression in *E. coli* and in *Agrobacterium tumefaciens* -mediated transformation as well. Glutenin genes as an example were isolated and their expression were derived by *P35S* using pCAMBIA-1304 (Pandey and Somssich, 2009).

Transient Overexpression of *FaWRKY1* & *MtNOOT* Genes in Strawberry Leaves Reduced Tissue Damage post *Macrophomina phasiolena* Inoculation

On the other hand, we evaluate the performance of our two genes; *FaWRKY1* and *MtNOOT* when transformed separately and in combination into Strawberry plant's tissue. Hence, the transformed plants were subjected to mechanical fungal infection. In such case, the expression of each gene is influenced by other transcription factor proteins in a definite pathway inside a complete defense network system.

pCP42::MtNOOT and pCambia1390::FaWRKY1 constructs; *MtNOOT* is cloned under the control of its own promoter and *FaWRKY* is cloned under

the control of p35S (Pnoot:: MtNOOT& *p35S*::FaWRKY), were transiently transformed into strawberry leaves *via Agrobacterium tumefaciens* followed by PCR confirmation (Fig. 2). The two expression cassettes were introduced into strawberry leaves *via* agrobacterium-infiltration procedure separately and in accomplishment with each other while the *Macrophomina phasiolena* mechanical infection was carried out two days post the agrobacterium-infiltration. The *FaWRKY1* & *MtNOOT* fragments and transcripts viability were confirmed by PCR and RT-PCR respectively third, fifth, and seventh days post mechanical infection with *Macrophomina phasiolena* (Fig. 3). The consequent symptoms were monitored on transiently transformed strawberry leaves and compared to the two controls; the healthy plant as negative control and the untransformed-infected strawberry leaves as a positive control for the disease. The control healthy plant is injected with an empty *Agrobacterium tumefaciens* to eliminate the effect of agrobacterium on strawberry leaves. While, the positive control strawberry plants were mechanically injured and sprayed with *Macrophomina phasiolena* spores but were not transiently transformed with any of the constructs or an empty agrobacterium. The data showed that three days post parasitic infection, welting and drying symptoms were observed in the positive control plants, and moreover the plants were eventually died after five days compared with uninfected control healthy plants (Fig. 4 A, B and C). Strawberry leaves transiently expressed *MtNOOT* and *FaWRKY* genes accompanied to each other displayed the highest resistance activity at the third, fifth, and seventh days post-inoculation with *Macrophomina phasiolena*, followed by the leaves transiently expressed *MtNOOT* however, leaves transiently overexpressed *FaWRKY1* were the most susceptible to the fungal infection but still more resistant than the positive control leaves (Fig. 4 D, E, F, G, H, I, J, K, and L).

Our data presented that the overexpression of *FaWRKY1* gene in strawberry plants considerably enhance defense mechanism against the fungal infection three, five, and seven days post mechanical infection. Likewise, ectopically expression of *Medicago truncatulla NOOT* gene in strawberry tissue derived a more protection pattern than that of *FaWRKY1*. In a previous data, the role of *FaWRKY1* in defense response against various pathogen attacks was proposed by dramatically up-regulated *FaWRKY1* transcript in strawberry (Casado-Diaz *et al*., 2006; Encinas-Villarejo *et al*., 2009). Similarly, *VvWRKY1* overexpression enhanced resistance against downy mildew disease caused by *Plasmopara viticola* in grapevines, *via* JA-pathway related genes induction (Marchive *et al*., 2013).

WRKY transcription factor family contains the WRKYGQK main sequence upstream a zinc-finger motif, have various roles during plant defense response and its can control its own expression positively and negatively self-regulation upon parasitic infection and in health condition respectively due to the presence of a W-box

within its promoter region (Asai *et al*., 2002; Robatzek and Somssich, 2002). WRKY is modulating defense plant either positively or negatively (Eulgem and Somssich, 2007). Contrarily to our result, *FaWRKY1* silencing is enhancing resistance against the pathogen *Colletotrichum acutatum* in strawberry (*Fragaria _ ananassa* cv. Primoris), so in that case *FaWRKY1* acts as a negative regulator of resistance activity (Higuera *et al*., 2019). In a similar way, the *GbWRKY1* silencing in cotton promoted plant resistance against *Verticillium dahlia* and *Botrytis cinerea* (Li *et al*., 2014).

The members of WRKY family can bind to the consensus sequence [(T)TGAC(C/T)] of the W-box found in the promoter of various stress-specific genes sequences, including PR proteins that responded to pathogens attacks and wounding (Ülker and Somssich, 2004). Thus, we transformed *MtNOOT*, the NPR1-family related gene, which can be influenced and positively activated by the overexpression of *FaWRK1* transcription factor during the parasitic attacks into strawberry tissue. NPR1 family compresses a BTB/POZ as well as ankyrin repeats domains in its sequence (Aravind and Koonin, 1999; Sedgwick and Smerdon, 1999), SAR is the plant defense action that is essentially regulated by NPR1. When the plants were subjected to biotic or abiotic stress, redox potential elicited by salicylic acid (SA) warning sign molecules accumulation is enhancing NPR1 to be converted from the oligomeric form to the monomeric form and hence can be translocate to the nucleus from the cytosol in order to interact with TGA transcription factors, (the TGACG specific binding protein sequence) that binds to SA-responsive elements. Various WRKYs were well-defined as SA-dependent responses regulators through the NPR1-dependent SAR initiation (Ishihama and Yoshioka, 2012).

The protection proposed by *FaWRKY*1 overexpression combined by heterologous overexpression *MtNOOT* in strawberry plants was ultimately better than when introducing each gene separately into plant's tissue. This is due to the fact that the sequences of W-box in promoter region of the *MtNOOT* are recognized precisely by SA-induced *FaWRKY* DNA binding proteins and hence *MtNOOT* expression was activated. The results proved that constitutive overexpression of *FaWRKY1* in strawberry tissue positively affected the ectopic expression of the *MtNOOT*-NPR1 related gene upon fungal infection. In a similar manner, Choi *et al*., (2004) conferred high resistance against fungus pathogen *A. alternata* and bacterial pathogen *P. syringae* in tobacco by fusing a promoter region of pea DRR206 gene, which contains the W-box and the wound/pathogen-inducible box (Palm *et al*., 1990), with *F. solani* DNase elicitor gene. Mutations in W-box sequence eliminated their recognition *via* WRKY DNA binding protein, and hence the promoter will not be able to activate the downstream protective genes (Yu *et al*, 2001). In addition, Viejobueno *et al*., (2021) induced defense response against *M. phaseolina* by treatments with *Azospi-*

rillum brasilense bacterial, the Plant Growth-Promoting Rhizobacteria (PGPR). This bacterium has a native antagonistic effects against parasitic agents and increased tolerance of strawberry plants to charcoal rot disease. This way of elevating the innate immune system of the plants is an ecological and environmentally friendly approach.

CONCLUSION

The results demonstrate that the *FaWRKY* transcript can be confirmed directly in *E. coli*, despite the fact that the main transcription sites seem not to be similar as in plants. The enhanced resistance through overexpression of *MtNOOT* & *FaWRKY1* genes in strawberry-transiently transformed leaves suggests that both genes activate plant defense mechanisms upon pathogen attack comparing to untransformed strawberry plants. Network regulation between *FaWRKY1* & MtNOOT were dedicated to ultimately increase strawberry defense response to *Macrophomina phasiolena* attack.

SUMMARY

Strawberry (*Fragaria x ananassa*) is one of the favorite fruit worldwide due to its wide health benefits, and distinct flavor and aroma. *FaWRKY1* gene was suggested as a significant element intermediate defense response against various pathogens attack in strawberry. Because of the influencing role of WRKY family involving in defense network, it has become a favorable candidate for improving crops quality. WRKY can precisely recognition and binding to the downstream promoters of transcription factors activating defense cascades. The ability of FaWRKY to enhance resistance against *Macrophomina phasiolena* was investigated by performing Agrobacterium-mediated transformation protocol for transient overexpression of *FaWRKY1* gene in strawberry leaves (Fragaria *x* ananassa cv. Camarosa) to evaluate its function upon the fungal infection. However, *MtNOOT* gene were transiently hetero-expressed in strawberry leaves separately and in accomplishment with the *FaWRKY*1. We demonstrated that the existing of W-box sequences within the *MtNOOT* (NPR1-like gene) promoter region, which are recognized definitely by SA-induced FaWRKY DNA binding protein, increased strawberry resistance activity when the two genes are transformed in combined to each other .The severity of leaf injury was observed at three, five, and seven days post pathogen inoculation on *FaWRKY1*, *MtNOOT*, and *FaWRKY1* & *MtNOOT* combination-transformed strawberry plants compared to the control untransformed infected plants as positive control and a healthy un-transformed non-infected plants as negative control . Susceptibility to fungal infection was obviously detected and showing that the two genes combination (*FaWRKY* & *MtNOOT*) revealed the best resistance against the pathogen fungal attack followed by *MtNOOT* and finally by the *FaWRKY1* transcription factor. Our results evidence that *FaWRKY1* gene acts upstream of the heterologous *MtNOOT*

(*NPR1-like gene)* and positively regulates its expression throughout plant defense activation during pathogen attack.

REFERENCES

Altindag M., Sahin M., Esitken A., Ercisli S., Guleryuz M., Donmez M. F. and Sahin F. (2006). Biological control of brown rot (*Moniliana laxa* Ehr.) on apricot (*Prunus armeniaca* L. cv. Hacıhaliloglu) by *Bacillus*, Burkholdria, and *Pseudomonas* application under *in vitro* and *in vivo* conditions. Biol. Control., 38:369-372.

Aravind L., and Koonin E. V. (1999). Fold prediction and evolutionary analysis of the POZ domain: Structural and evolutionary relationship with the potassium channel tetramerization domain. J. Mol. Biol., 285:1353-1361.

Asai T., Tena G., Plotnikova J., Willmann M. R., Wan-Ling C., Gomez-Gomez L., Boller T., Ausubel F. M. and Sheen J. (2002). MAP kinase signalling cascade in *Arabidopsis* innate immunity. Nature, 415:977-983.

Assaad F. F. and Signer E. R. (1990). Cauliflower mosaic virus *P35S* promoter activity in *Escherichia coli. Mol. Gen. Genet.,* 223: 517-520.

Benfey P. N., Ren L. and Chua N. H. (1989). The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J., 8: 2195-2202.

Browne G. T., Becherer H. E., McLaughlin S. T. and Wakeman R. J. (2002). Strategies for management of Phytophthora on California strawberries. The Pink Sheet: California Strawberry Commission Strawberry News Bulletin, 02-09.

Burkhardt A. K., Childs K. L., Wang J., Ramon M. L. and Martinm F. N. (2019). Assembly, annotation, and comparison of *Macrophomina phaseolina* isolates from strawberry and other hosts. BMC Genomics, 20:802.

Casado-Díaz A., Encinas-Villarejo S., Santos B. D. L., Schilirò E., Yubero-Serrano E. M. and Amil-Ruíz F. (2006). Analysis of strawberry genes differentially expressed in response to *Colletotrichum* infection. Physiol. Plant., 128: 633-650.

Chamorro M., Aguado A. and De los Santos B. (2015). First Report of Root and Crown Rot Caused by Pestalotiopsis clavispora (Neopestalotiopsis clavispora) on Strawberry in Spain. Plant Disease, 1-3.

Chern M., Canlas P. E., Fitzgerald H. A. and Ronald P. C. (2005). Rice NRR, a negative regulator of disease resistance, interacts with *Ara-*

bidopsis NPR1 and rice NH1. Plant J., 43:623-635.

Choi G. J., Jang K. S., Kim J. S., Lee S.W., Cho J. Y., Cho K. Y. and Kim J. C. (2004). *In vivo* antifungal activities of 57 plant Extracts against six plant pathogenic fungi. Plant Pathol. J., 3:184-191.

Couzigou J. M., Zhuko V., Mondy S., Abu el Heba G., Cosson V., Noel Ellis T.H., Ambrose M., Jiangqi Wen, Tadege M., Tikhonovich I., Mysore K. S., Putterill J., Hofer J., Borisov A. Y. and Rateta P. (2012). NODULE ROOT and COCHLEATA Maintain Nodule Development and Are Legume Orthologs of Arabidopsis BLADE-ON-PETIOLE Genes., 24: 4498-4510.

De Meyer G., and Höfte M. (1997). Salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 induces resistance to leaf infection by *Botrytis cinerea* on bean. Phytopathology 87:588-593.

Desveaux D., Maréchal A., and Brisson N. (2005). Whirly transcription factors: defense gene regulation and beyond. Trends Plant Sci., 10:95-102.

Diqiu Y. u., Chen C. and Chen Z. (2001). Evidence for an Important Role of WRKY DNA Binding Proteins in the Regulation of *NPR1* Gene Expression. Plant Cell, 13:1527-1540.

Encinas-Villarejo S., Maldonado A. M., Amil-Ruiz F., De Los Santos B., Romero F. and Pliego-Alfaro F. I. (2009). Evidence for a positive regulatory role of strawberry (*Fragaria* × *ananassa*) Fa WRKY1 and *Arabidopsis* at WRKY75 proteins in resistance. *J. Exp. Bot.*, 60:3043-3065.

Eulgem T. and I. Somssich E. (2007). Networks of WRKY transcription factors in defense signaling. Curr Opin Plant Biol., 10: 366-371.

Fu Z. Q., and Dong X. (2013). Systemic acquired resistance: turning local infection into global defense. Ann. Rev. Plant Biol., 64:839-863.

Garrido-Bigotes A., Figueroa N. E., Figueroa P. M. and Figueroa C. R. (2018). Jasmonate signaling pathway in strawberry: genome-wide identification, molecular characterization and expression of JAZs and MYCs during fruit development and ripening. PLoS One, 13:e0197118.

Giampieri F., Alvarez-Suarez J. M., Cordero M. D., Gasparrini M., Forbes-Hernandez T. Y., Afrin S., Santos-Buelga C., González-Paramás A. M., Astolfi P., Rubini C., Zizzi A., Tulipani S., Quiles J. L., Mezzetti B. and Battino M.

(2017). Strawberry consumption improves aging-associated impairments, mitochondrial biogenesis and functionality through the AMP-activated protein kinase signaling cascade. Food Chem., 234:464-471.

Higuera J. J., Garrido-Gala J., Lekhbou A., Arjona-Girona I., Amil-Ruiz F., Mercado J. A., Pliego-Alfaro F., Muñoz-Blanco J., C. J. López-Herrera and Caballero J. L. (2019). The Strawberry FaWRKY1 transcription factor negatively regulates resistance to Colletotrichum acutatum in Fruit upon Infection. Front. Plant Sci., 10:480.

Hussein G. M. H., Sayed M. A., Abdel-Rahman T. M. A. and Alwan A. H. (2012). Induction of acquired resistance in strawberry calli by elicitation of fungal homogenates. J. Food Agric. Environ., 10:750-756

Hussein G. M. H., Abdel-Rahman Tahany M. A. and Alwan A. H. (2016). Defense response enhancement in strawberry *via* elicitors. 3 Biotech, 6:130.

Ishihama, N., and H. Yoshioka (2012). Post-translational regulation of WRKY transcription factors in plant immunity. Curr. Opin. Plant Biol. 15:431–437.

Jacob D., Lewin A., Meister B. and Appel B. (2002). Plant-specific promoter sequences carry elements that are recognized by the eu-bacterial transcription machinery. Trans. Res., 11:291-303.

Jiang W., Wu J., Zhang Y., Yin L. and Lu J. (2015). Isolation of a WRKY30 gene from Muscadiniarotundifolia (Michx) and validation of its function under biotic and abiotic stresses. Protoplasma, 252:1361-1374.

Kapila J., Rycke R. D., Montagu M. V. and Angenon G. (1997). An Agrobacterium-mediated transient gene expression system for intact leaves. Plant Science, 122: 101-108.

Klessig, D. F. and Malamy J. (1994). The salicylic acid signal in plants. Plant Mol. Biol., 26:1439-1458.

Koncz C., Martini N., Mayerhofer R., Koncz-Kalman Z., Korber H., Redei G. P. and Schell J. (1989). High-frequency T-DNA-mediated gene tagging in plants. Proc. Natl. Acad. Sci. USA, 86:8467-8471.

Li C., He X., Luo X., Xu L., Liu L. and Min L. (2014). Cotton WRKY1 mediates the plant defense-to-development transition during infection of cotton by verticillium dahliae by activating JASMONATE ZIMDOMAIN1 expression. Plant Physiol., 166:2179-2194.

Liu X., Song Y., Xing F., Wang N., Wen F. and Zhu C. (2016). GhWRKY25, a group I WRKY

gene from cotton, confers differential tolerance to abiotic and biotic stresses in transgenic Nicotiana benthamiana. Protoplasma, 253: 1265-1281.

Makandar R., Essig J. S., Schapaugh M. A., Trick H. N. and Shah J. (2006). Genetically engineered resistance to Fusarium head blight in wheat by expression of *Arabidopsis* NPR1. Mol. Plant-Microbe Interact., 19: 123-129.

Mangano S., Gonzalez C. D. and Petruccelli S. (2014). *Agrobacterium* tumefaciens-Mediated Transient Transformation of Arabidopsis thaliana Leaves. Jose J. Sanchez-Serrano and Julio Salinas (eds.), Arabidopsis Protocols, Methods in Molecular Biology, vol. 1062.

Marchive C., Léon C., Kappel C., Coutos-Thévenot P., Corio-Costet M. F., Delrot S. and Lauvergeat V. (2013). Over-Expression of VvWRKY1 in grapevines induces expression of jasmonic acid pathway-related genes and confers higher tolerance to the downy mildew. PLoS One 8:e54185.

Millner P. D., (2006). Control of strawberry black root rot with compost socks. Online Plant Health Progr. doi: 10.1094/PHP-1016-02-RS.

Odell J. T., Nagy F. and Chua N. H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature, 313: 810-812.

Palm C. J., Costa M. A., An G. and Ryan C. A. (1990). Wound-inducible nuclear protein binds DNA fragments that regulate a proteinase inhibitor II gene from potato. Proc. Natl. Acad. Sci. USA., 87:603-607.

Pandey S. P., and Somssich I. E. (2009). The Role of WRKY transcription factors in plant immunity. Plant Physiol., 150:1648-1655.

Parikka P., (2004). Disease resistance in strawberry breeding programs: major pathogens in European strawberry production. Acta Hortic., 649:49-54

Phukan U. J., Mishra S. and Shukla R. K. (2015). Waterlogging and submergence stress: affects and acclimation. Crit. Rev. Biotechnol., 16:1-11.

Robatzek S. and Somssich I. E. (2002). Targets of AtWRKY6 regulation during plant senescence and pathogen defense. Genes and Development, 16:1139-1149.

Robert-Seilaniantz A., Grant M. and Jones J. D. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. Annu. Rev. Phytopathol., 49:317-343.

Sedgwick S. G. and Smerdon S. J. (1999). The ankyrin repeat: A diversity of interactions on a common structural framework. Trends Biochem. Sci., 24: 311-316.

Seo, E., Choi D. and Choi. (2015). Functional studies of transcription factors involved in plant defenses in the genomics era. Brief. Funct. Genomics 14: 260-267.

Sharma A. and Bhardwaj L. N. (2001). Influence of temperature, relative humidity and nutrient solution on sporangial germination and zoospore liberation in *Phytophthora cactorum* causing leather rot of strawberry. Plant Dis. Res., 16:243-246.

Spoel S. H., and Dong X. (2008). Making sense of hormone crosstalk during plant immune responses. Cell Host Microbe., 3:348-351.

Sukharev S. L., Klenchin V. A., Serov S. M., Chernomordik L. V. and Chizmadzhev Yu. A. (1992). Electroporation and electrophoretic DNA transfer into cells. The effect of DNA interaction with electropores, Biophys. J. © Biophysica Society, 63: 1320-1327.

Timudo-Torrevilla O. E., Everett K. R., Waipara N. W., Boyd-Wilson K. S. H., Weeds P., Langford G. I. and Walter M. (2005). Present status of strawberry fruit rot diseases in New Zealand. New Zealand Plant Prot., 58:74-79.

U¨ lker B. and Somssich I. E. (2004). WRKY transcription factors: from DNA binding towards biological function. Curr Opin Plant Biol., 7: 491-498.

Viejobueno J., Albornoz P. L., Camacho M., de los Santos B., Martínez-Zamora M. G., Salazar S. M. (2021). Protection of Strawberry Plants against Charcoal Rot Disease (*Macrophomina phaseolina*) Induced by *Azospirillum brasilense*. *Agronomy*, 11:195.

Yan, S. and Dong X. (2014). Perception of the plant immune signal salicylic acid. Plant Biol., 0: 64-68.

Yang, Y., Li R. and Qi M. (2000). *In vivo* analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. Plant J., 22:543-551.

Yu D., Chen C. and Chen Z. (2001). Evidence for an Important Role of WRKY DNA Binding Proteins in the Regulation of NPR1 gene expression. Plant Cell, 13:1527-40.

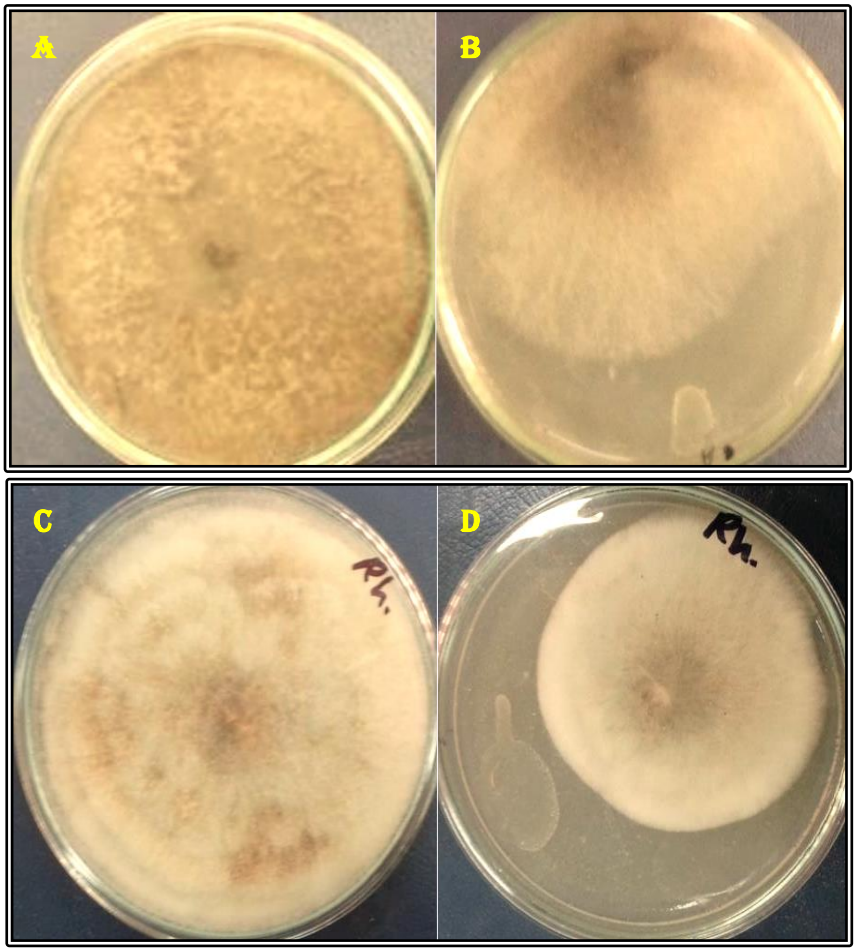

Fig. (1): Potato dextrose agar (PDA) plates inoculated with parasitic fungi; *Macrophomina phasiolena* grown on PDA as positive control (A), an inhibition zone was clearly observed on *Macrophomina phasiolena* PDA plates when inoculated with p35S::FaWRKY1 (B), *Rhizoctonia solani* grown on PDA as positive control (C), and an inhibition zone was clearly observed on *Rhizoctonia solani* PDA plates when inoculated with p35S::FaWRKY1 (D).

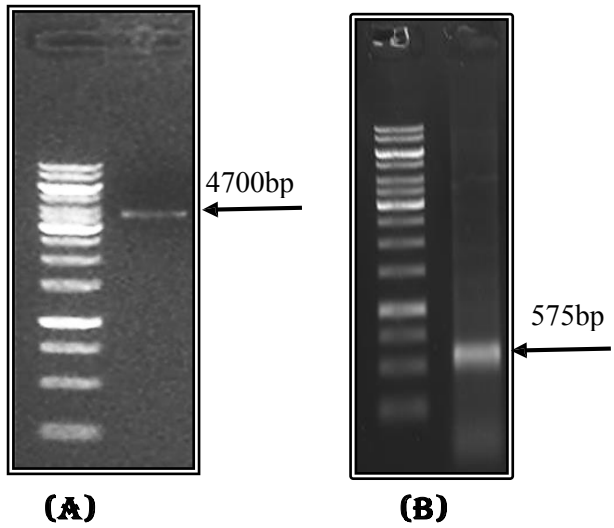

Fig. (2): Amplified fragment of 4700 bp including; 2811 bp of *MtNOOT* gene plus 1889 bp of its promoter region using Pnoot & nootR oligonucleotides primer (A), Amplified fragment of *FaWRKY1* giving a size of 575 bp using specific oligonucleotides primers*;* WR-F & WR-R (B).The two PCR were carried out to confirm the transformation step of the two genes into *Agrobacterium tumefaciens.* GeneRuler 1kb DNA ladder was used as DNA marker.

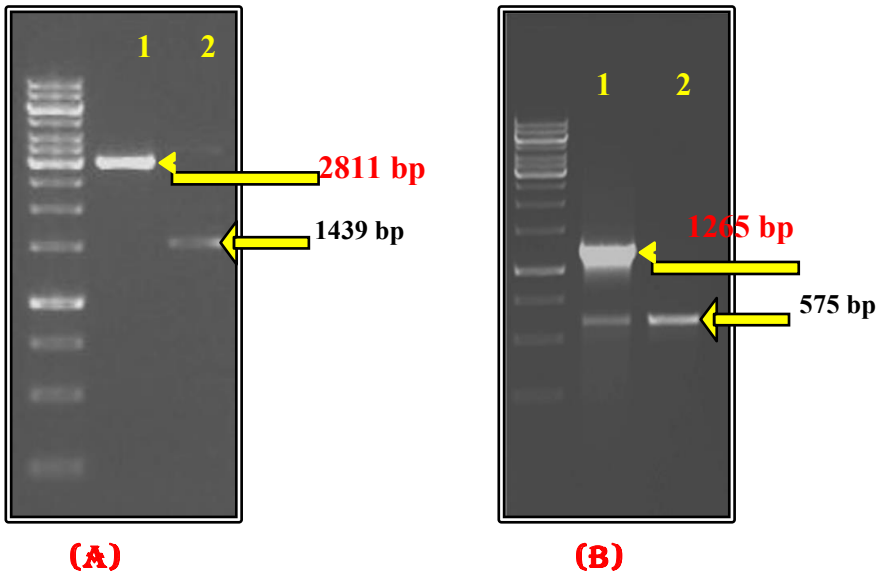

Fig. (3): PCR amplified fragment of 2811 bp for *MtNOOT*-ORF, RT- PCR amplified fragment of 1439 bp for *MtNOOT*, nootF&nootR oligonucleotides primer pair was used for PCR and RT-PCR (A1&2), Amplified two sizes fragments of *FaWRKY1*; 1265 bp fragment for the genomic *FaWRKY1* gene that is natively existing in strawberry plants, and 575 bp fragment for the transiently transformed *FaWRKY1* gene, RT-PCR amplified fragment of 575 bp for *FaWRKY1*, oligonucleotides primer pair WR-F&WR-R was used for PCR and RT- PCR (B1&2). The PCR and RT-PCR were carried out 3, 5, 7 days post mechanical infection with *Macrophomina phasiolena* to confirm the transient transformation and transcripts viability of the two genes in strawberry plants. GeneRuler 1kb DNA ladder was used as DNA marker.

Fig. (4): Transient overexpression of *MtNOOT* & *FaWRKY1* genes in strawberry was substantially altered susceptibility to *Macrophomina phasiolena* infection. Control healthy plant injected with empty agrobacterium (A). Disease symptoms appears strongly on the mechanically infected strawberry leaves three days post *Macrophomina phasiolena* infection (b). Strawberry infected plants were eventually died after five days post *Macrophomina phasiolena* infection compared with uninfected control healthy plants(C). Strawberry plants agro-infiltrated with (pCambia1390::FaWRKY1) displayed moderate infection three, five, and seven days post *Macrophomina phasiolena* inoculation (D, E, and F). Plants agro-infiltrated with (pCP42::MtNOOT) exhibited low to moderate symptoms three, five, and seven days post parasitic infection (G, H, and I). Plants agro-infiltrated with (pCambia1390::FaWRKY1& pCP42::MtNOOT) showed very mild disease symptoms three, five, and seven days post *Macrophomina phasiolena* inoculation compared to pCambia1390::FaWRKY1 and pCP42::MtNOOT separately-injected plants(J, K, and L).

MOLECULAR INSIGHT INTO WADI HAGUL RARE DIVERSITY:*Echinops spinosus* AND *fagonia molis*, PLANT SPECIES

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Keywords: DNA barcoding, Wadi Hagul, *Echinops. Spinosus*, *Fagonia. Mollis* and Karyotype.

Genus Echinops belongs to the family Asteraceae (*Compositae*), comprises about 120 species distributed through the Mediterranean region to central Asia and Tropical Africa (Kadereit and Jeffrey, 2007). In Egypt, *E. spinosus* L. is among five species representing this genus. It is common throughout the Sahara of Sinai and the Red Sea coast (Boulos, 2009).

In the Mediterranean and Saharo-Sindian regions, subtropical regions of North and South America and South Africa, the genus Fagonia comprises 40 species. Early evidence of (El Hadidy, 1966) described 18 species of Fagonia and constructed an artificial key for their identification (El Hadidy, 1966; Täckholm, 1974). Recent biosystematics research mostly depends on molecular methods, but karyology and morphology still necessary information of any organism (Peruzzi and Altınordu, 2014). The karyotype approach involved the size, number, and appearance of chromosomes of eukaryotic cell. Also, includes chromosomal measurements such characteristics, along with morphological and molecular data, which can be exploited to understand patterns and mechanisms of evolution and speciation in plants (Roma-Marzio *et al*., 2015). Karyotype evolution is a notable feature in the diversification of many plant species; until now there is a strong argument about the role of chromosomal alternations in the diversification process (Roalson *et al.*, 2007). Over the last decades, many karyological investigations had been performed and provided essential information for evolutionary rate and plant systematic (Stace, 2000). Variation in total and relative chromosomal size, morphology and stainable properties can be obtained from karyological analysis (Sharma and Sen, 2002). Indeed, the chromosome morphology characters have been proved to be a useful method to differentiate genomes at the generic and sub generic levels in

plants, animals and humans (Albers *et al.*, 2007).

DNA barcode is a molecular phylogeny method that utilizes short and standardized DNA sequences in a familiar gene to identify plants and other organism species (Xiwen *et al.,* 2015). Different varieties of phylogenetic markers have been applied, but the widely used is DNA barcoding markers due to their facility identifying problematic taxa (El-Sakaty *et al.*, 2014; El-Atroush *et al*., 2015). Recently, any credible plant barcode should be multi-locus with a coding region of *rbc*L because of its responsibility toward producing enzyme RuBisCo and evolving noncoding region (Kress and Erickson, 2007). The plastid gene *rbc*L gene consists of a 599 bp region at the 5′ end of the gene. It' is straightforward recovery and supplies a good backbone to the barcode dataset (Chase*et et al.,* 2005; Newmaster *et al.*, 2006).

The current investigation was aimed to identify and conserve *Echinops spinosus* and *Fagonia molli* and also to determine the chromosome numbers karyotype and ideograms for species.

DNA barcoding, as a tool primarily for species identification, can be used in two specific ways to address biodiversity conservation: 1) as a means of more accurate and eventually more rapid biodiversity monitoring both before and after conservation actions, and 2) by providing data that will assist in estimations of phylogenetic diversity for setting conservation priorities

MATERIAL AND METHODS

Plant materials

The plants were collected from the upstream site of Wadi Hagul within the two different seasons April (spring) at daily temperature 20ºC ± 2 and night temperature 10ºC ± 2 and October (autumn) at daily temperature 30ºC ± 2 and night temperature 19ºC ± 2.

Wadi Hagul is located in the northern portion of the Eastern Desert of Egypt within the Cairo-Suez district; it occupies approximately 350 km^2 representing about 0.16% of the Egyptian Eastern Desert (Abd El Aal, 2017). It is bounded by latitudes 29°48'28" - 29°57'43" N. and longitudes 32°09'32" - 32°17'27" E. Its main channel extends for about 40 km with a width of 6-10 km and it runs to debouch into the Suez Gulf.

Wadi Hagul lies within an arid desert climate with deficient rainfall, high temperature, and high evaporation rate. The geologic and vegetation structures of the Wadi Hagul led to the recognition of three main sections: upstream, middle stream and downstream sections (Zahran and Willis, 2009). These sections represent the natural xeric habitat that xerophytic plants mainly inhabit. The dominant species are *Fagonia mollis, Echinops spinosus* and other xerophytic plants.

Karyotype analysis

Seeds of the two studied plants (*Echinops spinosus* and *Fagonia mollis*) were collected from the upstream site in Wadi Hagul. The lateral roots, 1.5 - 2.0 cm length, were excised into glass vials containing 2 ml of 0.05% colchicine for three hours at room temperature. Fixation was done using ethanol-acetic acid (3: 1) fixative. Then, root samples were washed thoroughly with water and macerated with the enzymatic mixture (4% cellulase, 1 % pectenase, 75 mM KCl and 7.5 mM EDTA) on the glass slides in the moisture chamber at 37^{o}C for 40 min. The enzymatic mixture was then washed with distilled water and each root tip was chopped into fine pieces, flamed by forceps and squashed and stained by the acetoorcein solution. These stained samples were used to automatically scan the chromosome image of the examined material (Fukui and Kakeda, 1994). The samples were examined using the Image Processing Analysis System (KS - Chromo). Karyotype analysis was carried out using karyostar demo. & micro measure computer program in the c-metaphase stage and analyzed using the video test karyotype software (Reeves, 2001).

DNA barcode analysis

Fresh plant leaf samples from *F. mollis* and *E. spinosus* were used. DNA extraction was carried out using SIGMA® Plant High Molecular DNA extraction KIT®. DNA quality was tested using agarose gel electrophoresis, visualized by pre-added Red Safe® (5ul /100 ml) under UV light. The primer pairs for gene rbcLaF (5` -ATG TCA CCA CAA ACA GAG ACT AAA GC-3`) and rbcLaR (5`- GTA AAA TCA AGT CCA CCR CG- 3`) were used to amplify *rbc*L region (White *et al*., 1990). PCRs of 50 ul reaction mixture (1x Flexi buffer, 50ng DNA template, 2.5 mM $MgCl_2$, 10uM dNTPs, 0.4 uM of each primer, and1U Promega© Green Go Taq™ enzyme) were performed, standard PCR profile with 50ºC annealing temperature was used to amplify *rbc*L gene.

RESULTS AND DISCUSSIONS

The mitotic chromosome numbers of the studied plants, *Echinops spinosus* and *Fagonia mollis* are shown in Table (1), while the metaphase plates for these taxa are presented in Fig. (1). The karyotype analyses are represented in certain chromosomal features of both studied plants: chromosomal number, area of chromosomes, chromosomal length, arm ratio, the position of centromeres, and centromeric index.

The diploid chromosome number of *E. spinosus* is 2n=18, and the area of the chromosomes ranged between 4.66 µ to 0.61 µ, while the chromosomal length ranged between 5.63 µ to 1.53 µ while its long and short arm is varied between 3.04µ – 0.83µ and 2.60 µ - 0.71 µ, respectively. Consequently, the arm ratio of *E. spinosus* ranged between 1.17 - 1.11. Additionally, the centromeric index of *E. spinosus* is observed between ‘47.49 to 46.03’ and the position of centromeres is metacentric for all chromosomes (Fig. 1).

The diploid chromosome number of *F.mollis* is 2n=18 and its chromosomal area are fallen between 4.30 μ to 2.0 μ, while the chromosomal length ranged between 4.56μ to 2.07μ , while its long and short arm were ranged between 2.47 μ - 1.11 μ and 2.09 μ - 0.96 μ, respectively. Therefore, the arm ratio of *F. mollis* ranged between 1.41 - 1.10. Moreover, the centromeric index of *F. mollis* is observed between 47.63 - 45.52 and the position of centromeres is metacentric for all chromosomes (Fig.1).

The karyotype is the phenotypic feature of the chromosome complement seen at mitotic metaphase to give chromosomal information in evolution, plant systematic and ranges of possibilities to understand taxa affinities (Levin, 2002). In the present study of *E. spinosus* verified its chromosomal number as 2n=18 (Table 1), while Ismael *et al.*, (2009) have been analyzed the chromosomal number of different *Echinops* species and record *E. gmelini* chromosomal number being 2n=26 and its related *E. acantholepis* (2n=14) showed strongly divergent karyological patterns of difficult interpretation, along with *E. przewalskyi* counts its chromosomal number being 2n=32. Our results indicated that the *F. mollis* karyotype analysis was 2n=18 (Table 1), however, evidence of Kučera *et al.*, (2016) reported that the chromosomal number of India *F. cretica* was 2n=22, while Baquar (1970) described that the chromosomal number of this species outside of India are different being 2n = 18, 20 and 22. Other report recorded by Zaidi (2003), showed that the 2n of *F. schweinfurthii* being 20 and 22.

DNA barcode

In recent years, attention has been paid to the benefits of DNA barcoding for deciphering the phylogenetic relationships between closely related taxa. However, at lower taxonomic levels of medicinal plants, the problem is that no specific DNA barcoding is sufficient to resolve inter-and intraspecific relationships. We, therefore, turned our attention to the universal *rbc*L coding plastid genes. Therefore, the universal *rbc*L DNA barcode approach has been utilized to identify the plant species by using a short sequence of a universal standard part of a genome instead of using the whole genome. (Ali *et al.*, 2015). In the present study, both plants′ DNA barcode analysis revealed that *E. spinosus* tree (Fig.2) was divided into two main clads. The *rbc*L phylogenetic analysis showed that *E. spinosus* belonged to the family Asteraceae and supported *Echinops species*. On the other hand, *F. mollis* tree (Fig. 3) was divided into two clads, belonging to the family Zygophyllaceae and supported to *F. latistipulata* (bootstrap support of 93%). These results approved that *rbc*L region was effective in the identification of both studied plants. Recent evidence of Elsherbeny (2016) provided preliminary as-

sessment data to assist for more comprehensive DNA barcoding applications in wild medicinal plants. It was found that *rbc*L was helpful for barcoding some medicinal plant species in the family Labiatae. *rbc*L region should be involved as a standard for comparison to other barcoding due to the advantage that this gene is easily amplified and sequenced in most plants and it is regarded as a benchmark locus in phylogenetic investigations by providing a reliable placement of a species into plant family and/or genus (Hassel *et. al.*, 2013).

Karyotype analysis and DNA barcode of Egyptian flora have been utilized to study the plant evolution and systematic of some plant groups as well to form a genetic database of natural plants which is endangered and susceptible to become rare (Badr and Sharawy, 2007). In this consideration, karyotype analysis and DNA barcoding have been performed for *Echinops spinosus* and *Fagonia mollis*. To the best of our knowledge, there is no literature have been reported for the chromosomal karyotype and DNA barcode of the two studied plants in Egypt, hence it will be essential to discuss and report this work.

Chromosomes in the Egyptian flora karyotype analysis of plant have been applied to address some plant group′s systematic, so it was essential to study some plants′ karyotype in Wadi Hagul as participation in documenting the chromosomal number and shape of these plants. Karyotyping was performed for *Echinops spinosus* and *Fagonia mollis* plants growing in Wadi Hagul. To our knowledge, no literature was available, dealing with a chromosomal karyotype of the two studied plants, so it was necessary to do; however, no polyploid chromosome are recorded in these plants. So, this work aims to provide a base for nature conservation and other applied programs for a genetic study.

On the other hand, a few molecular phylogenies at taxonomic levels have been assessed through RAPD, SSR, ISSR, AFLP and IRAP markers applied for germplasm identification and genetic diversity. The increasing demands for direct genome analysis based on DNA barcodes are currently one of the powerful techniques existing to employ in specific fields particularly in systematic and taxonomy. In the current study, the studied plants have been used to determine rbcL sequence divergence can reasonably guide gene choice in phylogenetic across abroad scale in Egyptian plants. DNA barcoding not only helps in the identification of species but can also define species boundaries, flagging of new species and species delimitation.

SUMMARY

Genetic studies have been performed to distinguish two plants inhabiting a natural valley renowned as Wadi Hagul of Egypt throwing karyotype and DNA barcode analysis. The two plants were morphologically identified as *Echinops spinosus* and *Fagonia mollis*. As a result, the karyotype approach exhibited a significant variant among the certain

chromosomal features involving chromosomal number, area of chromosomes, chromosomal length, arm ratio, the position of centromeres, and centromeric index. The chromosomal number of both plants was 2n=18, while the DNA barcode identified their species as *E. spinosus* of the family *Asteraceae* and *F. mollis* of the family *Zygophyllaceae* based on *rbc*L phylogenetic analysis.

Results indicated that, *F. mollis* evolutionary tree was rooted into two clads, belonging to family *Zygophyllaceae* and supported to *Fagonia latistipulata* (bootstrap support of 93%). Indeed, analysis of *rbc*L sequence will *permit* to *evaluate* the taxonomy and systematic of different plants recovered with a good performance in *clarifying* genetic diversity within and between populations in the studied plants. In particular, *Echinops spinosus* and *Fagonia mollis* showed a minor divergent from the common species, therefore an urgent survey for these genotypes and their conservation is required.

REFERENCES

Abdelaal M. (2017). Current statue of the floristic composition in Wadi Hagul, Northwest Suez Gulf, Egypt. Rend. Fis. Acc. Lincei , 28: 81-92.

Albers F., J. Van der Walt J. and Geraniace (2007). The families and genera of vascular plants. Springer Berlin Heid.157-167.

Ali Z., Abulfaraj A., Idris A., Ali S., Tashkandi M. and Mahfouz M. (2015). CRISPR/Cas9-mediated viral interference in plants. Genome Biol., 16:238.

Badr A. and Sharawy S. (2007). Karyotype analysis and systematic relationships in the *Egyptian Astragalus* L. (Fabaceae). Int. J. Bot.,, 3: 147-159.

Baquar S. (1970). Natural tetraploidy in the genus *Fagonia* L. Curr. Sci., 36:172.

Boulos L. (2009). Flora of Egypt ChecklistRevised Annotated Edition, Al Hadara Publishing, Cairo, Egypt, pp. 221.

Chase M. W., Salamin N., Wilkinson M., and Dunwell J. M. (2005). Land plants and DNA barcodes: short-term and long-term goals. Philos. Trans. R. Soc. Lond. B Biol. Sci., 360: 1889-1895.

El Atroush H., Magdi M.. and Werner O. (2015). DNA Barcoding of two endangered medicinal plants from Abou Galoom protectorate. Life Sci. J., 12: 101-109.

EL Hadidy M. N. (1966). The genus *Fagonia* L. in Egypt. Candollea, 21: 13 - 54.

El-Sakaty S. I. A., Magdi M., El-Atroush H., Mohamed M. M., Abou-Salama

U. (2014). DNA barcoding of three *Bryaceae –Musci*. Egypt. J. Bot., special issue for the fourth international conference of Botany and Microbiology Sciences, 53-63

Elsherbeny Esraa A. (2016). DNA barcoding of some medicinal plants, family Labiatae. Egypt. J. Exp. Biol. (Bot.)., 12: 175-180.

Fukui K. and Kakeda K. (1994). Dynamic changes in the morphology of barley chromosomes during the mitotic metaphase stage. Jap. J. Gen., 69:545-554.

Hassel K., Segreto R., and Ekrem T. (2013). Restricted variation in plant barcoding markers limits identification in closely related bryophyte species. Mol. Ecol. Res., 13: 1047-1057.

Ismael S., Georgy A., Oriane H. and Teresa G. (2009). Molecular systematics of *Echinops* L. (*Asteraceae*, *Cynareae*): A phylogeny based on ITS and trnL-trnF sequences with emphasis on sectional delimitation. Taxon., 59:698-708.

Kadereit J. W. and Jeffrey C. (2007). Flowering plants. Eudicots. In: "The Families and Genera of Vascular Plants. (Kubitzki K. Ed.)". Springer - Verlag, Berlin, Heidelberg, pp. 61-128.

Kress W. and Erickson D.. (2007). A two-locus global DNA barcode for land plants: the coding *rbcL* gene complements the non-coding trnHpsbA spacer region. PLoS One., 2: e508.

Kucera J., Martonfiova L., Stubnova E. and Slovak M. (2016). Picris. IAPT/IOPB chromosome data 21. Taxon., 65 : 675.

Levin D. (2002). The role of chromosomal changes in plant evolution. Oxford Univ. Press.

Newmaster S., Fazekas A. and Ragupathy S. (2006). DNA barcoding in land plants: evaluation of *rbc*L in a multigene tiered approach. Canad. J. of Bot., 84: 335-441.

Peruzzi L. and Altınordu F. (2014). A proposal for a multivariate quantitative approach to infer karyological relationships among taxa. Comp. Cytogen., 8: 337-349.

Reeves A. (2001). Micro Measure: a new computer program for the collection and analysis of cytogenetic data. Genome., 44:439-443.

Roalson E., Mccubbin A. and Whitkus G. (2007). Chromosome evolution in *Cyperales*. Ran. San. Ana. Bot. Gard., 23: 62-71.

Roma-Marzio F., Astuti G. and Peruzzi L. (2015). Taxonomy, typification and

karyology of *Crepislacera* (Asteraceae). Phyto. 208:45-54.

Sharma A., and Sen S. (2002). Chromosome botany. Enfield (NH): Science Publishers.

Stace C. (2000). Cytology and cytogenetics as a fundamental resource for the 20th and 21st centuries. Taxon., 49: 451-477.

Täckholm V. (1974). Student Flora of Egypt, 2nd edition. University Publishing, pp. 888.

White T. J., Bruns T., Lee S., Taylor J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetic. In: "PCR Protocols: a guide to methods and applications. (Innis M.A., Gelfand D.H., Sninsky J.J., White T.J., eds)". Academic Press, New York, USA, pp. 315-322.

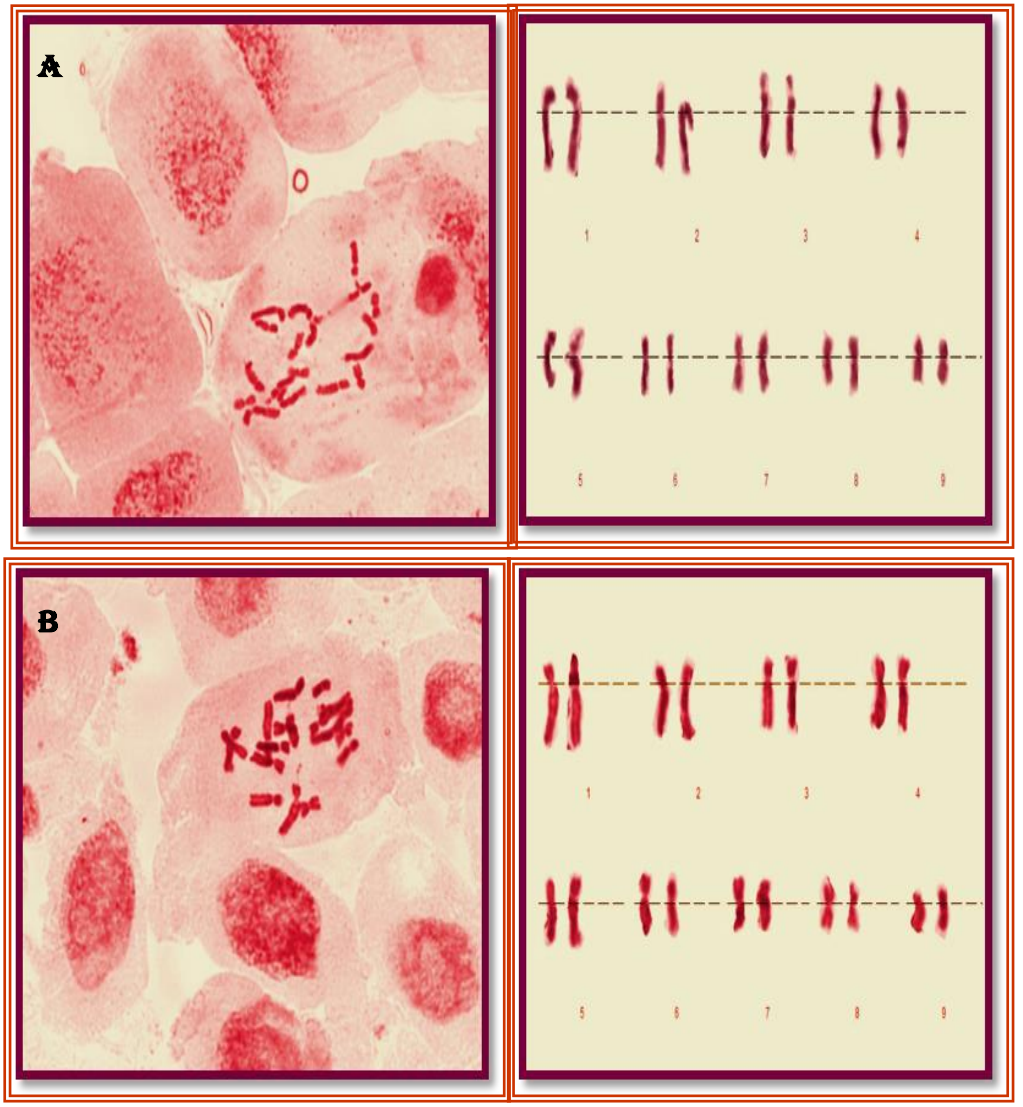
Xiwen L., Yang Y., Robert J., Maurizio R., Yitao W and Shilin C. (2015). Plant DNA barcoding: from gene to genome. Biol. Rev., 90: 157-166.

Zahran M. A. and Willis A. J. (2009). The Vegetation of Egypt. 2nd ed. Springer, Netherland, pp. 437.

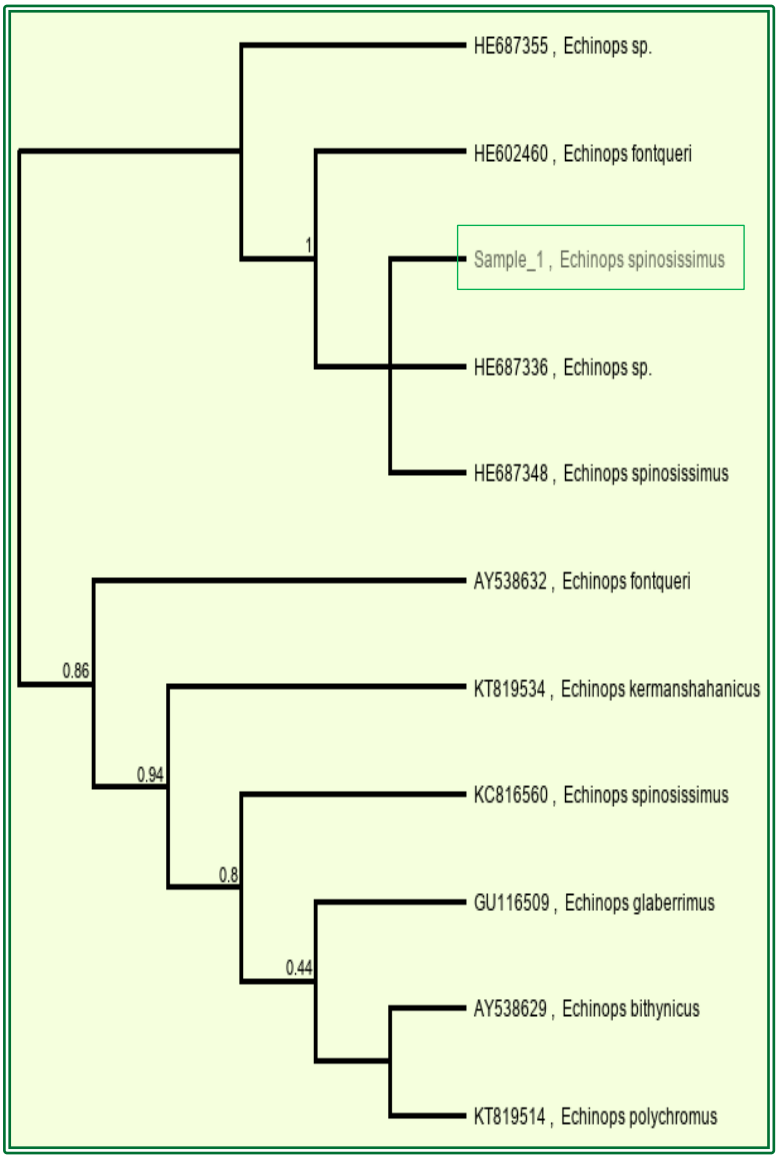
Zaidi S. (2003). Effects of electromagnetic fields (created by high tension lines) on the indigenous floral biodiversity in the vicinity of Karachi - I: Studies on PMC meiosis, meiotic products and pollen fertility. Pakistan J. Bot., 35: 743-755.

Table (1): Chromosome parameters of *Echinops spinosus* and *Fagonia mollis* are Chr. No.: chromosome pair number, CA: chromosome area, CL: chromosome length (µm), LA: long arm, SA: short arm, AR: arm ratio, CI: Centromeric index, PC: position of centromere and M: Metacentric chromosome.

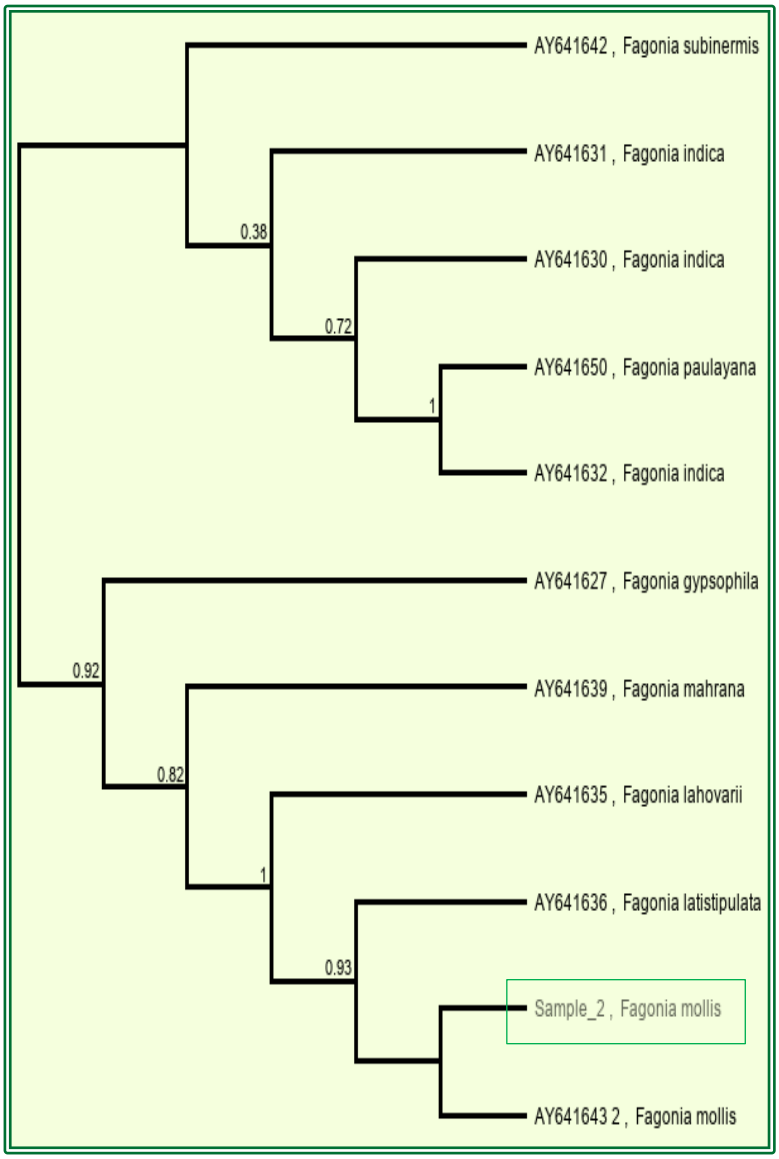
Species	***Echinops spinosus***						
Chr. No.	CA	CL	LA	SA	AR	CI	PC
1	4.66	5.63	3.04	2.60	1.17	46.10	M
2	4.53	5.43	2.89	2.55	1.13	46.85	M
3	4.46	5.36	2.89	2.47	1.17	46.03	M
4	4.39	5.14	2.71	2.42	1.12	47.17	M
5	2.88	3.57	1.89	1.68	1.12	47.07	M
6	2.78	3.49	1.86	1.63	1.14	46.70	M
7	3.19	3.53	1.89	1.65	1.15	46.60	M
8	2.62	3.30	1.73	1.57	1.11	47.49	M
9	0.61	1.53	0.83	0.71	1.17	46.11	M
species	***Fagonia mollis***						
Chr. No.	CA	CL	LA	SA	AR	CI	PC
1	4.30	4.56	2.47	2.09	1.18	45.83	M
2	4.11	4.31	2.26	2.05	1.10	47.63	M
3	3.83	4.11	2.41	1.70	1.41	41.44	M
4	3.51	3.67	1.98	1.69	1.18	45.95	M
5	3.20	3.47	1.89	1.58	1.20	45.52	M
6	3.00	3.18	1.68	1.51	1.11	47.35	M
7	2.49	3.05	1.62	1.44	1.12	47.10	M
8	2.19	2.31	1.23	1.08	1.14	46.75	M
9	2.00	2.07	1.11	0.96	1.16	46.22	M



(Fig. 1): Karyotype characteristics and metaphase chromosome of *E. spinosus* (A) and *F. mollis* (B). (9 pairs each).



(Fig. 2): Dendrogram of *E. spinosus* based on *rbc*L gene.



(Fig. 3): Dendrogram of *F. mollis* based on *rbc*L gene.

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