Studying the Possibility of Indirect Metabolite Microorganisms' Classification Using Electronic Tongue and Multivariate Data Analysis

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Dedication

This study is wholeheartedly dedicated to my beloved parents Ali and Najah Abu Rumaila, who have been my source of inspiration and taught me the value of hard work, who continually provide their moral, spiritual, emotional and financial support.

It is as well dedicated to my beloved seven brothers and ten sisters all by their names and value in my heart, to my beloved finance Khaled manasra and my best friends who meant and continue to mean so much to me, who shared words, advice, encouragement and energy to stand all my good and bad emotions to finish this study.

Lastly, this work is dedicated to everyone who had good influence or gave me some of their time to teach, motivate and solve a problem during the work. Also, to every challenge, obstacle and hard work I had to face through the past two and half years.

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Loving thanks to my dear brothers and sisters, my beloved finance, my best friends, my learning partners and colleges, who played such important roles along the journey, as we mutually engaged in making sense of the various challenges we faced and in providing encouragement to each other at those times when it seemed impossible to continue.

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List of Abbreviations or Symbols

The abbreviation	Abbreviation description
˚C	Celsius
µg/mL	Microgram per milliliter
µL	Microliter
Ag/AgCl	Silver-silver chloride reference electrode
ANN	Artificial neural network
bp	Base pairs
ca.	Circa
Cat #	Catalog number
$CE\text{-}MS^n$	Capillary electrophoresis coupled with multistage accurate mass spectrometry
ChemFETs	Chemical sensitive field effect transistor
cm	Centimetre
CTAB	Cetyltrimethyl ammonium bromide
D.W.	Distilled water
DAMD	Directed amplification of minisatellite-region DNA
DFA	Discrimination function analysis
Di	Discrimination index
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribonucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
ET	Electronic tongue
FCM	Fuzzyc means
g	Gram
$GC\text{-}MS^n$	Gas chromatography coupled with multistage accurate mass spectrometry
h	Hour/ hours
HCA	Hierarchical cluster analysis
ISFET	Ion-sensitive field-effect transistor
ISSR	Inter simple sequence repeats
ISSR-PCR	Inter-simple sequence repeats related polymerase chain reaction
KARC	Kadoorie Agricultural Research Center
kbp	Kilo-base pair
KCl	Potassium chloride
L	liter
lag phase	Lagging phase
$LC\text{-}MS^n$	Liquid chromatography coupled with multistage accurate mass spectrometry
LDA	Linear discriminate analysis
log phase	Logarithmic phase

LVQ	Learning vector quantization
M	Molar
$MgCl_2$	Magnesium chloride
min	Minute/ minutes
mL	Millitre
mm	Millimetre
mM	Millimolar
MVDA	Multivariate data analysis
NA	Nutrient agar
NaCl	Sodium chloride
NaOAc	Sodium acetate
NB	Nutrient broth
NH_4OAc	Ammonium acetate
NMR	Nuclear magnetic resonance spectroscopy
PCA	Principle component analysis
PCR	Polymerase chain reaction
PCs	Principal components
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PLS	Partial least square
PNN	Probabilistic neural net-work
psi	Pounds per square inch absolute
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Round per minute
rRNA	Ribosomal RNA
RTU	Ready to use
SDS	Sodium dodecyl sulfate
SIMCA	Soft independent model class analogy
SPAR	Single primer amplification reaction
TBE	Tris borate EDTA
TE	Tris EDTA
TRI reagent	TRIzol reagent
Tris	Tris(hydroxymethyl)aminomethane
Tris-HCl	Tris- hydrochloric acid
UBC-ISSR	University of British Columbia- inter simple sequence repeats primer
unit/µL	Unit per microliter
UPGMA	Unweighted pair groups method using average
UV	Ultra violet
Ver	Version
xg	Relative centrifugal force (RCF) or G-Force

Studying the Possibility of Indirect Metabolite Microorganisms' Classification Using Electronic Tongue and Multivariate Data Analysis

Researched by: Basima Abu Rumaila

Supervised by: Dr. Nawaf Abu-Khalaf

Abstract

Chemical sensor systems become increasingly popular and promising analytical tools for various liquid analyses. One of these systems is the electronic tongue (ET), which is based on a multi-sensor array set with high cross-sensitivity and low selectivity characteristics. This research is a trial to investigate the possibility of using a potentiometric ET as a fast and alternative assessment tool for (complex and native state) bimolecular microorganism's (bacterial and fungal species) foot-printing in a liquid media.

The study was carried out by collecting 44 different fungal and bacterial isolates. These microorganisms were cultivated on suitable liquid media, where the filtrated media were then analyzed using Astree II Alpha MOS ET during their growth cycle. After that, the collected data were analyzed using multivariate data analysis MVDA methods (basically using principal component analysis PCA) for microbial clustering according to their similarities and/ or differences among each other and to follow their growth

rate. In the meantime, the clustering patterns of these microorganisms were validated using molecular phylogenetic tree.

The results of this study were promising, since ET's used sensors array showed high discrimination power between samples ranged from 0.927 to 1 for fungi and from 0.960 to 0.999 for bacteria at the end of testing period. In which, a PCA scores plot with 98 and 96 discrimination index (Di) for fungi and 95 Di for bacterial clustering patterns were indicated. Moreover, the similarity test revealed a high similarity of 27.18% Di among F5 and F6 fungal isolates in group 1, 70.52% Di among F14 and F21 in group 2 fungal isolates, 3.18% Di among B3 and B11 in group 1 bacterial isolates and 12.91% Di among B13 and B22 in group 2 bacterial isolates.

Also, the PCA clustering patterns were very similar to the validated molecular phylogenetic tree showing the relationship between isolates. Furthermore, ET could follow microbial growth and overlapping (stop of chemical change) in the liquid media.

1 Introduction

1.1 General introduction

Microscopic organisms have thoroughly been focused for their interactions and influence on the surrounding environments, where plenty of studies are still being established to illustrate their functions and varieties (Borkowski *et al.*, 2018; Deveau *et al.*, 2018; Schwab, Terra, & Baldani, 2018; Zhang, Hu, Ren, & Zhang, 2018). In order to diagnose microorganisms (*i.e.* bacteria and fungi) both genetic interaction among these microorganisms and their interactions with the surrounding atmospheres are taken into account. Although, molecular identification using genetic data bases (*i.e.* DNA, RNA and protein sequences) and their different related techniques (*i.e.* polymerase chain reaction (PCR) dependent or non-PCR dependent techniques) are essential, these techniques need pre-sequence knowledge, sample preparation, different molecular instruments and numerous hazardous chemicals during the process (Gan *et al.*, 2013; Rai, Phulwaria, & Shekhawat, 2013; Somervuo, Koskela, Pennanen, Nilsson, & Ovaskainen, 2016; Thangavelu, Kumar, Devi, & Mustaffa, 2012; Zhu, Qu, & Zhu, 1993). Moreover, for industrial bio-production of amino acids, antibiotics, enzymes, vitamins, bulk chemicals, bio-pharmaceuticals and projects that need characterizing complex distributed metabolic interactions, molecular techniques are costly and time consuming (Cai *et al.*, 2018; Mashego *et al.*, 2007; Mosier *et al.*, 2013; Purves *et al.*, 2016).

According to Roessner & Bowne (2009) metabolomics science is considered as the fourth level of molecular illustration (*i.e.* Genomic, Transcriptomics and Proteomics studies). This science refers to the comprehensive (qualitative and quantitative) information extraction and data interpretation of the complete range of the exometabolome and endometabolome presented from growing cells (under defined time and conditions) using sophisticated analytical technologies, with applied statistics and multi-variant methods (Boughton *et al.*, 2011; Cai *et al.*, 2018; Kenny *et al.*, 2005; Paczia *et al.*, 2012).

Numerous analysis terminologies have been improved to differentiate metabolites qualitative analysis into both metabolite foot-printing and finger-printing that deals with extracellular and intercellular metabolite analyses, respectively. However, target analysis is referred to quantitative analysis of predefined metabolites. This approach thus complements genomics, transcriptomics, proteomics and also fluxomics data that facilitate metabolic-engineering systems. Also, it has the possibility to provide closer insights into the function and ecology of microbial communities (Brown *et al.*, 2005; Jadhav *et al.*, 2015; Kell *et al.*, 2005; Roessner & Bowne, 2009). Furthermore, since metabolites production (during different growth phases) is considered as species-specific, it can be used as genetic markers to differentiate between different micro-species and even different strains (Beck, Knoop, Axmann, & Steuer, 2012; Jensen,

Williams, Oh, Zeigler, & Fenical, 2007; Jensen, 2016; Koo *et al.*, 2014; Purves *et al.*, 2016). Yet, these complex mixtures of metabolites remain largely ambiguous, due to the difficulty of searching for each component individually.

Routine metabolites detection and quantification techniques are mainly enzyme based techniques and/or using modern high-tech hyphenated analytical protocols, mostly chromatographic techniques coupled to mass spectrometry (*i.e.* liquid chromatography coupled with multistage accurate mass spectrometry (LC-MS^n), gas chromatography–mass spectrometry (GC-MS^n), capillary electrophoresis–mass spectrometry (CE-MS^n)), and nuclear magnetic resonance spectroscopy (NMR). In which, these techniques need pre-rapid sample collection, instant quenching of microbial metabolic activity and extraction of relevant metabolites to be measured (Beck *et al.*, 2012; Bertrand *et al.*, 2014; Fauvelle, Mazzella, Morin, Delest, & Budzinski, 2015; Gros, Rodríguez-Mozaz, & Barceló, 2012; Liu, Pan, Li, Cai, & Miao, 2014; Paczia *et al.*, 2012).

Chemical sensor systems become increasingly popular analytical tools for liquid analysis (Momeni & Sedaghati, 2018; Pavase *et al.*, 2018; Terbouche *et al.*, 2018). One of these systems is the electronic tongue (ET) (and also called taste sensor) that seems to be a promising tool for analysis of various complex liquids. It is based on a multi-sensor array system with high cross-sensitivity and low selectivity characteristics. During the process signals

obtained are processed with multivariate data analysis (MVDA) techniques, such as principle component analysis (PCA), partial least square (PLS), discrimination function analysis (DFA) and soft independent model class analogy (SIMCA), which revealed qualitative and/ or quantitative information on the analyzed samples (Abu-Khalaf & Iversen, 2007a; Feng *et al.*, 2016; Kirsanov, Korepanov, Dorovenko, Legin, & Legin, 2016). The system was successfully applied for analysis of various foods, drinks and pharmaceuticals applications, environmental researches , fermentable microbial uses and microorganisms' studies (Abu-Khalaf & Iversen, 2007b; Abu-Khalaf, Khayat, & French, 2015; Ceto, Voelcker, & Prieto-Simon, 2016; Kutyla-Olesiuk, Wawrzyniak, Ciosek, & Wróblewski, 2014; Lorenz, Reo, Hendl, Worthington, & Petrossian, 2009; Soderstrom, oren, inquist, & Krantz-Rulcker, 2003).

Although, the direct ways for microorganisms' classification can't be neglected, using ET as a new technique for metabolomics qualitative analysis will offer many advantages, as it is label-free, easy to handle, relatively low cost technique compared with other sophisticated and multi-instrumental techniques, deals with a whole mixture in fast way and need little sample preparation or testing it in their native state (Kumar *et al.*, 2016; Soderstrom *et al.*, 2003; Wesoly & Ciosek, 2018; Zabadaj, Szuplewska, Kalinowska, Chudy, & Ciosek-Skibinska, 2018). To our best

knowledge this is the first study for metabolite analysis using ET in Palestine.

1.2 Aim

The aim of this research is to investigate the possibility of using potentiometric chemical sensor based system (*i.e.* ET) to help as an alternative fast tool for (complex and native state) microorganisms' (bacterial and fungal species) bimolecular foot-printing relying on their secreted metabolite in a liquid media.

1.3 Objectives

The objectives of this study are:

1- To use ET for metabolites qualitative (foot-printing) analysis in a complex liquid media,
2- To have insights on liquid media chemical changes due to microbial primary metabolite production through growth phases, and
3- To observe species specification through secondary metabolite production and differentiation during stationary phase.

2 Literature review

2.1 Microorganism classification

The microbe's category includes a massive range of microscopic organisms that are often illustrated as unicellular organisms, including bacteria, fungi, viruses, algae, archaea and protozoa. In last decades, these microorganisms have thoroughly been studied for their interaction within their niches and with surrounding environments (*i.e.* plants, animals and humans). The studies where carried out in order to fulfill their functions, life cycles, mutations, survival patterns, influence, bio-products and their varieties (Andreevskaya *et al.*, 2018; Bahram, Vanderpool, Pent, Hiltunen, & Ryberg, 2018; Gonzalez–Mula *et al.*, 2018; Rangel, Finlay, Hallsworth, Dadachova, & Gadd, 2018; Strullu-Derrien, 2018; Wang *et al.*, 2018).

2.1.1 Fungal molecular identification

There are many molecular methods for identifying the genetic diversity of microorganisms using genetic data bases. Some of these bases for fungi, plant and animal molecular classification are DNA segments called inter-simple sequence repeats (ISSR) (Abadio *et al.*, 2012). In which, these targeted bases are 100-3000 base pairs (bp) nucleotides located dissimilarly between identical, adjacent and oppositely oriented microsatellite regions (Al-Turki & Basahi, 2015; Lagkouvardos *et al.*, 2016; Soliman, Zaghloul, & Heikal, 2014).

The technique of inter-simple sequence repeats related polymerase chain

reaction (ISSR-PCR) is a simple and quick method called single primer amplification reaction (SPAR) where the amplification is occurred directly (also called directed amplification of minisatellite-region DNA (DAMD)). This technique depends on using about 16-25 bp in length single primer that is either unanchored primer (designed through the core motif microsatellite) in di-, tri-, tetra-nucleotide repeats only, or anchored primer with 1- 4 selective nucleotides at 3' or 5' end (Albayrak, Yörük, Gazdagli, & Sharifnabi, 2016; Rai *et al.*, 2013; Salahlou, Safaie, & Shams-Bakhsh, 2016; Thangavelu *et al.*, 2012).

Those designed micro-primers is considered as multi-locus markers that can create, after being separated and scored by gel electrophoresis, from 10 to 60 highly polymorphic fragment profile according to the presence or absence of a particular sized fragments. The advantages of ISSR analysis applications are reported in many studies and it includes: the stability of the designed primers, the consideration that it is fast and single PCR protocol and it is useful for studying genetic identity, genetic diversity, phylogeny, parentage, gene tagging, strain identification, genome mapping and studying evolutionary taxonomy (Nirmaladevi *et al.*, 2016; Rampersad, 2013; Shao, Xu, & Chen, 2011; Sornakili, Rathinam, Thiruvengadum, & Kuppusamy, 2017).

However, ISSR can have reproducibility problems as other genetic markers such as random amplification of polymorphic DNA (RAPD). Moreover,

ISSR fragments may have some misleading results of similar sized non-homologous bands (Abadio *et al.*, 2012; Aiyaz *et al.*, 2016; Sudmoon, Chaveerach, & Tanee, 2016; Thangavelu *et al.*, 2012).

2.1.2 Bacterial molecular identification

Over the past years, a number of molecular markers for phylogenetic classification have been evaluated. Currently, one of the most used techniques for prokaryotic molecular identification is the amplification of specific housekeeping marker region of the 16S ribosomal RNA (rRNA) genes (Takahashi, Tomita, Nishioka, Hisada, & Nishijima, 2014; Thijs *et al.*, 2017; Vetrovský & Baldrian, 2013).

The 16S rRNA gene, is approximately 1500 bp gene coding for a catalytic RNA that is part of the 30S ribosomal subunit and it is presented in all prokaryotic cells and includes differently rated highly conserved and variable sequence regions. Generally, the conserved region is used for universal specification, while sequencing of PCR amplicons of the genetic differences in the variable regions allows the assignment of close relationships at the species and even at genus level between different races of microorganisms (Lagkouvardos *et al.*, 2016; Nguyen, Warnow, Pop, & White, 2016; Yang, Wang, & Qian, 2016; Yarza *et al.*, 2014).

Universal designed PCR primers targeting the conserved regions of 16S make it possible to amplify the gene in a wide range. This technique considered as constantly valid, relatively fast, cost-efficient and used for

multiple applications (*i.e.* microbial identification, phylogeny, diversity analysis, determination of new species and metagenomics). Moreover, the availability of huge full length 16S data bases sequences for a large number of strains, make it easy to compare an unknown strain with pre-identified sequences (D'Amore *et al.*, 2016; Fadrosh *et al.*, 2014; Meola *et al.*, 2018; Tremblay *et al.*, 2015).

However, universal PCR primers selection is critical point in 16S rRNA gene amplification. In which, incorrect specification conclusions can be created due to inappropriate primers selection; related to the fact that the current designed universal 16S rRNA gene primers are based on the conserved sequences of previously identified microbes that sometimes cannot detect some mismatched species (Giusti *et al.*, 2017; Hahn, Jezberová, Koll, Saueressig-Beck, & Schmidt, 2016; Mori *et al.*, 2013).

2.2 Microbial metabolites

In recent years, microbial metabolites (*i.e.* the intermediates and products of metabolism) have received much attention due to their critical rules to global processes. Metabolites are typically characterized as small molecules with various functions, which are categorized into both primary and secondary metabolites (Mashego *et al.*, 2007; Robertson, Robertson, & Bahnemann, 2012; Ross, Morgan, & Hill, 2002).

2.2.1 Primary metabolites

Primary metabolites (*i.e.* the central metabolite) are formed during all growth phases (Figure 1) as a result for energy metabolism and are deemed essential for proper growth, development and reproduction of the organism and it is a key component in maintaining normal physiological processes (Brockman & Prather, 2015; Piotrowska-Niczyporuk, Bajguz, Talarek, Bralska, & Zambrzycka, 2015; Ziemert *et al.*, 2012).

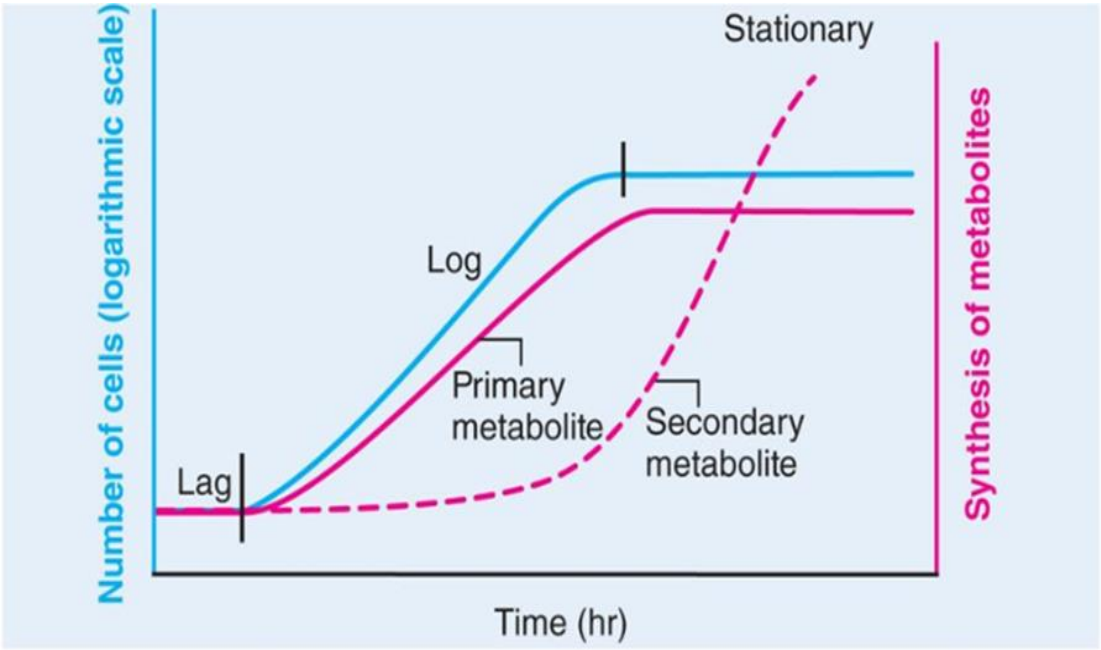

Figure 1. Primary and secondary metabolites production rate during different growth phases (*i.e.* lag, log and stationary phases). (Goodwin, H., 2018).

2.2.2 Secondary metabolites

Meanwhile, secondary metabolites are organic compounds produced through the modification of primary metabolite synthesis. They are typically formed during the end or near the stationary phase of growth (Figure 1) (Demain & Fang, 2000; Macheleidt *et al.*, 2016).

Many of the known secondary metabolites have a role in:

- Defense, such as antibiotics,
- Competition against other bacteria, fungi, amoebae, plants, insects and large animals,
- Transpiration,
- Symbiosis, as an agent between microbes and plants, nematodes, insects and higher animals,
- Mating, as sexual hormones,
- Differentiation, as effectors,
- Stimulating spore formation, when favorable conditions for growth are existed, and
- Inhibiting growth until less competitive environments are present

(Beccari, Senatore, Tini, Sulyok, & Covarelli, 2018; Bertrand *et al.*, 2014; Deveau *et al.*, 2016; Ziemert *et al.*, 2012).

Studies have been carried out in order to categorize the importance of secondary metabolite for different microorganisms, to illustrate their role in protecting and adjusting these microbes with the surrounding environment and to characterize the specificity of different antibiotics with certain microorganisms (Blin *et al.*, 2013; Deveau *et al.*, 2016; Gan *et al.*, 2013; Mosier *et al.*, 2013; Parastar, Jalali-Heravi, Sereshti, & Mani-Varnosfaderani, 2012; Ziemert *et al.*, 2012). Moreover, secondary metabolites production has been recognized to be strain specific, where the

structural diversity of these metabolite coupled with gene probing and phylogenetic analyses are considered as a lateral gene transfer evolution (Jensen *et al.*, 2007; Marliere, 2016; Wink, 2003).

However, due to the limited knowledge of microbial metabolites, the difficulty of identifying metabolites from complex samples and the inability to link metabolites directly to community members; have been proven to be major limitations in developing advance systems interactions (Demain & Fang, 2000; Gika, Wilson, & Theodoridis, 2014).

2.3 Electronic tongue (ET)

ET is a simple liquid analytical instrument consists of four parts (Figure 2): A: an auto-sampler for repeatable sensor measurement, B: a chemical multi-sensor system, C: signal acquisition system and D: a multivariate (chemometric) software and the instrument control on a PC.

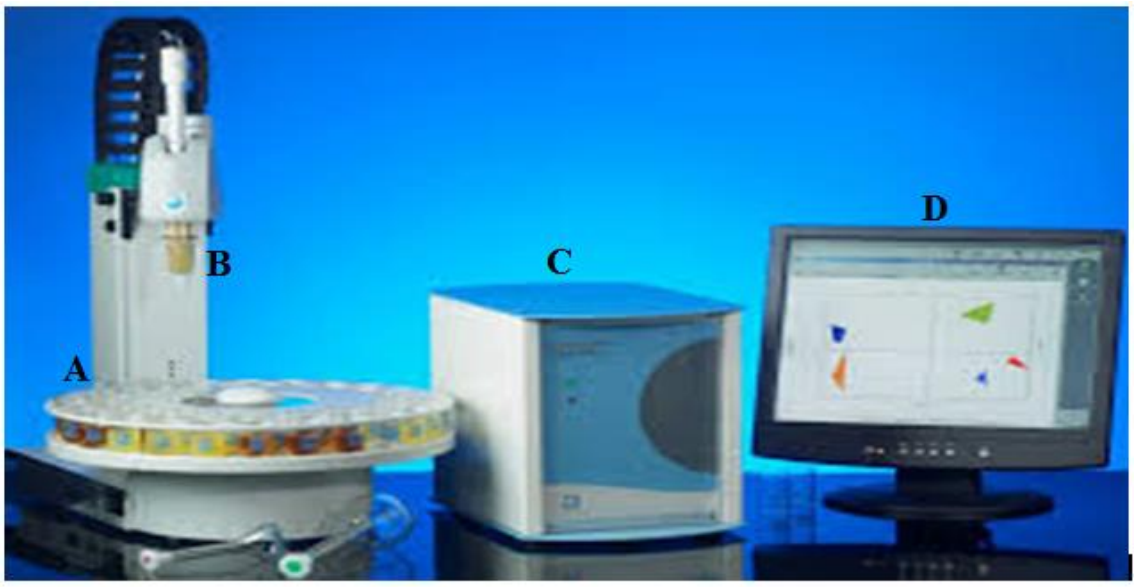

Figure 2. A typical Astree II electronic tongue package. A: auto-sampler, B: array of liquid sensors, C: electronic unit and D: advances chemometric software (Alpha Soft ver. 14) (Alpha MOS, 2009).

The principal of this sensing technology depends on transforming information of chemical composition (*i.e.* taste substances) using chemical multi-sensor system having partial specificity into electrical signals that is coupled then with chemometric analysis methods. In which, the whole system is capable of recognizing quantitative and qualitative composition of liquids and provides an objective low selective and high cross sensitive evaluation of tasted nonspecific complex solutions (Jiang, Zhang, Bhandari, & Adhikari, 2018; Power & Morrin, 2013).

2.3.1 Types of ET

According to the different sensor array working principals, ETs are divided into three common types: potentiometric, volumetric and taste sensor (*i.e.* lipid/ polymer membrane) (Faura, Gonzalez-Calabuig, & Valle, 2016; Tahri *et al.*, 2018).

In this study, an Astree II potentiometric ET is used. The system consists of seven modified chemical sensitive field effect transistor solid electrochemical sensors (ChemFETs). In which, these sensors patently have been developed by Alpha MOS company (Alpha MOS, 2009). The type of Astree's sensors is ion-sensitive field-effect transistor (ISFET), which categorized in two essential parts, one of them is a seven sensing transducers with a chemically coated sensitive layer and the other is Ag/AgCl reference electrode (*i.e.* silver-silver chloride reference electrode),

where the system measurement consists of the potentiometric difference between each individual sensor compared with the reference one.

The potentiometric measurement of ISFET sensor changes by the trapping of ions or molecules on its chemically sensitive layer that generates a change in the membrane potential. Where, this change leads to a final variation of potential between source and drain region of the field effect transistor of the sensor, which consider as an electronic signal (Figure 3).

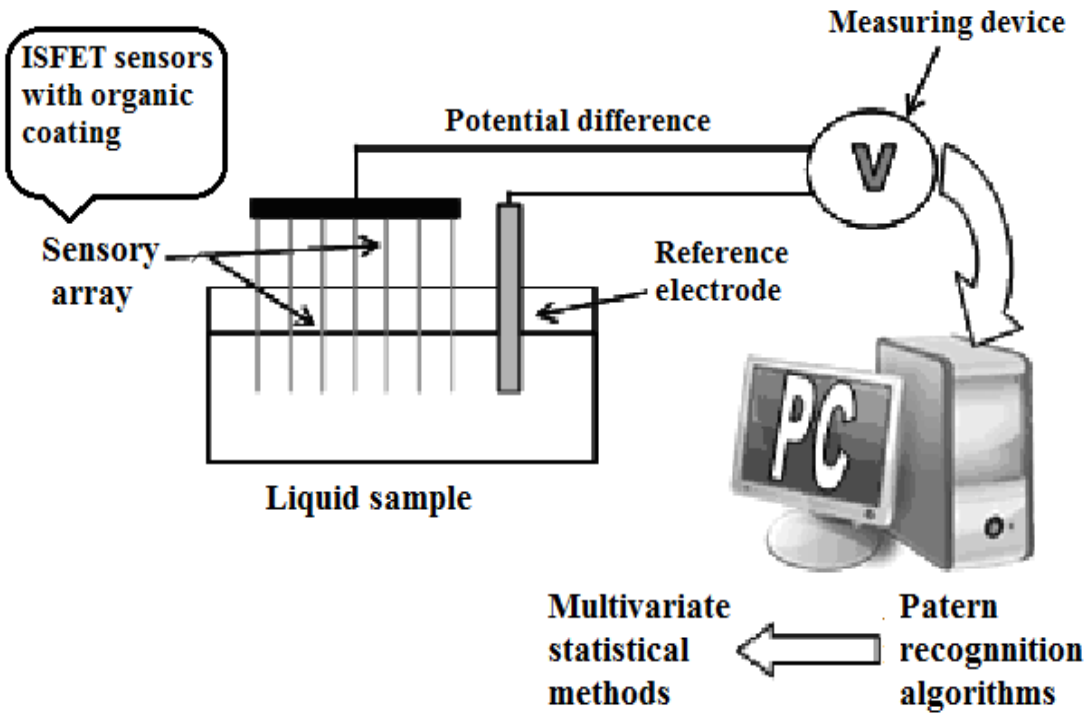

Figure 3. Schematic diagram of a potentiometric type electronic tongue, showing seven ISFET sensors immersed in complex liquid sample, the potential difference signals are sent to a signal processor system to be analyzed using pattern recognition algorithms (Kovács, Szöllosi, & Fekete, 2009).

Moreover, the specificity of this organic coating has been developed to ensure good reproducibility and to govern sensitivity and selectivity of each

individual sensor, according the chemical composition and the dissolved compounds in liquid media (Kutyla-Olesiuk *et al.*, 2014; Voitechovic, Korepanov, Kirsanov, & Legin, 2018). An ET equipped with potentiometric sensors is considered as one of the devices having ease of construction and miniaturization properties and has the privilege of rapid, reproducible, sensitive and selective response. Also, it can be used for toxicological analysis for its clean, simple and nondestructive methods of measurements and it has the possibility of obtaining sensors selective to various species. However, the main disadvantage of this type of ETs is temperature dependence and the adsorption of solution component can easily affect the nature of charge transfer (Veloso, Sousa, Estevinho, Dias, & Peres, 2018; Woertz, Tissen, Kleinebudde, & Breitkreutz, 2011).

2.3.2 Principal component analysis (PCA)

The MVDA software is used to transfer information acquired by sensors to a distinguishable patterns of interest that can be analyzed and make reasonable decisions about categories of the pattern (Jiang *et al.*, 2018; Voitechovic *et al.*, 2018). For specification, the electro chemical responses are used to form databases that are subjected to unsupervised and/ or supervised MVDA methods. Some of these methods include principal component analysis (PCA), linear discriminate analysis (LDA), partial least squares (PLS), hierarchical cluster analysis (HCA), support vector machine (SVM) and artificial neural network (ANN). Moreover, different

recognition methods have different applicable approaches. In which, the PCA, PLS, LDA and HCA are bases for linear approaches, but the ANN and SVM are regarded as non-linear methods. However, the unsupervised methods (also known as exploratory data analysis method) do not need any prior knowledge about the class structure of the data, but instead it produce grouping (Ceto *et al.*, 2016; Pavase *et al.*, 2018; Tahri *et al.*, 2018).

The PCA is one of the most widely used unsupervised linear techniques and it is often the first step in data analysis to verify patterns in measured data for qualitative purposes. It is used for converting and reducing multidimensional primary variables from a dataset to new independent lower dimensional approximation variables called principal components (PCs). The projections of the points from the original data space on PCs are called scores plot. Hence, PCA simplify the interpretation of the data by the PCs (PC1, PC2, … PCn) and preserve most of the variance in the data. It successively provides a set of orthogonal axes indicating the direction of the largest variance in the data. In which, The first principal component (PC1) accounts for the maximum of the total variance, the second (PC2) is orthogonal to the first and lies in the direction of the largest remaining variation, and so on, until the total variance is explained by *ca.* 100%. Each principal component contains different sources of information allowing them to be visualized, while maintaining as much information as possible from the original data (Scholz, 2006; Yaroshenko *et al.*, 2015).

On other words, the basic idea behind using PCA, as an unsupervised linear technique, is to have qualitative clustering of samples through the conversion of data from a high dimensional space to a low dimensional space and to visualize it graphically as scores plot, which shows the relation between samples. Objects or samples that are similar tend to cluster in the score plots, while different objects tend to be separated (Figure 4) (Esteki *et al.*, 2018; Feng *et al.*, 2016; Yaroshenko *et al.*, 2015).

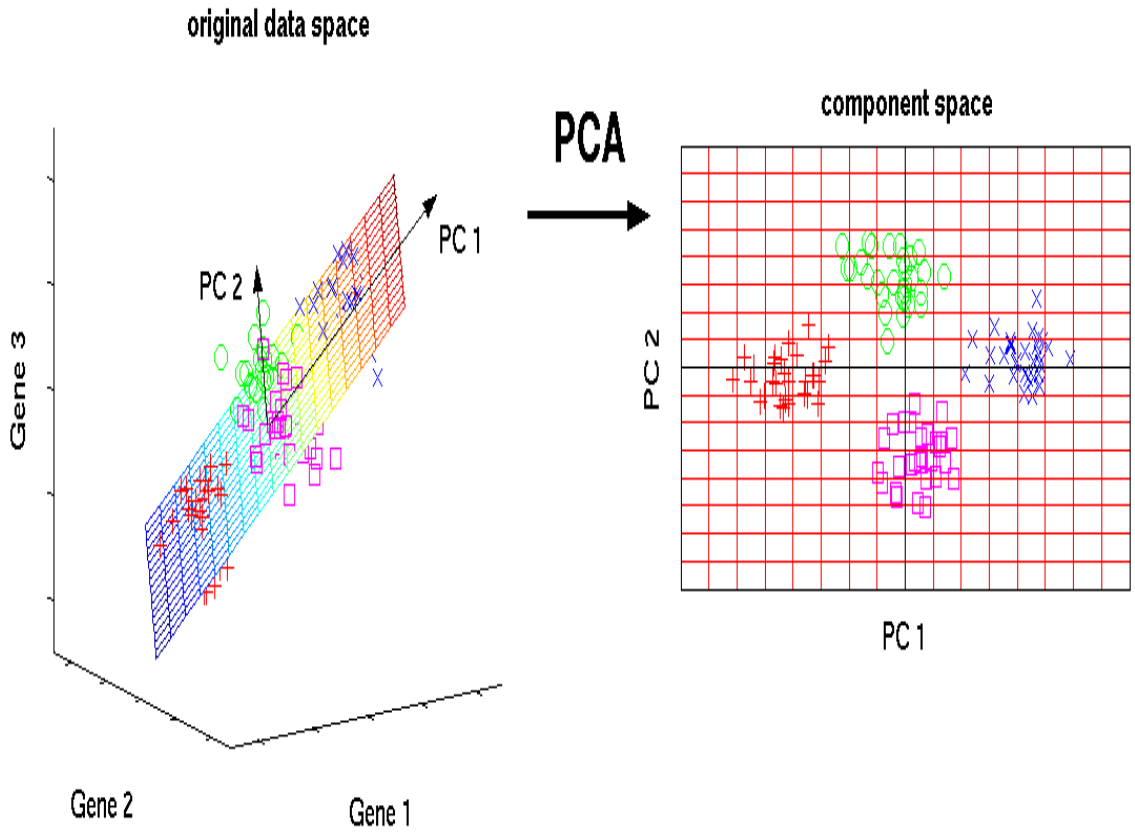

Figure 4. Representative chart of PCA unsupervised linear technique for qualitative data analysis. Showing how it reduces multidimensional primary variables from a dataset (original data space) to new independent lower dimensional approximation variables (component space, also called scores plot) called principal components (PCs) (Scholz, 2006).

2.3.3 ET biological scope of applications

Sensors were successfully used for monitoring and sensing the activity of microorganisms, in which promising results were obtained (Bougrini *et al.*, 2016; Brockman & Prather, 2015). Also, ET has number of applications that showed great solutions to many biotechnology and biomedical problems. It is useful for a wide variety of industries ranging from environmental control to blood analysis (Ceto *et al.*, 2016; Esteki *et al.*, 2018; Jiang *et al.*, 2018). Furthermore, it has been used in many biotechnology applications; one of these was to discriminate edible fungi varieties and evaluate their umami intensities (Feng *et al.*, 2016). The results of this study indicate that ET has a great potential in qualitative and quantitative analysis of edible fungi. Likewise, it was used to analyze mold growth in liquid media (Soderstrom *et al.*, 2003), and to recognize six microbial different fungal species (Soderstrom, Winquist, & Krantz-Rulcker, 2003). After that, two electronic tongues (potentiometric and voltammetry) were applied to differentiate between four *Aspergillus* species and one *Zygosaccharomyces* based on different measurement techniques, where promising results were achieved (Soderstrom, Rudnitskaya, Legin, & Krantz-Rulcker, 2005). Additionally, it was used for monitoring citric acid production by *Aspergillus niger* (Kutyla-Olesiuk *et al.*, 2014). As well , it was applied for fermentation monitoring, beverage, pharmaceuticals and taste masking (Ha *et al.*, 2015; Medina-Plaza *et al.*,

2015; Peris & Escuder-Gilabert, 2016; Yaroshenko *et al.*, 2015). Pharmaceutically, it was used for in vivo evaluation of the taste of commercially available clarithromycin oral pharmaceutical suspensions in Palestinian market. Plus, it was utilized to evaluate the taste, total phenols and antioxidant for fresh, roasted, shade dried and boiled leaves of edible *Arum palaestinum* bioss (Abu-Khalaf *et al.*, 2018; Qneibi *et al.*, 2018). Besides that, it was used for quantification of immobilized proteins using potentiometric multisensory array (Voitechovic *et al.*, 2018) and it was considered as safe and objective alternative for drugs' taste assessment in some pharmaceutical studies (Woertz *et al.*, 2011). Furthermore, in a recent study ET was used as an accurate, fast and cost-effective analytical technique for honey adulterations assessment, in addition to the classical techniques (*e.g.* physicochemical analysis, microscopy, chromatography, immunoassay, DNA metabarcoding and spectroscopy) (Veloso *et al.*, 2018).

3 Material and methods

3.1 Fungal experiment

3.1.1 Samples collecting and maintenance

Fungi samples were collected by spreading small portion of the rusted spoiled area from decayed fruits and vegetables (Figure 5) and from contaminated samples at Kadoorie Agricultural Research Center's (KARC) laboratories (Figure 6) on prepared potato dextrose agar (PDA) media. The media was prepared by completely dissolve 39 g of PDA powder in 1 L distilled water (D.W.) with heating. After that, the suspension was autoclaved at 121℃ for 15 min, followed by 15 psi for 15 min, and then suspended in 9 cm petri-dishes, where each plate contained approximately 12 mL of the powered media and then it was allowed to solidify.

After samples purification, 22 different pure fungal isolates were obtained and divided into two groups (Table 1). These cultures were maintained by dual culturing on PDA media every three weeks, through placing four 8 mm diameter discs of previously cultured growth oppositely over the media with incubation at 28℃ in dark to obtain full growth.

Samples were grouped into two groups: group 1 that labeled as F1, F2, … and F11 (Figure 7) and group 2 labeled as F12, F13, … and F22 (Figure 8).

Figure 5. Some photos of different spoiled vegetables, fruits, cheese, and bread samples. The rusted spoiled area was spread on prepared PDA and NA media for obtaining different fungal and bacterial cultures.

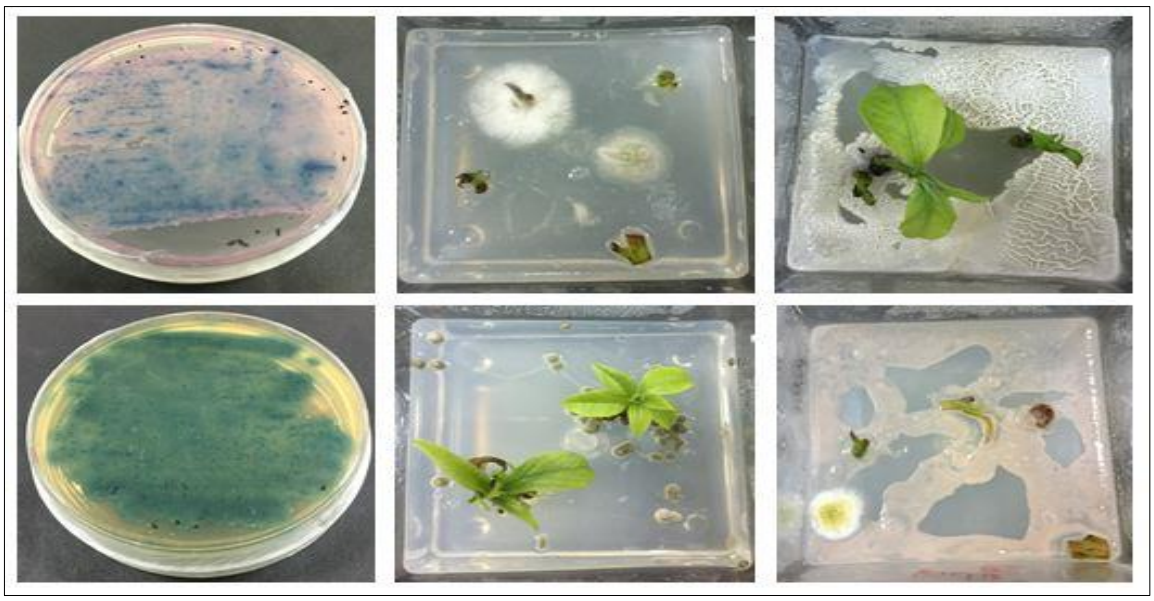

Figure 6. Some photos of different contaminated samples at KARC's laboratories. The different contaminated cultures were isolated on prepared PDA and NA media for purification and obtaining different fungal and bacterial samples.

For liquid growth, four 8 mm diameter discs of pure cultured fungi samples were cultivated in potato dextrose broth (PDB) media. The media was prepared by completely dissolve 24 g of PDB powder in 1 L D.W. with heating. After that, the suspension was suspended in 250 mL flask where each flask contained approximately 100 mL of powered media that were then autoclaved at 121℃ for 15 min followed by 15 psi for 15 min then lifted to cool down for the culturing process.

Table 1. List of fungal (pure isolates) different labeling for molecular and ET experiments.

Group no.	**no.**	**Molecular sample name**	**ET name for day 0**	**ET name for day 1**	**ET name for day 2**	**ET name for day 3**	**ET name for day 4**	**ET name for day 5**
Group 1 (F1-F11)	1	F1	F1D0	F1D1	F1D2	F1D3	F1D4	F1D5
	2	F2	F2D0	F2D1	F2D2	F2D3	F2D4	F2D5
	3	F3	F3D0	F3D1	F3D2	F3D3	F3D4	F3D5
	4	F4	F4D0	F4D1	F4D2	F4D3	F4D4	F4D5
	5	F5	F5D0	F5D1	F5D2	F5D3	F5D4	F5D5
	6	F6	F6D0	F6D1	F6D2	F6D3	F6D4	F6D5
	7	F7	F7D0	F7D1	F7D2	F7D3	F7D4	F7D5
	8	F8	F8D0	F8D1	F8D2	F8D3	F8D4	F8D5
	9	F9	F9D0	F9D1	F9D2	F9D3	F9D4	F9D5
	10	F10	F10D0	F10D1	F10D2	F10D3	F10D4	F10D5
	11	F11	F11D0	F11D1	F11D2	F11D3	F11D4	F11D5
Group 2 (F12-F22)	12	F12	F12D0	F12D1	F12D2	F12D3	F12D4	F12D5
	13	F13	F13D0	F13D1	F13D2	F13D3	F13D4	F13D5
	14	F14	F14D0	F14D1	F14D2	F14D3	F14D4	F14D5
	15	F15	F15D0	F15D1	F15D2	F15D3	F15D4	F15D5
	16	F16	F16D0	F16D1	F16D2	F16D3	F16D4	F16D5
	17	F17	F17D0	F17D1	F17D2	F17D3	F17D4	F17D5
	18	F18	F18D0	F18D1	F18D2	F18D3	F18D4	F18D5
	19	F19	F19D0	F19D1	F19D2	F19D3	F19D4	F19D5
	20	F20	F20D0	F20D1	F20D2	F20D3	F20D4	F20D5
	21	F21	F21D0	F21D1	F21D2	F21D3	F21D4	F21D5
	22	F22	F22D0	F22D1	F22D2	F22D3	F22D4	F22D5

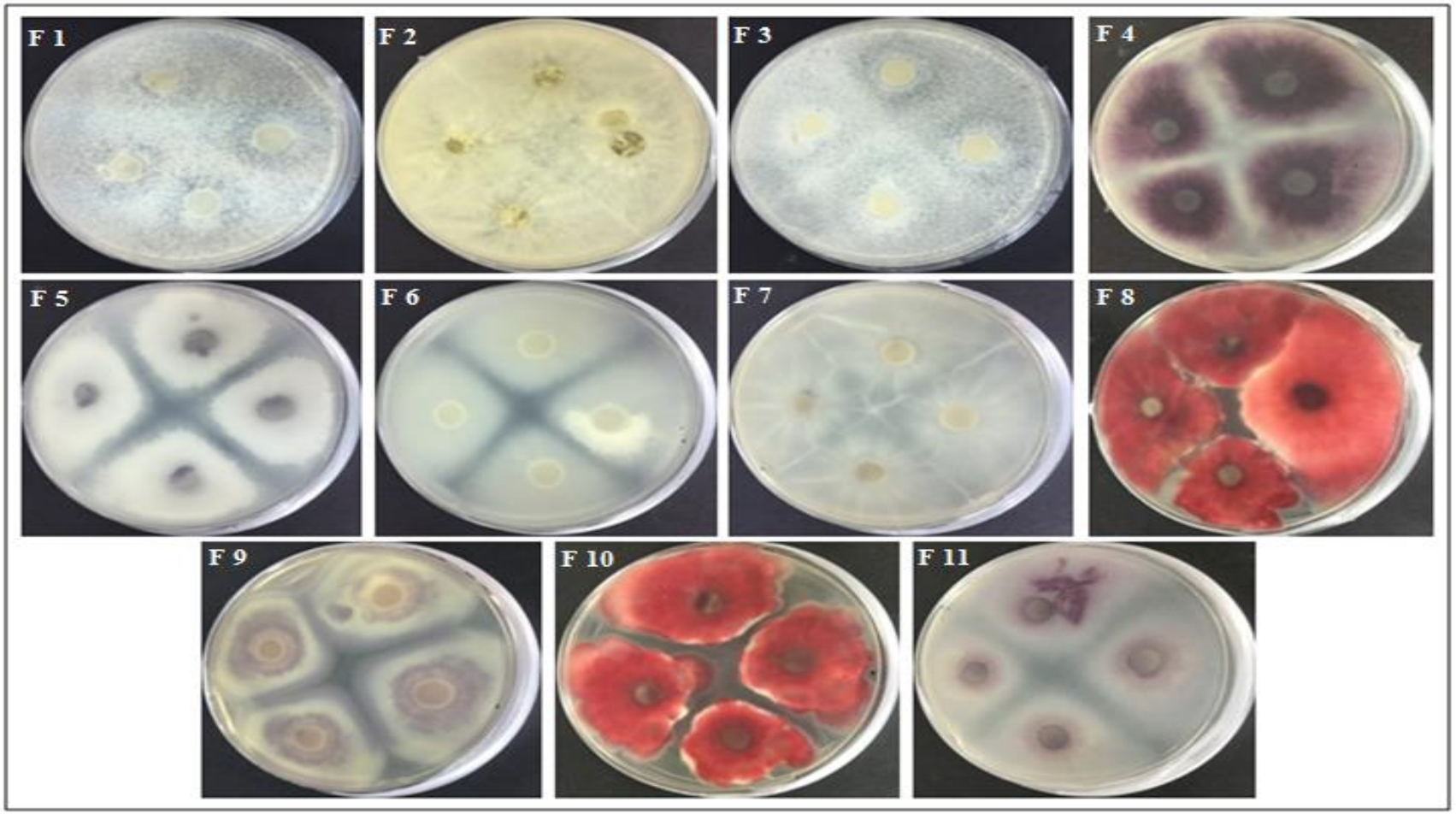

Figure 7. Group 1of purified different fungal isolates (F1-F11), which are cultured on prepared PDA media with labeling.

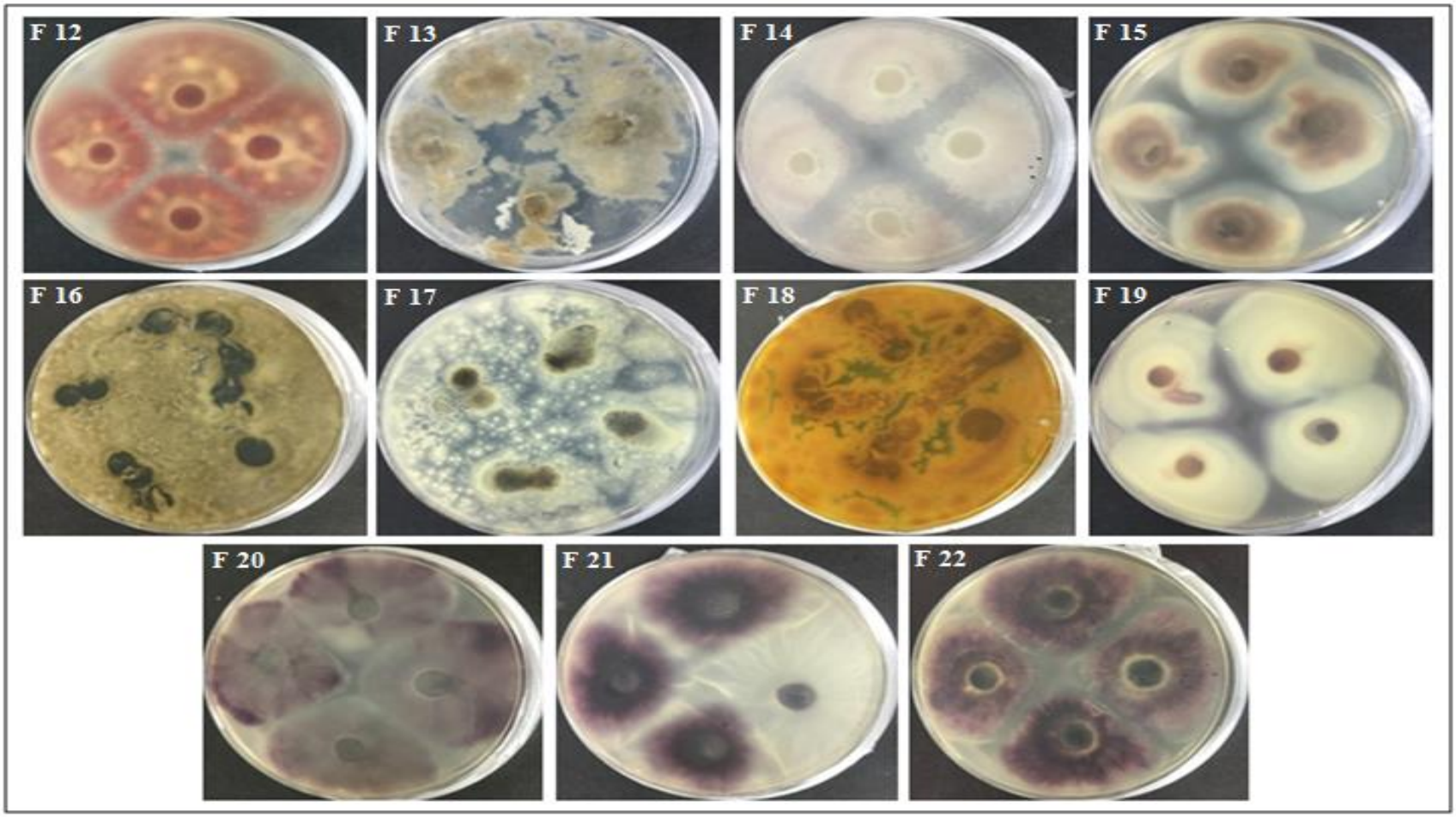

Figure 8. Group 2 of purified different fungal isolates (F12-F22), which are cultured on prepared PDA media with labeling.

3.1.2 Fungal molecular phylogenetic

3.1.2.1 Fungal DNA isolation

Fungal genomic material was isolated using the CTAB DNA isolation method (Zhu *et al*., 1993) with slightly modifications. The procedure started by collecting a (*ca*. 50-100 mg) mycelia of three days freshly grown fungi on prepared PDB media, which was placed in 1.5 mL microfuge tube containing sterile sea sand (*ca*. 100 mg). Then, 500 µL of extraction buffer (100 mM Tris-HCl, 10 mM EDTA, 2% SDS, 100 µg/mL proteinase K, and 1% B-mercaptoethanol) was added to each tube.

After that, samples were grind into slurry using pellet pestles homogenizer with sterilized tips (cat # 3110) and incubated at 60℃ for one hour with dual shaking every 3-4 min. Afterward, the salt concentration of the homogenate was adjusted to 1.4 M by adding 200 µL of 5 M NaCl, and then 0.1 of the resulted volume was added from 10% CTAB solution followed by an incubation period for 10 min at 65℃.

After incubation, one volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to each tube that were gently emulsified by inversion, and incubated at 0℃ for 30 min to be spun after that at 12000 rpm, 4℃ for 10 min. At that point, the top phase was transferred to new 1.5 mL microfuge tube and half the transferred volume was added with 5 M NH_4OAc and

mixed gently. Tubes then were incubated at 0℃ for 60 min and spun at 4℃, 12000 rpm for 15 min. The supernatant was transferred to new 1.5 mL eppindorf tube, followed by the addition of RNase solution to have a final concentration of 0.02 µg/mL and 0.55 of the resulted volume with cold isopropanol that was then mixed gently.

The mixture was spun at 1000 rpm for 5 min with discarding the supernatant without disrupting the collected pellet. Later on, pellets were suspended in 200 µL TE buffer (10 mM Tris base at pH.8 and 1 mM EDTA) and 20 µL of 3 M NaOAc at pH 7. Then for pellet washing, 2.5 of the resulted volume was added with cold 75% ethanol that was gently mixed and span at 10000 rpm for 5 min (this step was repeated twice). At the end, pellets were resuspended in 50 µL TE buffer after being completely dry from ethanol residues and stored at -20℃ for further uses.

3.1.2.2 Inter simple sequence repeats (ISSR) sequences amplification, electrophoresis and data analysis

- ISSR sequences amplification reaction mixture

A total of six University of British Columbia- inter simple sequence repeats primer (UBC-ISSR) primers were used for PCR amplification of DNA templates. The ISSR sequences were amplified according to Abadio *et al.*

(2012) with modifications. Where, primers were dissolved in sterilized distilled and DNase free water at concentration of 100 µM.

The amplification reaction was performed in a volume of 20 µL using Red taq DNA polymerase ready mix (Lot # SLBF8650V), that contained 2 µL of 10X red taq PCR reaction buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 11 mM $MgCl_2$ and 0.1% gelatin), 0.25 µL of 10 mM dNTPs (200 µL of each dNTPs), 0.2 µL of 100 µM primer, 0.36 µL of 50 mM $MgCl_2$, 15.19 µL of free DNase water, 1 µL of red taq DNA polymerase (1 unit/µL) and 1 µL of (30-50 ng) DNA template.

- PCR amplification program

The amplification program was performed using Verti^{TM} 96 well thermal cycler (Cat. #: 4375786) (Applied Biosystems company, California, USA), that was programed to perform an initial denaturation cycle of 94°C for 5 min, and 35 cycles of 94°C for 1 min, 1 min at the primer annealing temperature, which varied according to the used primer (Table 5 (in result section)), and at 72°C for 2 min, followed by a final extension cycle of 72°C for 7 min.

To ensure reproducibility of the amplified DNA fragments, all PCRs were performed in duplication for each isolate and reactions without DNA were performed for DNA contamination determination.

- Gel electrophoresis

ISSR products were separated by electrophoresis according to their molecular weight using 2% agarose gel. That was prepared by weighting 9 g of agarose powder that was completely dissolved in 450 mL of 1X TBE electrophoresis buffer (0.089 M Tris base, 0.089 M Boric acid and 0.002 M EDTA) by thermal heating using microwave, then the mixture was cool to 60℃. After that, 8 µL of 1000X Gel Red DNA stain (Cat. # 41003) was added with stirring. The suspension was then powered and allowed to solidify in (20 x 20) tray with 46 wells comp. After submerging the gel in 1X TBE buffer and loading 5 µL of PCR products, the device was run for three hours at 80 volt.

DNA fragments were visualized using 10000X Gel Red DNA stain and UV-illuminator and documented using SynGene gene tool system (Synoptics Ltd., Cambridge C, UK) for image acquisition and documentation. Also, for estimating the size of the amplified DNA fragments, a 100 bp DNA RTU ladder (Cat. # DM001-R500) was used as a molecular size marker.

- Data analysis

To ensure the reproducibility and reliability of the ISSR markers, PCR reactions were repeated twice for each primer. Only reproducible band were considered for analysis.

For data analysis, each DNA fragments with a different electrophoretic mobility were used to assign loci for each primer. Bands were scored as diallelic for each assigned locus (1= band present and 0= band absent) (scoring table can be seen in results and discussion section, Table 4).

A dendogram was constructed through using unweighted pair groups' method average (UPGMA) cluster analysis based on a Dice coefficient, using gel SynGene Ver. 4.3.5 (Synoptics Ltd., Cambridge C, UK) analysis software. The similarity matrix was calculated among the exanimated samples accessions based on Dice genetic distance. In this study, Dice coefficient was used because it is the suitable measure for haploids with co-dominant markers.

3.1.3 ET measurements of fungal metabolites

A liquid taste analyzer Astree II ET (Alpha MOS company, Toulouse, France), composed of a sensor array of seven sensors (CA, JB, HA, ZZ, BB, JE and GA) with an Ag/AgCl reference electrode, was used to follow up metabolite consumption and production during growth of microorganisms in PDB from day zero until the fifth day of inoculation.

3.1.3.1 Fungal broth samples preparation

Two rounds of fungal samples were measured using ET. In each round, 11 fungi with a PDB sample (control) were tested in triplicate. In which, fungal

samples were grown in PDB media that was prepared by completely dissolving 24 g of PDB powder in 1 L D.W. with heating, the mixture was then suspended in 250 mL Erlenmeyer flasks each flask contained 100 mL of broth media (72 flask were prepared for each round), the suspended flasks were then autoclaved sterilized at 121℃ for 15 min followed by 15 psi for 15 min.

Then four discs (8 mm diameter) of each cultured fungi (Table 1) from PDA media were inoculated in each flask with proper labeling and incubated at 28℃ in dark with shaking. For each tested fungi, six flasks were prepared for every tested day (0, 1, 2, 3, 4 and 5 days) including a control PDB media sample for the comparison.

3.1.3.2 ET sequence preparation and auto-sampler samples loading

A binomial way of labeling was used to create a sequence for ET method, where samples names' have two parts, one for the number of fungi and the other for the tested day (*e.g.* F0D0, F0D1, …, F11D5) (Table 1) this sequence was created for each day of measurements. For the first ET measurement round, only 11 fungi (group 1) with F0 as a control sample were measured daily after 0, 24, 48, 72, 96 and 120 h of inoculation (*i.e.* 0-5 days).

Each fungal growth was filtered from mycelia using white cheese cloth to obtain approximately 80 mL of each broth that was placed on the ET's 16-position auto-sampler (with an automatic stirrer) and broth samples were separated by four water samples for cleaning ET sensors after each test (Figure 9).

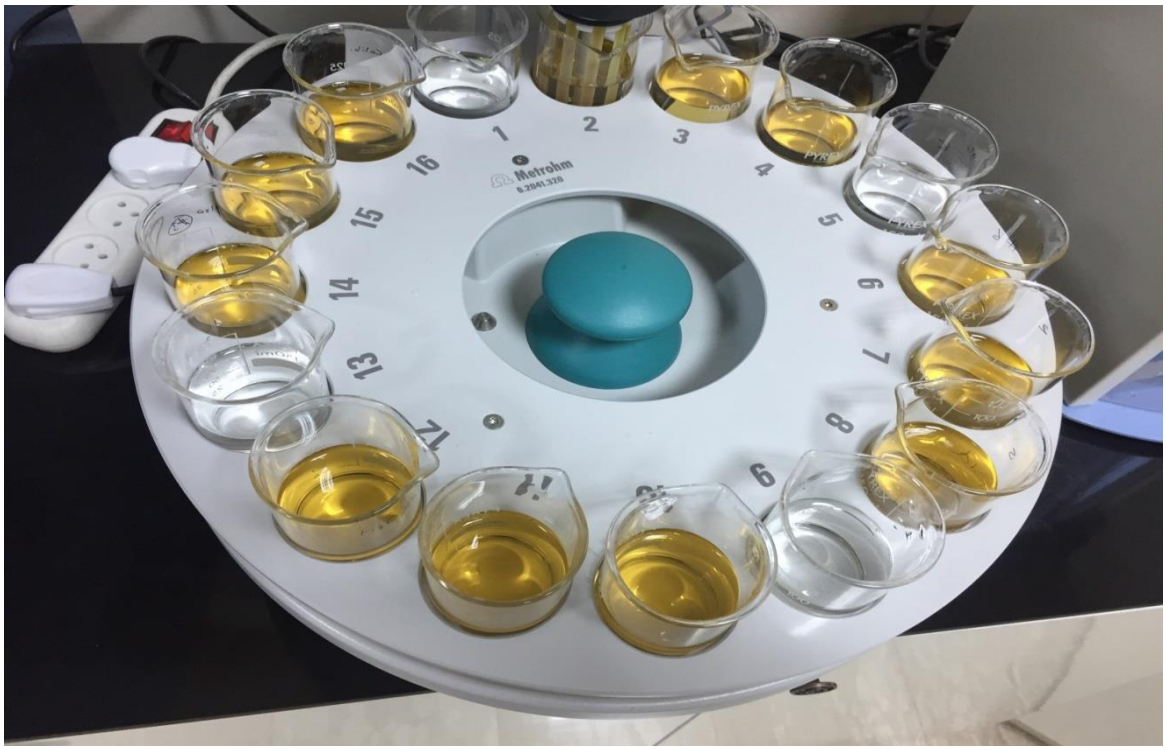

Figure 9. Auto-sampler samples' distripution, the first round for ET mesurmet, consists of 11 different PDB fillterated extarct of fungal growth (in positions 3, 4, 6, 7, 8, 10, 11, 12, 14, 15 and 16) and a control PDB sample (in position 2), seperated by four D.W. samples (in position 1, 5, 9 and 13) for sensor cleaning processes after each measurment.

For the second round, fungal samples were labeled as F12, F13, …, F21 and F22, with F0 as a control sample. In which those samples were also measured daily after 0, 24, 48, 72, 96 and 120 h of inoculation following the

same previously mentioned process for sequence labeling (such as F0D0, F0D1, …, F22D5) (Table 1).

This second round of measurements was done in order to be sure of the created measurement, to observe the change in the collected data according to the change of tested samples and to have a broad spectrum of different fungi.

3.1.3.3 ET data library creation

After each measurement the obtained data from each sensor were collected in a folder categorized by fungal sequence and the date of measurement for each round after creating a library of the experiment (as stated in Alpha MOS ASTREE manual, 2009).

3.1.3.4 ET data analysis

The collected data from analyzed sensors were analyzed using Alpha Soft Ver. 12.4 (Alpha MOS, Toulouse, France) multivariate data analysis (MVDA) software package, such as PCA scores plot that was used for automatically collecting and storing the sensors' outputs and studying the relationship among samples.

3.2 Bacterial experiment

3.2.1 Samples collecting and maintenance

Bacterial samples were collected by screening small portion of the spoiled area on fruits and vegetables (Figure 5), and contaminated samples in Kadoorie Agricultural Research Center's (KARC) laboratories (Figure 6) on prepared nutrient agar (NA) media. The media was prepared by completely dissolve 23 g of NA powder in 1 L D.W. with heating. After that, the suspension was autoclaved at 121℃ for 15 min followed by 15 psi for 15 min that was set aside to cool down and suspended in 9 cm petri-dishes where each plate contained approximately 12 mL of powered media and allowed to solidify.

After purification, 22 different pure bacterial cultures were obtained with proper labeling that was divided into two groups according to the auto-sampler capacity (Table 2).

These cultures were maintained with dual culturing on NA media every two weeks by spreading small bacterial inoculum of previously cultured growth over a new NA media and incubated at 28℃ in dark for full growth.

Group 1 labeled as B1, B2, … and B11 (Figure 10) and group 2 labeled as B12, B13, … and B22 (Figure 11).

For liquid growth, small inoculum of pure cultured bacteria was grown in nutrient broth (NB) media. The media was prepared by completely dissolving 13 g of NB powder in 1 L D.W. with heating, the mixture was then suspended in 250 mL flask where each flask contained approximately 100 mL of the powered media, then the suspension was autoclaved at 121℃ for 15 min followed by 15 psi for 15 min and allowed to cool down for the culturing process.

Table 2. List of bacterial (pure isolates) different labeling for molecular and ET experiments.

Group no.	no.	Molecular sample name	ET name for Day 0	ET name for Day 1	ET name for Day 2	ET name for Day 3	ET name for Day 4	ET name for Day 5
Group 1 B1-B11	1	B1	B1D0	B1D1	B1D2	B1D3	B1D4	B1D5
	2	B2	B2D0	B2D1	B2D2	B2D3	B2D4	B2D5
	3	B3	B3D0	B3D1	B3D2	B3D3	B3D4	B3D5
	4	B4	B4D0	B4D1	B4D2	B4D3	B4D4	B4D5
	5	B5	B5D0	B5D1	B5D2	B5D3	B5D4	B5D5
	6	B6	B6D0	B6D1	B6D2	B6D3	B6D4	B6D5
	7	B7	B7D0	B7D1	B7D2	B7D3	B7D4	B7D5
	8	B8	B8D0	B8D1	B8D2	B8D3	B8D4	B8D5
	9	B9	B9D0	B9D1	B9D2	B9D3	B9D4	B9D5
	10	B10	B10D0	B10D1	B10D2	B10D3	B10D4	B10D5
	11	B11	B11D0	B11D1	B11D2	B11D3	B11D4	B11D5
Group 2 B12-B22	12	B12	B12D0	B12D1	B12D2	B12D3	B12D4	B12D5
	13	B13	B13D0	B13D1	B13D2	B13D3	B13D4	B13D5
	14	B14	B14D0	B14D1	B14D2	B14D3	B14D4	B14D5
	15	B15	B15D0	B15D1	B15D2	B15D3	B15D4	B15D5
	16	B16	B16D0	B16D1	B16D2	B16D3	B16D4	B16D5
	17	B17	B17D0	B17D1	B17D2	B17D3	B17D4	B17D5
	18	B18	B18D0	B18D1	B18D2	B18D3	B18D4	B18D5
	19	B19	B19D0	B19D1	B19D2	B19D3	B19D4	B19D5
	20	B20	B20D0	B20D1	B20D2	B20D3	B20D4	B20D5
	21	B21	B21D0	B21D1	B21D2	B21D3	B21D4	B21D5
	22	B22	B22D0	B22D1	B22D2	B22D3	B22D4	B22D5

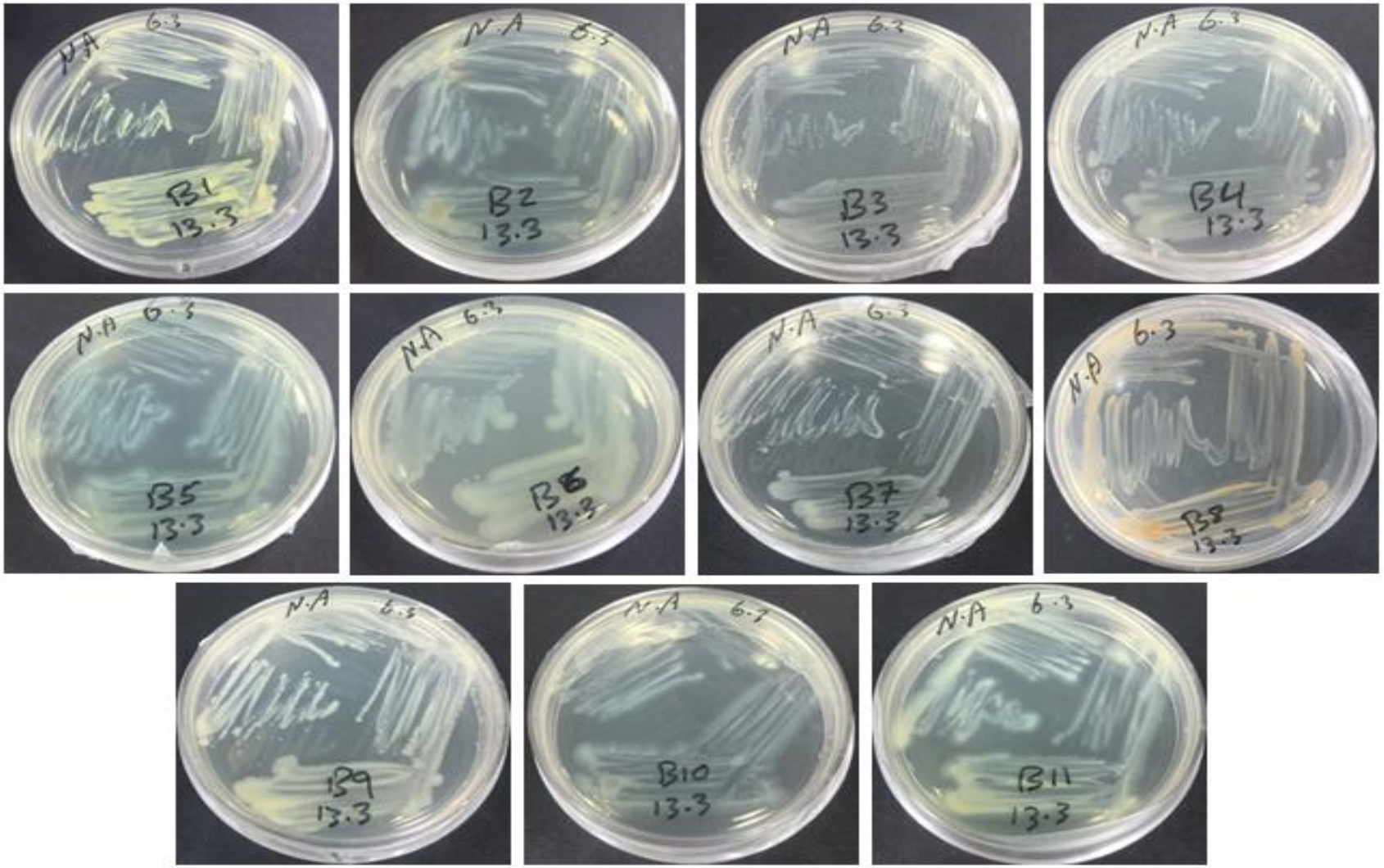

Figure 10. Group 1 of purified bacterial isolates (B1-B11), which are cultured on prepared NA media with labeling.

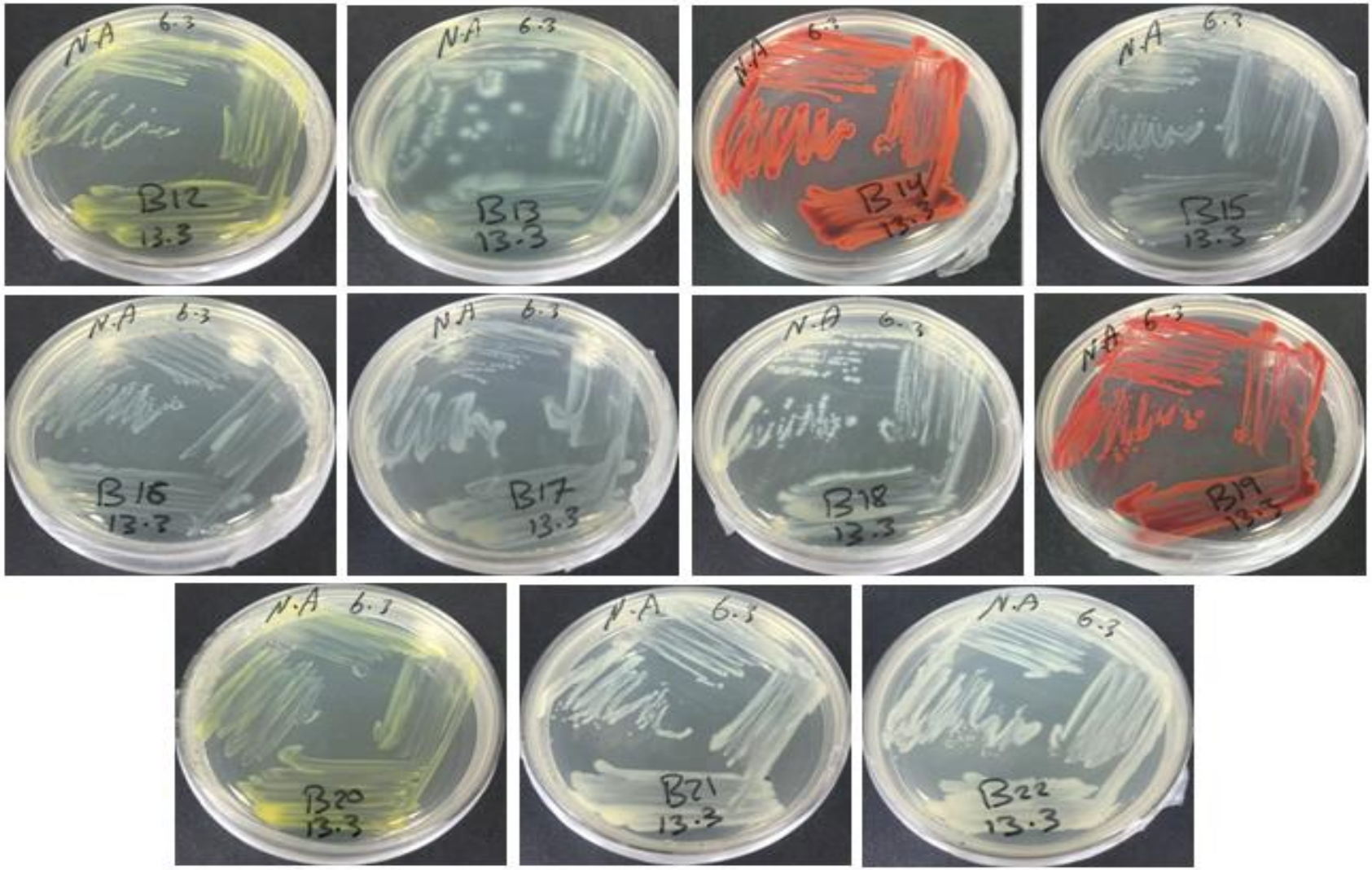

Figure 11. Group 2 of purified bacterial isolates (B12-B22), which are cultured on prepared NA media with labeling.

3.2.2 Bacterial molecular phylogenetic

3.2.2.1 Bacterial DNA isolation

Bacterial DNA isolation procedure was done according to TRIzol reagent manual (TRI reagent) (Cat. # T942). In which, a small freshly grown bacterial portion (grown in NA media) was dissolved in 1 mL of TRI reagent contained in 1.5 mL microfuge tubes through variously shacking using vortex. Samples were allowed to stand for 5 min at room temperature, 200 µL of absolute cold chloroform was added per mL of TRI reagent, that tubes were then shacked vigorously for 15 sec and left to stand for 15 min at room temperature.

The resulted mixture was centrifuged afterwards at 12000 xg (11573 rpm) for 10 min at 4°C that gave three phases:

- Colorless upper phase (RNA),
- Inter phase (DNA), and
- Red organic phase (protein lower phase).

After that, the aqueous overlying phase was removed and discarded, and 300 µL of cold 100% ethanol was added per mL of TRI reagent. Tubes then were mixed by inversion, let to stand for 3 min at room temperature and centrifuged at 2000 xg (4730 rpm) for 5 min at 4°C. Then, the supernatant was removed and saved for protein isolation (if it is needed).

DNA pellets were washed twice using 1 mL of cold 0.1 M Trisodium cetrate in 10% ethanol solution per mL of TRI reagent. After that, samples were allowed to stand with occasionally mixing for at least 30 min. Followed by, centrifugation at 2000 xg (4730 rpm) for 5 min at 4℃, pellets were then resuspended with cold 75% ethanol by adding 1.5 mL and allowed to stand for 20 min at room temperature before being centrifuged at 2000 xg (4730 rpm) for 5 min at 4℃ with discarding the resulted supernatant. At the end, pellets were dried for 10 min under vacuum, dissolved in 50 µL of TE buffer and stored at -20℃ for further uses.

3.2.2.2 Sequences amplification, electrophoresis and data analysis

- 16S rRNA sequences amplification reaction

A total of four universal bacterial 16S primers were used for PCR amplification of DNA templates. Where, primers were dissolved in sterilized distilled and DNase free water at concentration of 100 µM.

Amplification mixture was done using thermo-scientific 2X ready mix PCR master mix with 1.5 mm $MgCl_2$ (Cat. # AB-0575/DC/LD/A). In which, a 25 µL PCR reaction mixture containing 12.5 µL of 2X ready mix PCR master mix (0.625 U thermo prime taq DNA polymerase, 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs), 0.125 µL of 100 µM

forward primer, 0.125 µL of 100 µM reverse primer, 0.5 µL of 50 mM $MgCl_2$, 10.75 µL of free DNase water and 1 µL of DNA template.

-The PCR amplification program

The amplification program was performed using Verti^{TM} 96 well thermal cycler (Cat. # 4375786) (Applied Biosystems company, California, USA), programed to perform an initial denaturation cycle at 94℃ for 3 min, then a 35 cycles of 94℃ for 45 sec, the annealing temperature for different primer combination (Table 3) for 50 sec, and 72℃ for 1 min, and then an extension cycle of 72℃ for 7 min.

To ensure reproducibility of the DNA amplified fragments, all PCRs were duplicated for each isolate, and a tube sample without DNA was performed to determine if the DNA was contaminated (as negative control).

- Gel electrophoresis

The 16S rRNA products were separated by electrophoresis according to their molecular weight on 2% agarose gel. The gel was prepared by weighting 9 g of agarose powder that was completely dissolved in 450 mL of 1X TBE buffer using microwave, then the mixture was cooled to 60℃. After that, 8 µL of 1000X Gel Red DNA stain (Cat. # 41003) was added with stirring. The suspension was then powered and allowed to solidify in (20 x 20) tray

with 46 wells comp. After submerging the gel in 1X TBE buffer and loading 5 µL of PCR products, the device was run for three hours at 80 volt.

DNA fragments were visualized by 10000X Gel Red DNA stain and UV-illuminator and documented by using SynGene gene tool system (Synoptics Ltd., Cambridge C, UK) for image acquisition and documentation. And to estimate the size of the amplified DNA fragments, a 100 bp DNA RTU ladder (Cat. # DM001-R500) was used as a molecular size marker.

- Data analysis

To ensure the reproducibility and reliability of the 16S rRNA coded primers, PCR reactions were repeated twice for each primer. Only reproducible bands were considered for analysis. For data analysis, each DNA fragments with a different electrophoretic mobility were used to assign loci for each primer. Bands were diallelicly scored for each assigned locus as (1= band present and 0= band absent) (scoring table can be seen in results and discussion section Table 10).

A dendogram was constructed by UPGMA cluster analysis based on a Dice coefficient, using gel SynGene Ver. 4.3.5. analysis software (Synoptics Ltd., Cambridge C, UK). The similarity matrix was calculated among the exanimated samples accessions based on Dice genetic distance.

Table 3. PCR used primers' codes, combinations and their annealing tempereature for 16S ribosomal RNA sequence amplification.

Prime code	**Forward (F) primer**	**F primer sequence 5'-3'**	**Reverse (R) primer**	**R primer sequence 5'-3'**	**Annealing temperature (˚C)**
16S RNA1	Bakt 341F	CCTACGGGNGGCAGCAGCAG	Bakt 805R	GACTACNVGGGTATCTAATCC	53
16S RNA2	27F	AGATTTGATCTGGCTCAG	1492R	TACGGTTACCTTGTTACGACTT	51
16S RNA3	Bakt 341F	CCTACGGGNGGCAGCAGCAG	1492R	TACGGTTACCTTGTTACGACTT	55
16S RNA3	27F	AGATTTGATCTGGCTCAG	Bakt 805R	GACTACNVGGGTATCTAATCC	52

N= A, T, G and C. V = A, C and G.

3.2.3 ET measurements of bacterial metabolites

A liquid taste analyzer Astree II ET (Alpha MOS company, Toulouse, France), composed of a sensor array of seven sensors (CA, JB, HA, ZZ, BB, JE and GA) with an Ag/AgCl reference electrode, was used to follow up metabolite consumption and production during growth of microorganisms in PDB from day zero until the fourth day of inoculation.

3.2.3.1 Bacterial broth samples preparation

Two rounds of bacterial samples were measured using ET. In each round, 11 bacteria with a NB sample (control) were tested in triplicate. The media was prepared by completely dissolving 13 g of the powder in 1 L D.W. with heating, the mixture was then suspended in 250 mL erlenmeyer flasks. Each flask contained 100 mL of broth media (60 flasks were prepared for each round), the suspended flasks were then autoclave sterilized at 121℃ for 15 min followed by 15 psi for 15 min. After that, small inoculum (around $125x10^{-14}$) of each cultured bacteria were inoculated in each flask with proper labeling, which was incubated at 28℃ with shaking in dark condition. For each tested bacteria, five flasks were prepared for every tested day (0, 1, 2, 3 and 4 days) including a control NB media sample for the comparing issues.

3.2.3.2 ET sequence preparation

To create a sequence a binomial way of labeling was used, where samples names' has two parts. One for the number of bacteria and the other for the testing day (*i.e.* B0D0, B0D1, …, B11D4) (Table 2) this sequence was created for each day of measurements.

For the first ET measurement round only group 1 of bacteria with B0 as a control sample were tested. In which, those samples were measured daily after 0, 24, 48, 72 and 96 h of inoculation.

Each bacterial growth was filtered using white cheese cloth to obtain approximately 80 mL of each broth to be placed on ET's 16-position auto-sampler, with an automatic stirrer, after creating the sequence. Samples were separated by four water samples for cleaning ET sensors after each test.

For the second round, bacterial samples of group 2 with B0 as a control sample were tested. In which, those samples were also measured daily after 0, 24, 48, 72 and 96 h of inoculation following the same previously mentioned process with proper labeling.

This second round of measurements was done to be sure of the created system of measurement, to observe the change in the collected data according to the change of tested samples and to have a broad spectrum of different bacteria.

3.2.3.3 ET data library creation

After each measurement the resulted data from each sensor were collected in a folder categorized by bacterial sequence and the date of measurement for each round after creating a library of the experiment as mentioned earlier.

3.2.3.4 ET data analysis

The collected data from analyzed sensors were analyzed using Alpha Soft Ver. 14 (Alpha MOS, Toulouse, France) multivariate data analysis (MVDA) software package, as PCA scores plot was used for scoring the collected and stored sensors' outputs and studying the relationship among samples.

4 Results and discussion

4.1 Fungal experiment results

4.1.1 Fungal DNA data analysis

The total DNA extraction of 22 different fungi using CTAB isolation method is shown in Figure 12 that was grouped into two groups according to the final comparison with ET measured capacity of the auto-sampler. The total six used ISSR primers resulted in polymorphic ISSR profiles, primers include poly (GA) unanchored dinucleotide as ISSR 807, 3' anchored primers as ISSR 808, 816 and 840 and 5' anchored primers as ISSR 885 and 890 (Table 5).

The number of bands produced by the used primers ranged between 9 for primer 890 ($HVH(GA)_7$) and 12 for the primer 840 ($((GA)_8YT$). The total number of alleles produced by all primers is 62 including 54 polymorphic markers with only 8 monomorphic markers (Table 5). A 100% polymorphism was scored for ISSR 808 ($((GA)_8T$) and 807 ($((GA)_8$). Meanwhile, the lowest polymorphism of 66.7% was scored for primer 840 ($((GA)_8YT$). The other three primers produced polymorphism ranging between 80% for primer 885 ($BHB(GA)_7$) and 90% for primer 816 ($((CA)_8T$). The total percentage of polymorphic markers for all primers in the

examined 22 genotypes is 87.6%; this indicated high level of genetic variation among the examined fungal genotypes.

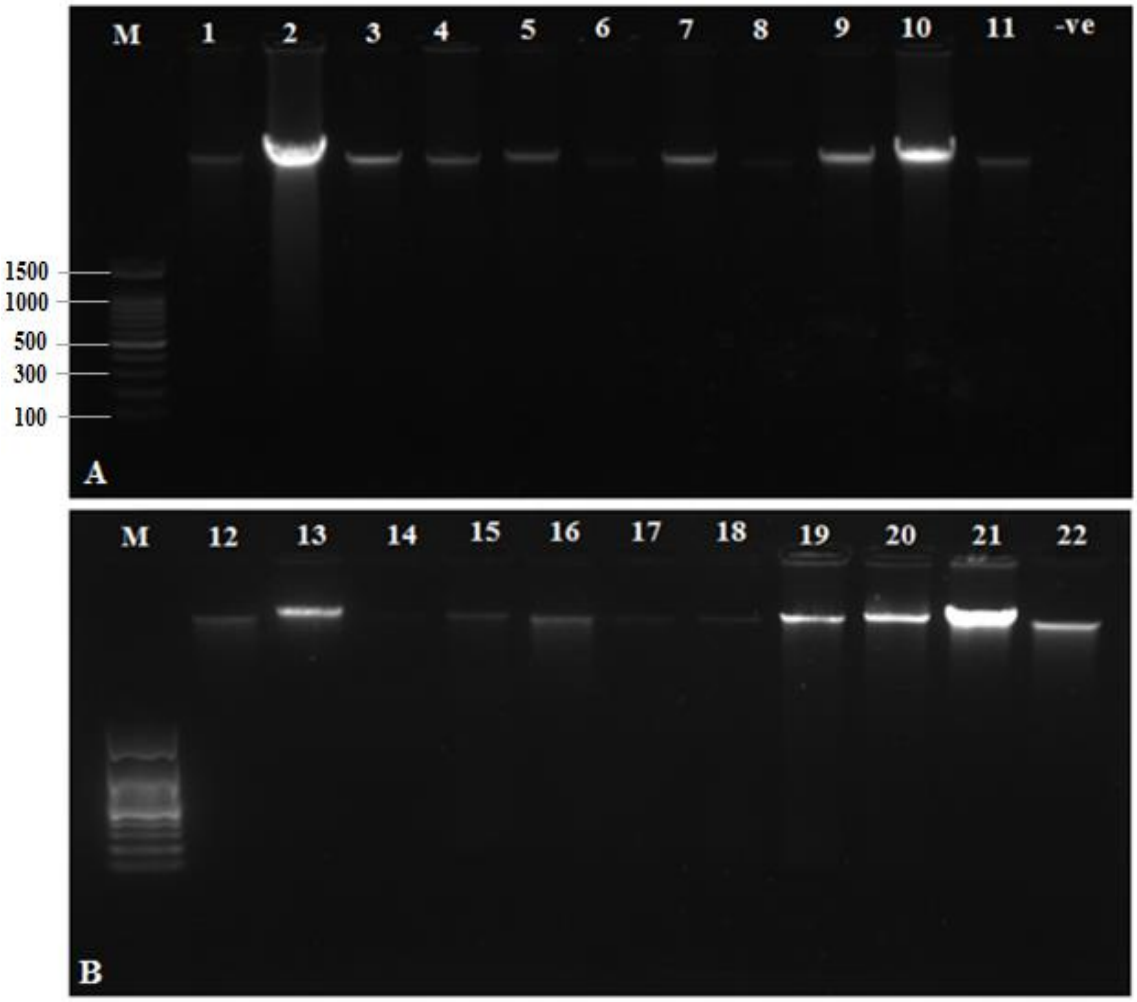

Figure 12. Gel electrophoresis documented photos of total DNA isolated from fungal samples using CTAB method for genomic isolation. **A**: represents group 1 fungal samples from F1-F11 as lanes from 1-11. **B**: repents group 2 fungal samples from F12-F22 as lanes from 12-22. M= 100 bp ladder as a molecular size marker. –ve= represents a negative control sample.

The number of alleles varied per primer between 9 for primer 890 and 12 for primer 840 with a mean of 10.3. Photographs illustrating the ISSR finger-

printing of selected six primers for the 22 fungal genotypes are shown in Figure 13 A-F. Where the largest produced fragment was approximately 1.5 Kbp and the smallest recognized produced fragment was approximately 0.2 Kbp (Table 5).

Table 4. ISSR bands' profile scores for 808 primer of 22 different fungal genotypes. Where, 1= present band and 0= absent band.

Primer 808 (bp)	Sample No.																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1400	1	0	0	1	1	1	0	0	1	0	1	0	0	1	1	0	0	0	1	1	1	1
1200	0	0	0	1	1	1	1	0	1	0	1	0	0	1	0	0	0	0	1	1	1	1
1000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
900	1	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0
700	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
600	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
500	1	1	1	0	0	0	1	1	1	1	1	1	0	0	1	1	1	0	1	0	0	0
400	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0
300	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0
200	0	0	0	1	1	1	1	0	0	0	0	0	0	1	0	1	1	1	1	1	1	0
Total bands	4	1	3	4	4	3	3	3	3	3	3	3	2	4	3	3	2	2	4	3	3	2

Table 5. Base sequence of the six used ISSR primers which produced polymorphic finger-printing in 22 different fungal genotypes, number of total alleles, number of amplified monomorphic and polymorphic bands as well as the percentage of polymorphism.

Primer code	Sequence (5'-3')	Annealing temperature (°C)	Size range (bp)	Total No. of alleles	No. of monomorphic bands	No. of polymorphic bands	%Polymorphism
UBCISSR 808	$(AG)_8T$	50	200-1400	10	0	10	100
UBCISSR 807	$(AG)_8$	50	250-1100	11	0	11	100
UBCISSR 816	$(CA)_8T$	50	200-1400	10	1	9	90
UBCISSR 840	$(GA)_8YT$	51	200-1500	12	4	8	66.7
UBCISSR 885	$BHB(GA)_7$	52	200-1500	10	2	8	80
UBCISSR 890	$HVH(GA)_7$	52	200-1000	9	1	8	88.9
Total				62	8	54	525.6
Average				10.3	1.3	9	87.6

Y = C and T. B = C, G and T. H = A, C and T. V = A, C and G.

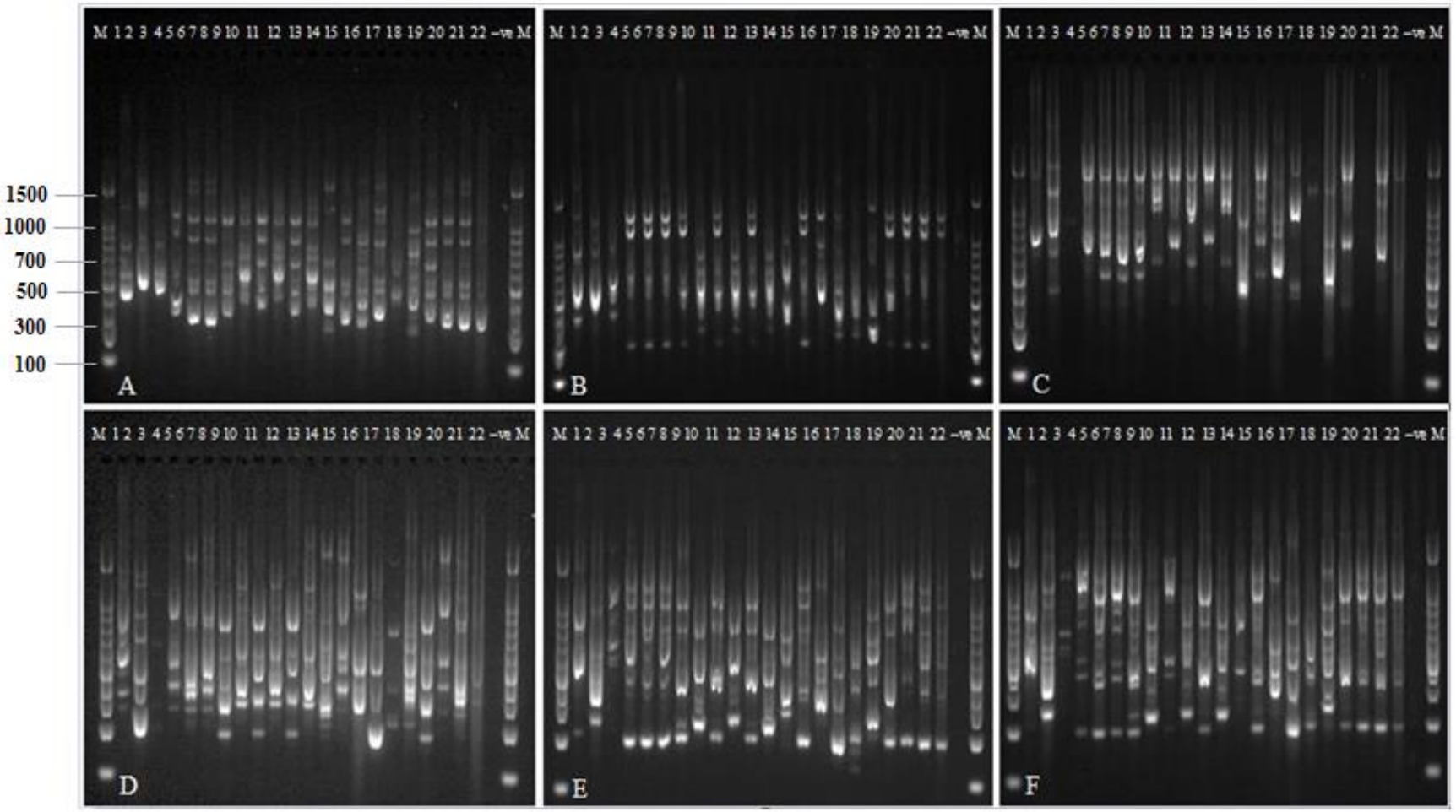

Figure 13. Gel electrophoresis documented photos for the ISSR fingerprinting amplification in 22 different fungal isolates using six different UBCISSR primers. A: primer 808, B: primer 807, C: primer 816, D: primer 840, E: primer 885 and F: primer 890. M= 100 bp ladder as a molecular size marker. –ve= represents negative control sample.

The pair-wise genetic similarity estimates, for one of the 100% polymorphism primers (*i.e.* primer 808), based on Dice similarity coefficient, for group 1 of fungal isolates (F1-F11) that used in this study are given in Table 6. The similarity coefficient ranged from 0.018 to 0.992. In which, the highest similarity (*i.e.* 0.992) was observed between fungal genotypes numbered 8 and 10. The second highest similarity (*i.e.* 0.989) was between genotypes numbered 5 and 6 and the third highest similarity value (*i.e.* 0.986) was the same between genotypes numbered 1 and 5, the two genotypes numbered 1 and 6 and genotypes 9 and 11. Meanwhile, the genetic similarity values ranged from 0.018 to 0.937 among other isolates in group 1 (Table 6).

For group 2 of fungal isolates (F12-F22), the similarity coefficient ranged from 0.001 to 0.991. Where, the highest value of similarity (*i.e.* 0.991) was recorded between genotypes numbered 12 and 18, followed by (*i.e.* 0.965) between isolate numbered 12 and 13. Also, the third highest value (*i.e.* 0.947) was between 12 and 18 samples (Table 7).

In the meantime, the genetic similarity values ranged from 0.001 to 0.913 amongst other isolates in group 2 (Table 7).

The genetic tree was demonstrated using cluster analysis by the UPGMA method through the SynGene software.

Table 6. Similarity matrix, based on Dice coefficient distance, for group 1 fungal isolates (F1-F11) based on ISSR marker variation.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**M**
1	1.000											
2	0.000	1.000										
3	0.000	0.932	1.000									
4	0.088	0.521	0.272	1.000								
5	0.986	0.000	0.000	0.084	1.000							
6	0.986	0.000	0.000	0.018	0.989	1.000						
7	0.708	0.393	0.299	0.380	0.674	0.673	1.000					
8	0.031	0.855	0.776	0.802	0.000	0.000	0.443	1.000				
9	0.637	0.545	0.499	0.472	0.611	0.608	0.959	0.598	1.000			
10	0.020	0.866	0.799	0.769	0.000	0.000	0.445	0.992	0.597	1.000		
11	0.571	0.571	0.527	0.498	0.551	0.545	0.937	0.623	0.986	0.622	1.000	
M	0.000	0.179	0.291	0.055	0.000	0.000	0.000	0.041	0.000	0.048	0.000	1.000

Table 7. Similarity matrix, based on Dice coefficient distance, for group 2 fungal isolates (F12-F22) based on ISSR marker variation.

	12	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**M**
12	1.000											
13	0.965	1.000										
14	0.913	0.887	1.000									
15	0.001	0.064	0.092	1.000								
16	0.891	0.876	0.855	0.000	1.000							
17	0.039	0.040	0.341	0.500	0.000	1.000						
18	0.991	0.947	0.827	0.076	0.730	0.067	1.000					
19	0.697	0.704	0.882	0.351	0.645	0.589	0.726	1.000				
20	0.005	0.134	0.278	0.044	0.365	0.043	0.000	0.110	1.000			
21	0.845	0.823	0.801	0.021	0.786	0.230	0.773	0.610	0.269	1.000		
22	0.007	0.088	0.169	0.161	0.236	0.077	0.014	0.045	0.863	0.307	1.000	
M	0.102	0.188	0.537	0.153	0.621	0.485	0.110	0.526	0.473	0.611	0.603	1.000

The UPGMA genetic tree for group 1 fungal samples (F1-F11) showed two main subgroups: A and B with a distance of 20 according to distance scale (Figure 14). In subgroup A, the isolate numbered 4 was clearly different from clusters I and II in a distance of 59. Cluster I composed of two isolates numbered 8 and 10 with a Dice close similarity value of 0.992 and distance of 99.2. Cluster II also consists of two isolates numbered 2 and 3 with a 0.932 value of similarity. In subgroup B, two clusters are observed, cluster III composed of three isolates numbered 1, 5 and 6 with a range of 0.989 to 0.986 coefficient value in the similarity matrix. Cluster IV contains also three fungal isolates numbered 7, 9 and 11 having a Dice similarity value range of 0.986 to 0.959 (Table 6).

In group 2 fungal samples (F12-F22), the tree revealed two main subgroups C and D with a distance of 12 according to distance scale (Figure 15). Group C consists of three clusters, where cluster I composed of five fungal isolates, numbered 12, 13, 16, 18 and 21 including the highest Dice coefficient value of 0.991 and ranged to 0.773 among other isolates in the cluster. Genotypes numbered 14 and 19 are included in cluster II with a similarity value of 0.882. Also, 0.863 value was between genotypes numbered 20 and 22 included in cluster III. Meanwhile, group D contain only two fungal isolates (numbered 15 and 17) with a distance of 50.

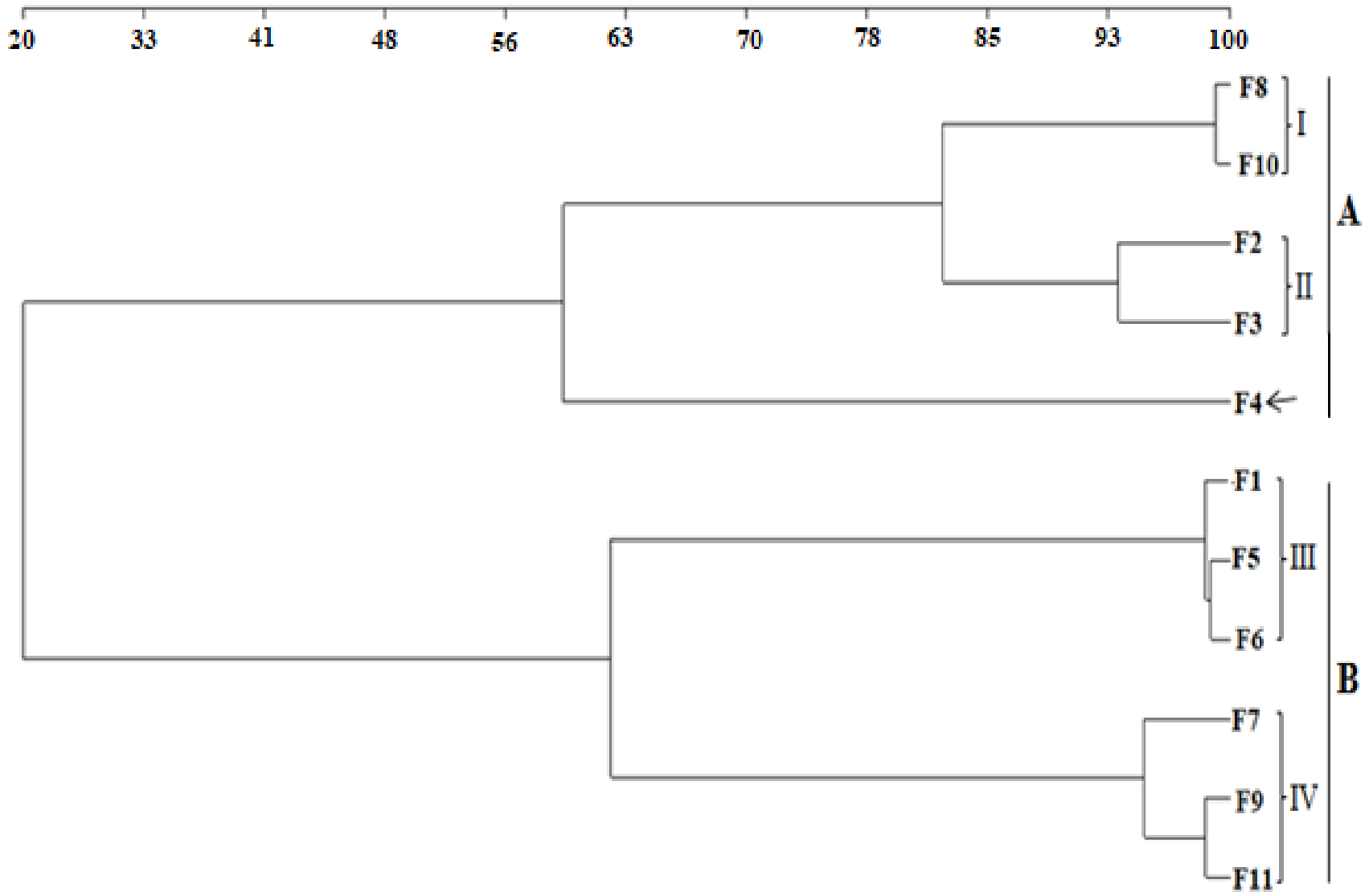

Figure 14. UPGMA tree demonstrating the genetic diversity among group 1 different fungal genotypes (F1-F11) based on ISSR markers and constructed using the SynGene software.

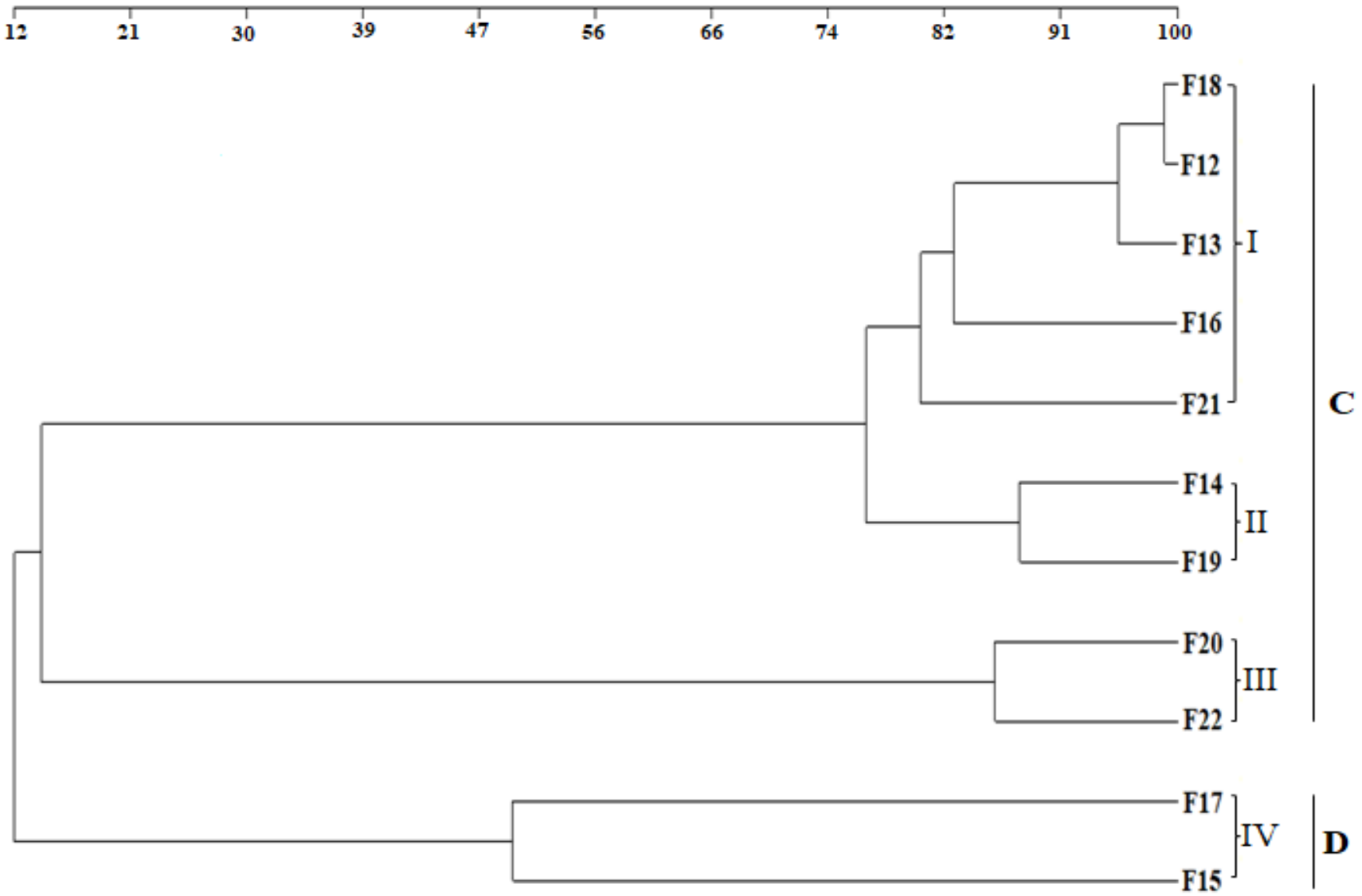

Figure 15. UPGMA tree illustrating the genetic diversity among group 2 different fungal genotypes (F12-F22) based on ISSR markers and constructed using the SynGene software.

4.1.2 Fungal ET data analysis

Evaluation of tasted metabolites by ET revealed a significant discrimination, not only on grouping levels for each day, but also between each sample in the same tested period. ET used sensors array (*i.e.* CA, JB, HA, ZZ, BB, JE and GA) showed different discrimination power during the tested period, this power value indicates the ability of each sensor to discriminate each sample from others. In group 1 fungal samples (F1-F11) the discrimination powers for the sensors array are shown in Figure 16.

A

Index	Sensors	Discrimination power
4	CA	0.304
7	JB	0.120
6	HA	0.110
1	ZZ	0.104
3	BB	0.096
2	JE	0.092
5	GA	0.028

B

Index	Sensors	Discrimination power
1	ZZ	0.999
5	GA	0.984
2	JE	0.947
3	BB	0.927
6	HA	0.833
7	JB	0.294
4	CA	0.260

Figure 16. Aatree II ET's sensors array discrimination power for group 1 fungal samples (F1-F11). A: shows the discrimination power for each sensor of PDB inoculated with fungi at the day of inoculation. B: shows the discrimination power for each sensor of PDB inoculated with fungi after 120 h of growth (day 5).

The discrimination power for each sensor according to tested PDB media at the inoculation day (Figure 16-A) ranged from 0.028 for GA sensor to 0.304

for CA in the same test, which considered as low discrimination power. Also, it shows how the discrimination power changed after 120 h of inoculation (Day 5) (Figure 16-B), that represent a very close discrimination range of 0.927 to 0.999 for four sensors (*i.e.* ZZ, GA, JE and BB) that are considered the highest discriminative sensors, followed by HA sensor with 0.833 recorded discriminative power and leaving JB and CA sensors with the lowest discrimination power for group 1 samples at the end of the fifth day.

For group 2 fungal samples (F12-F22), the discrimination power for the same sensors array are shown in Figure 17.

A

Index	Sensors	Discrimination power
2	JE	0.097
1	ZZ	0.088
3	BB	0.083
5	GA	0.080
6	HA	0.067
4	CA	0.043
7	JB	0.030

B

Index	Sensors	Discrimination power
1	ZZ	1.000
5	GA	0.998
2	JE	0.997
6	HA	0.997
3	BB	0.993
7	JB	0.982
4	CA	0.323

Figure 17. Astree II ET's sensors array discrimination power for group 2 fungal samples (F12-F22). A: shows the discrimination power for each sensor of PDB inoculated with fungi at the day of inoculation. B: shows the discrimination power for each sensor of PDB inoculated with fungi after 120 h of growth (day 5).

This figure shows different discrimination patterns at the same testing periods (day 0 to day 5 of inoculation) according to the changed of tested fungal samples. The discrimination power was lower than group 1 (F1-F11) for all sensors' reading in group 2 fungal isolates (F12-F22), the discrimination power ranged below (*i.e.* 0.1) in the day of inoculation (Figure 17-A). On the other hand, in the fifth day of inoculation (Figure 17-B) sensors readings shows the highest discriminative power value (*i.e.* 1) for sensor ZZ, followed by five sensors with a discrimination power ranged from 0.982 to 0.998, leaving CA with the lowest discrimination power (*i.e.* 0.323).

Moreover, PCA is used to analyze the data by searching for axes along which the samples are scattered, it is the first analysis performed. Where the discrimination effectiveness is assessed based upon the discrimination index, the dispersion and grouping of samples on the PCA scores plot.

The discrimination index (Di) gives the discrimination quality through an indication of the surface (non-Euclidean) between fungal samples (*i.e.* each sample is considered by the grouping of the three measurements of each fungus).

The Di value between samples is calculated according to the following equations 1 and 2. When samples are distinct, the Di is calculated according to equation 1, while when clusters overlap, the Di is calculated according to equation 2 (Alpha MOS, 2009).

$$Di = 100\,x \left[1 - \left[\frac{\Sigma\ group\ surface}{Total\ surface}\right]\right] \ldots\ldots\ldots (1)$$

$$Di = -(\Sigma\ Intersection\ Surface/Total\ surface)\ x\ 100 \ldots\ldots\ldots (2)$$

The discrimination is confirmed if each fungal three reading are grouped together to form one sample or cluster, and if there is no intersection between various fungal clusters. In which, the Di indicate how distanced each fungal cluster from the other. However, when there is a large variability within a cluster, the cluster will be spread and it will decrease the Di value. So, even if the discrimination is improved, the Di value will be still low (Alpha MOS, 2009).

Moreover, the Di value can be positive or negative, where the positive value indicates that there is no intersection between fungal samples, the tested samples are different from each other and these samples have the greatest distance between each other. While, the negative value indicates the intersection and overlapping between fungal samples or clusters, the tested

clusters are similar to each other and these clusters have the smallest distance between each other. Furthermore, the maximum positive Di value is 100 revealing that fungal clusters are completely distant from each other. But, there is no minimum value for the negative Di indication (Alpha MOS, 2009).

In this study, the evaluation of the Di revealed a significant difference between the centers of gravity and dispersion of each cluster. Where, this index changed in each tested day following the change in PDB media according to fungal consumption of the media for their growth or the release of secondary metabolites for their survival. In which, a linear unsupervised pattern of recognition technique (*i.e.* PCA) was used to help showing the change in the Di from the day of inoculation to the fifth day of fungal growth. This PCA had the ability to present groups' clusters following each day, where two principal components (PC1 and PC2) were able to show approximately 100% of the data variation (the sum of both PCs) of the generated data as shown in Figure 18 to Figure 23.

Furthermore, during the day of inoculation (D0) all samples grouped together with a Di of -1820 (Figure 18). Then fungal clusters stared to separate with slightly Di change to -55 after 24 h of inoculation (D1) (Figure 19). After the second day of inoculation until the fifth day, the Di value

continue rising up ranging from 91 to 95 as shown in Figures 20, 21, 22 and 23 indicating a well separated and clustered fungal groups.

For group 2 of fungal samples (F12 to F22), the PCA and the change in the Di value of each measured day are shown in appendix (A) Figures 43- 48.

Where, the first Di value at the day of inoculation was -1519 revealing that all samples have the same properties (Figure 43). Fungal samples started to be separated and discriminated but with negative Di value, that continued after 24 h and 48 h of fungal growth as -28 and -13, respectively (Figure 44 and Figure 45). Then, after 72 h of growth each sample has different properties that was indicated by the positive Di value that continued until the 120 h of growth (*i.e.* 96) (Figure 48).

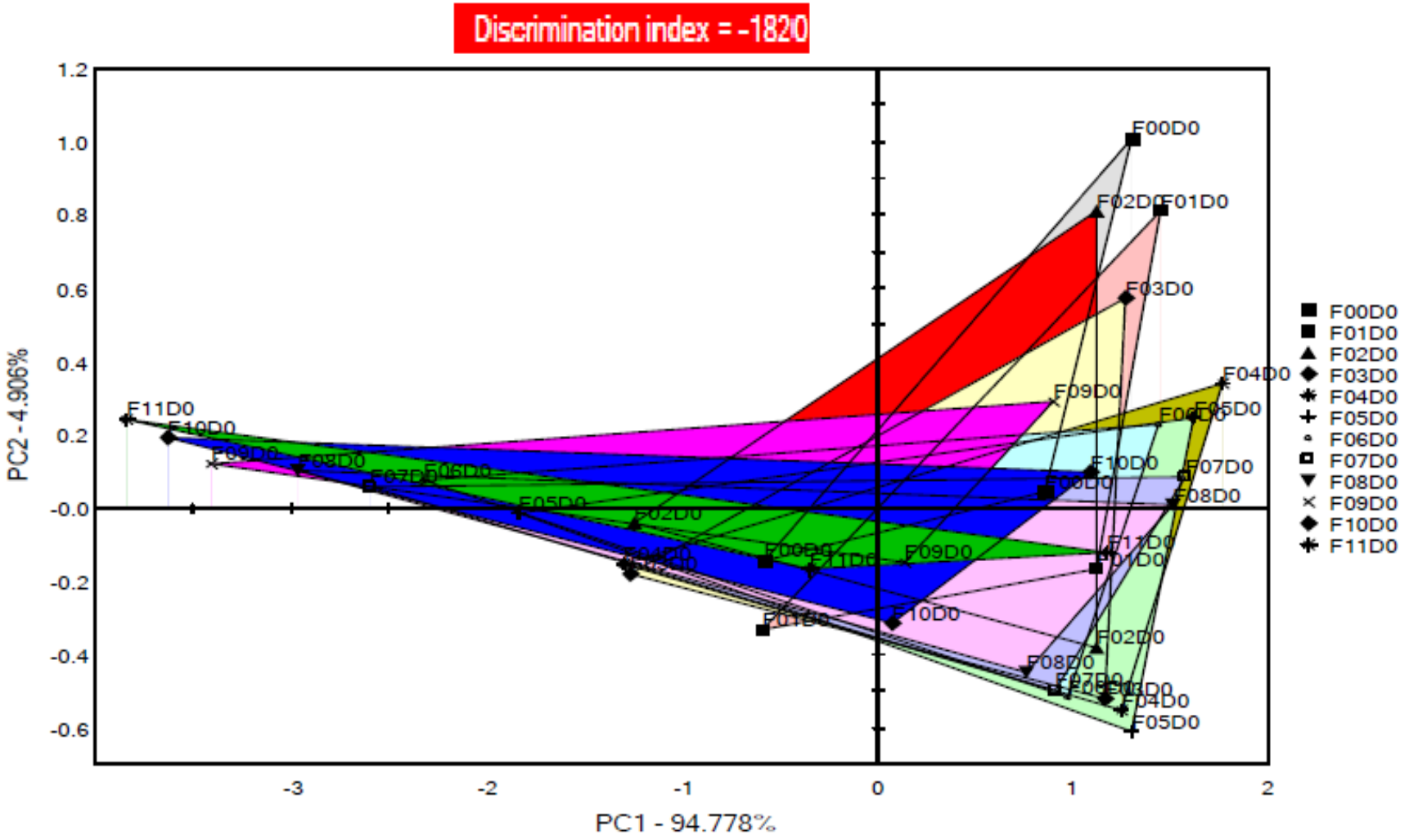

Figure 18. PCA scores plot of group 1 fungal samples (F1- F11 with F0 as control) on the day of inoculation (D0), showing no discrimination with an index value of -1820 between samples. PC1 and PC2 explain about 100% of the total variation.

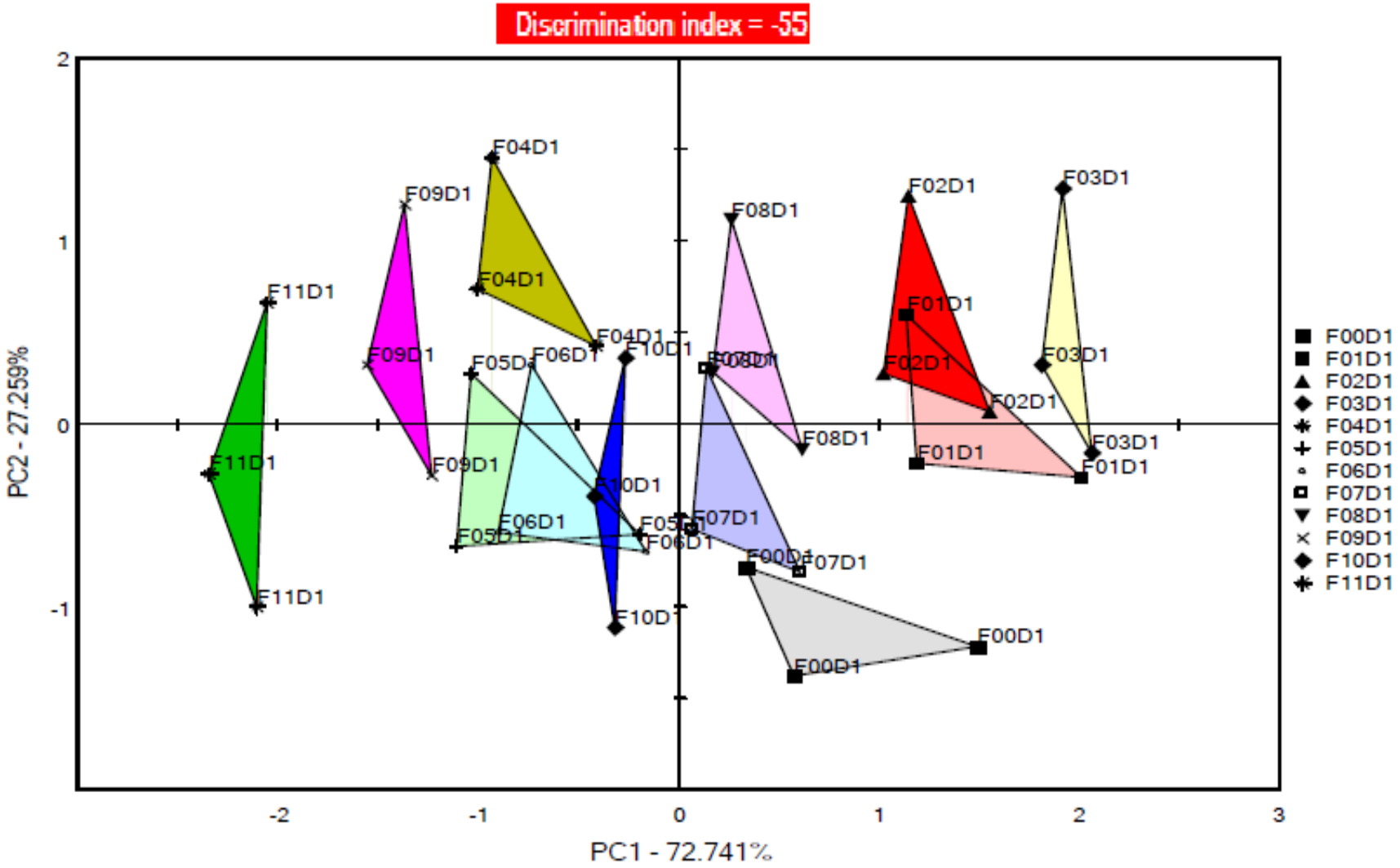

Figure 19. PCA scores plot of group 1 fungal samples (F1- F11 with F0 as control) after 24 h of inoculation (D1), showing changed discrimination index value to -55 between samples. PC1 and PC2 explain 100% of the total variation.

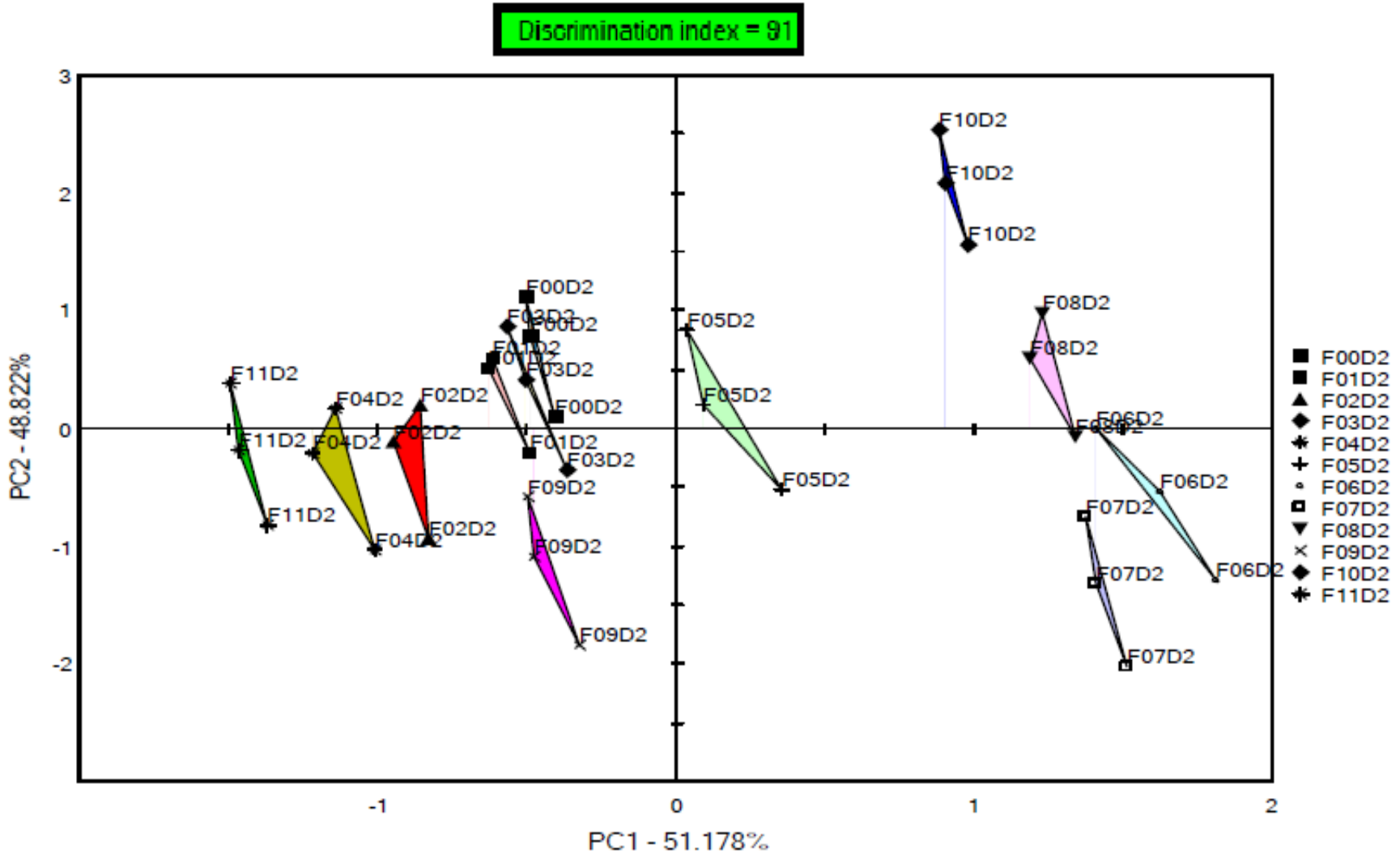

Figure 20. PCA scores plot of group 1 fungal samples (F1- F11 with F0 as control) after 48 h of inoculation (D2), showing a high discrimination index value of 91 between samples, that starts to be clearly separated. PC1 and PC2 explain 100% of the total variation.

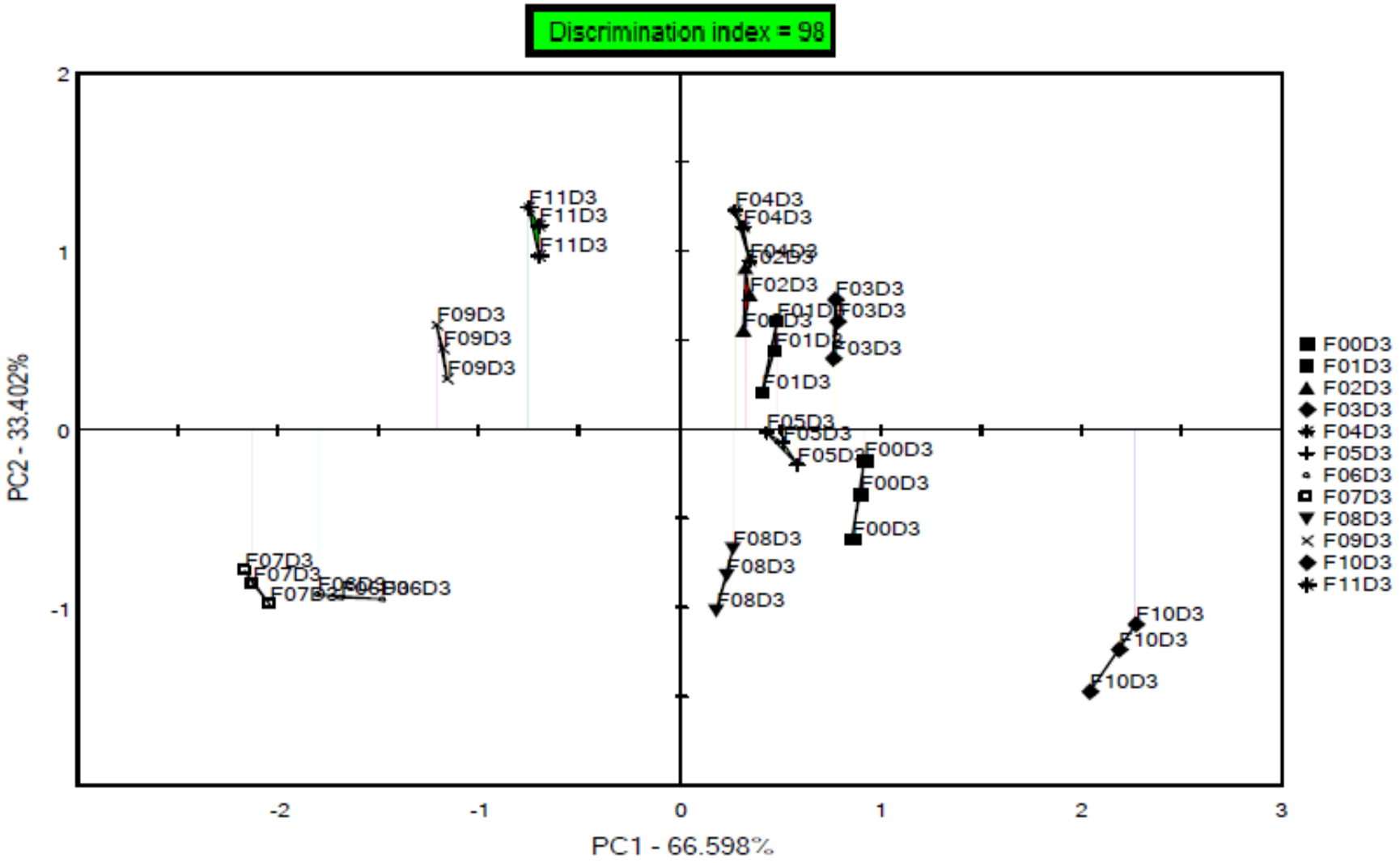

Figure 21. PCA scores plot of group 1 fungal samples (F1- F11 with F0 as control) after 72 h of inoculation (D3), showing a high discrimination index value of 98 between samples. PC1 and PC2 explain 100% of the total variation.

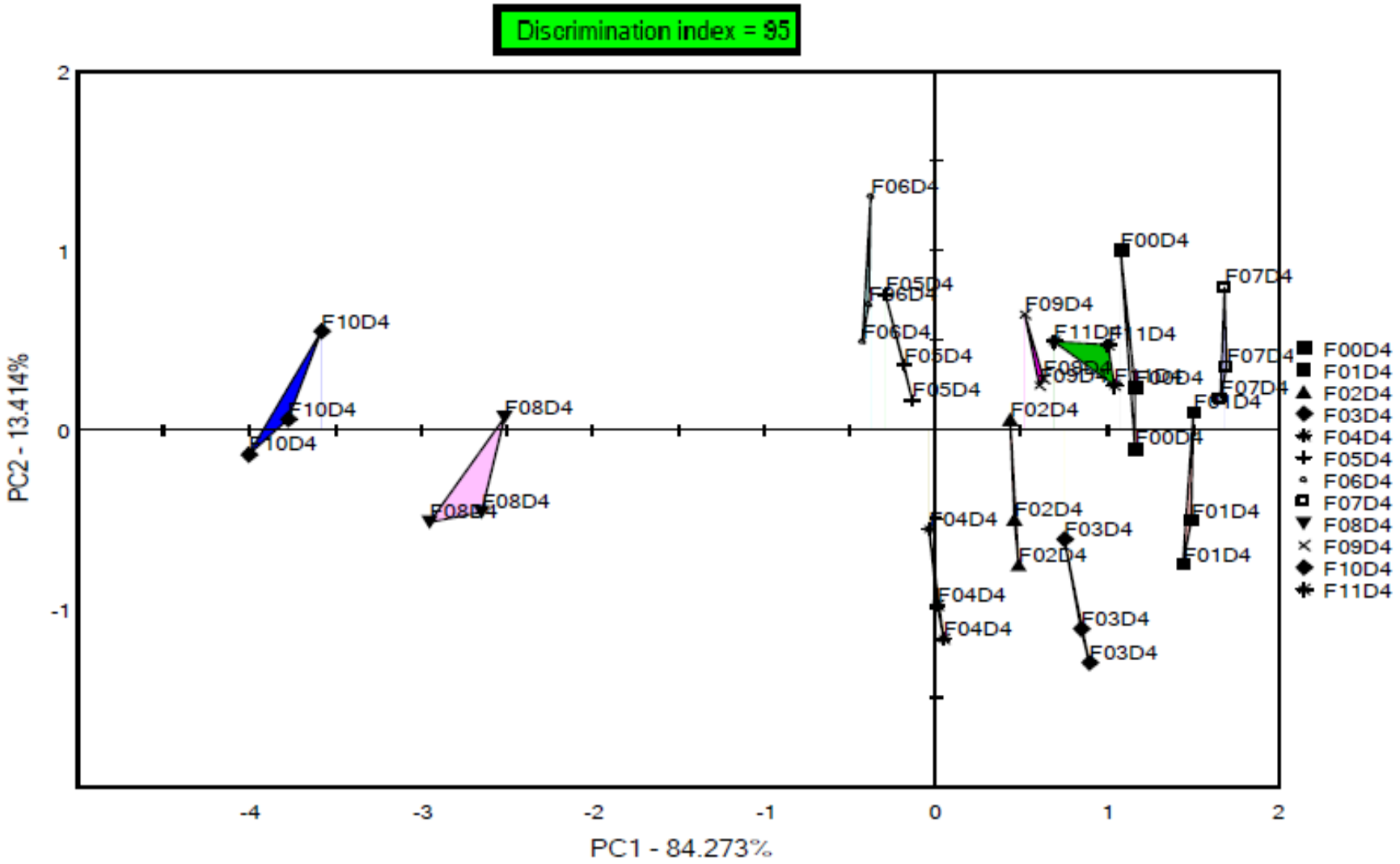

Figure 22. PCA scores plot of group 1 fungal samples (F1- F11 with F0 as control) after 96 h of inoculation (D4), showing a high discrimination index value of 95 between samples. PC1 and PC2 explain about 100% of the total variation.

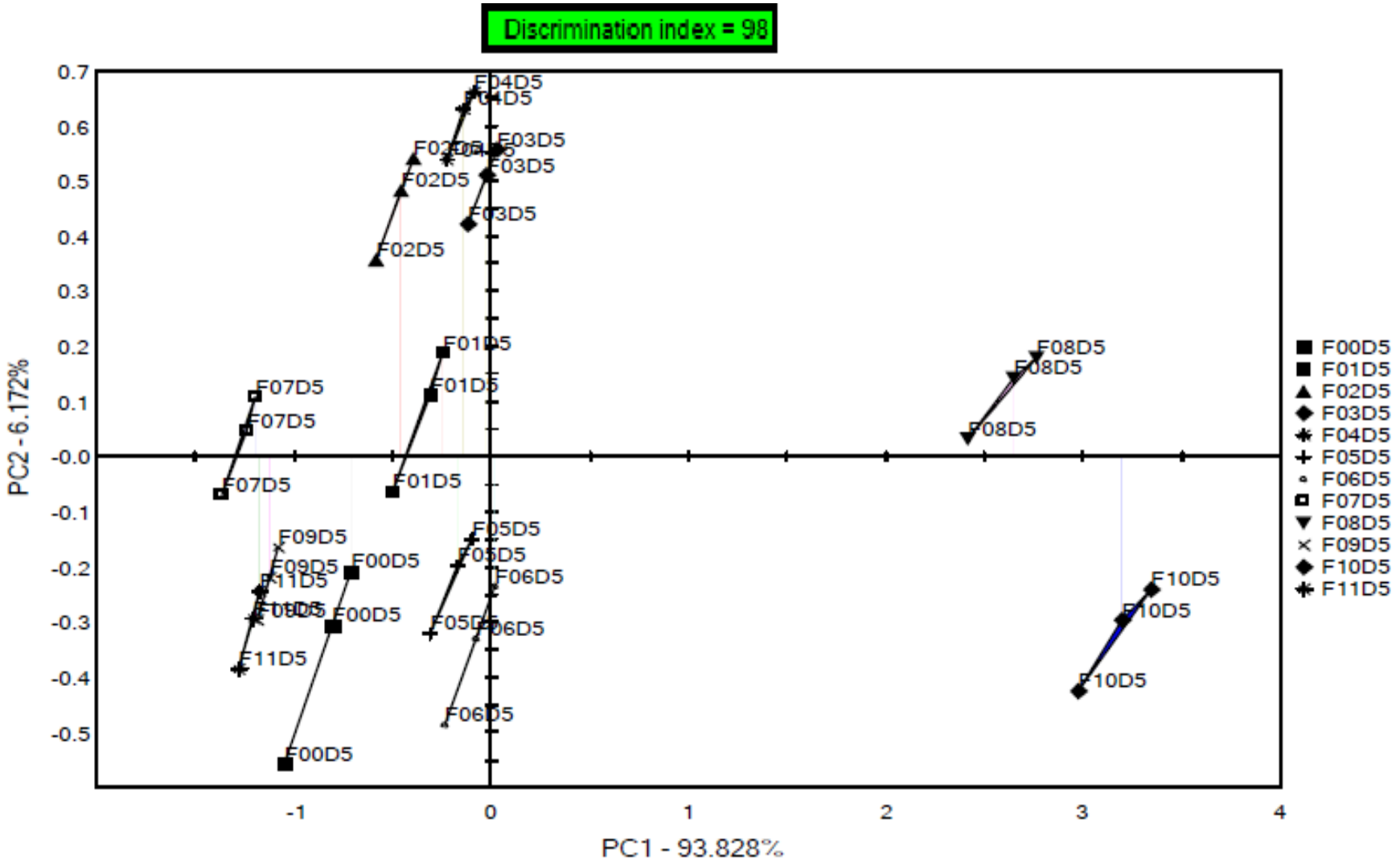

Figure 23. PCA scores plot of group 1 fungal samples (F1- F11 with F0 as control) after 120 h of inoculation (D5), showing a high discrimination index value of 98 between groups. PC1 and PC2 explain 100% of the total variation.

ET not only discriminates and cluster data or samples in the same measured day, it exceeds that to discriminate between samples in different individual day. PCA scores plot can follow the grouping between different tested days, in order to identify were they overlapped (*i.e.* stop changing). So far in group 1 fungal samples (F1-F11), day's discrimination started to overlap after 96 h of inoculation (D4 and D5) (Figure 24), which means that groups clustering stabilized and can be used for further analysis and evaluations.

In group 2 fungal samples (F12-F22), days' overlapping started after 48 h inoculation period and continued till the 120 h of inoculation (D2- D5) (Figure 25). This indicates that the differences between fungal samples in group two are clear.

According to previous analysis, PDB media with fungal cultures having at least 96 h of growth rate can be suitable candidate for group clustering directly without being tested each day. In this study, D5 collected data for both fungal groups 1 and 2 were used to build a table with the similarity distance test (*i.e.* Euclidian test) between the centroids of the defined clusters and generate the corresponding clustering (Figure 23 and Figure 48). The group distance is a practical means of evaluating the similarity or difference between two groups (Alpha MOS, 2009).

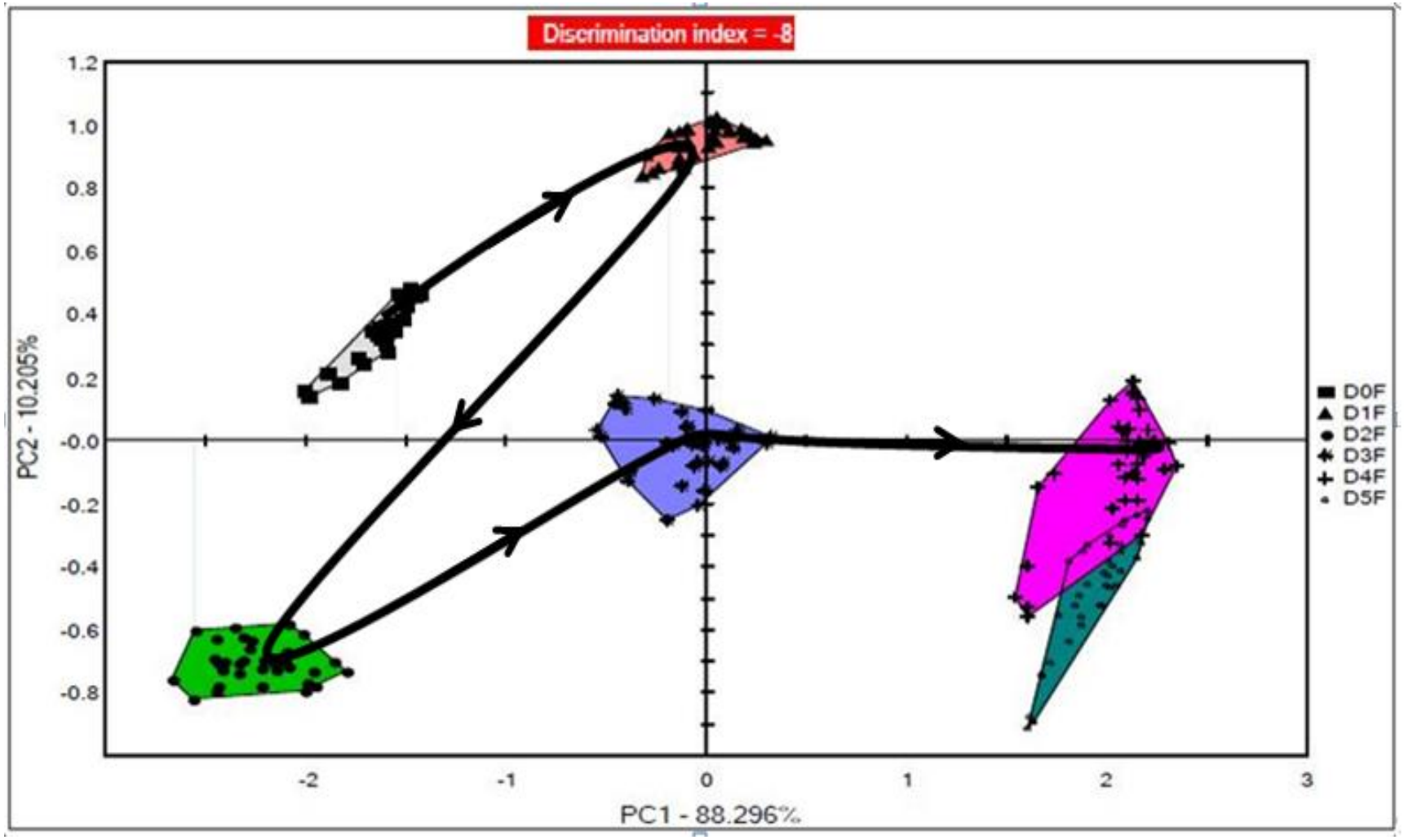

Figure 24. PCA scores plot for gathered data according to tested day for group 1 of fungal samples (F1-F11). Showing each tested day grouping and day's overlapping after 96 h of fungal inoculation. PC1 and PC2 explain about 100% of the total variation.

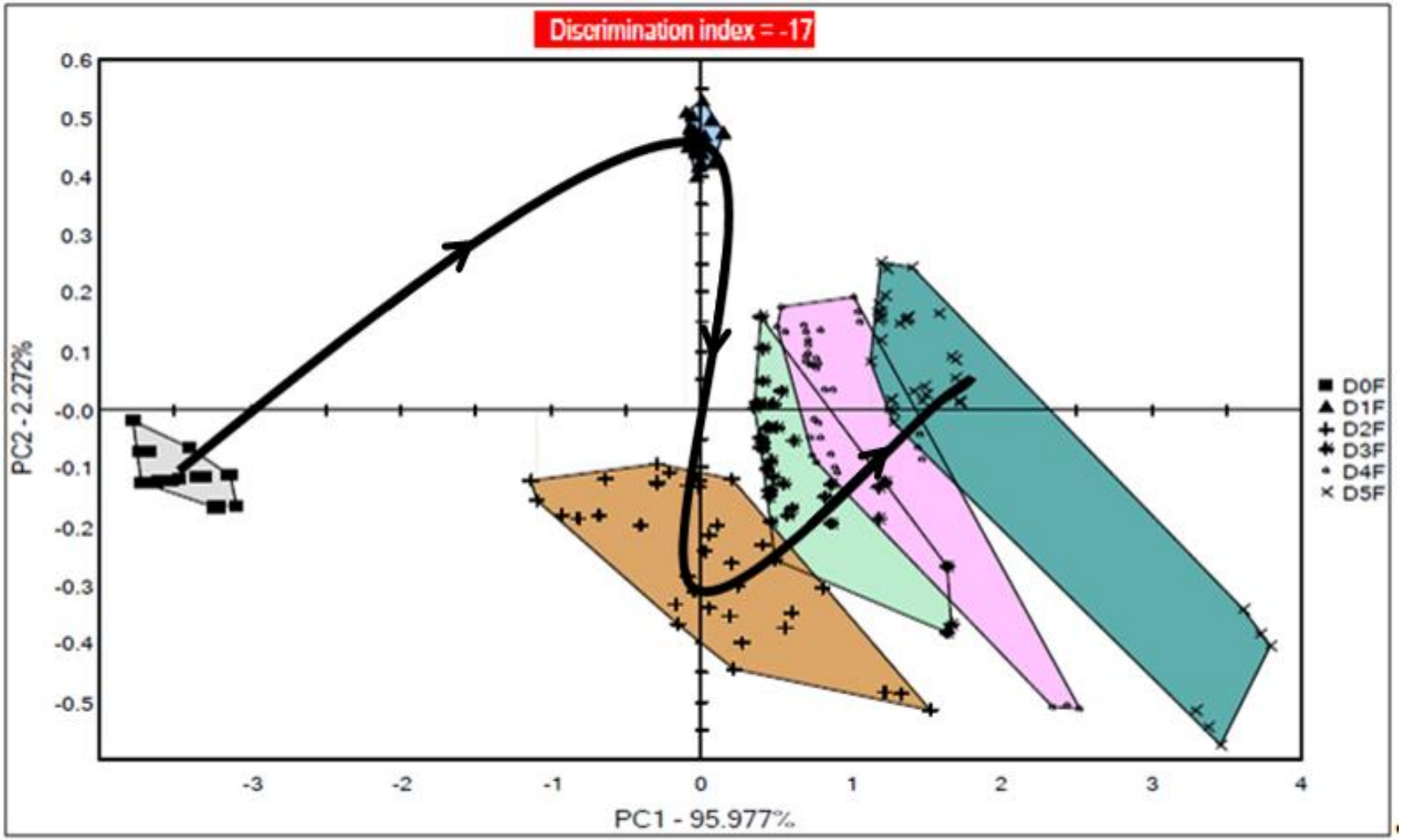

Figure 25. PCA scores plot for gathered data according to tested day for group 2 of fungal samples (F12-F22). Showing each tested day grouping and day's overlapping after 48 h of fungal inoculation. PC1 and PC2 explain almost 100% the total variation.

Table 8 shows the results of similarity distance test for group1 of fungal samples (F1-F11) to evaluate the difference and the similarity among different samples. The test describes three parameters: group Euclidian distance, probability value (P-value) and pattern discrimination index (%). In which, the Euclidian distance between two clusters is a convenient way to assess the similarity between them. Where, the greater the Euclidian distance amongst the center of gravity of each cluster, the better the differences between groups are. But, this value does not take into account groups' dispersion. Where, this dispersion happens due to inappropriate selection of sensors, poor reproducibility, sensor failure to discriminate the samples and the presence of too wide sample to sample variability. As a result, if two groups are widely dispersed and have a relatively large distance among their center of gravity, they don't consider significantly different. Moreover, the P-value is a good means for evaluating the discrimination of the compared clusters. Wherein, the smallest the P-value is (near to 0), the higher the probability that groups are discriminated. This P-value calculation is based on multivariate analysis of variance algorithm technique (MANOVA), this technique is used for assessing group differences across multiple non-metric dependent variables simultaneously.

In a conclusion, as pattern index finger-prints indicator takes into account

the difference between the centers of gravity and also the dispersion of each cluster. It is wise to look first at the index of discrimination, then the distance and P value. Where, P value can be only considered as an alarm value which helps to determine whether the reproducibility and/or discrimination between groups are correct or not. It may also demonstrate if the sensors selection were good enough (Alpha MOS, 2009).

In general, in this study the following similarity distance test table shows the pattern discrimination index (%) (two by two). In which, each sample (as a product sample) was compared to another one (as a reference sample). This pattern discrimination index ranged for group 1 of fungal samples (F1-F11) on day 5, from 27.18% between F05 and F06 to 99.64% between F10 and F11, revealing the highest and lowest similar fungal samples, respectively (Table 8). Where, the P-value generally decreased as the discrimination value increased except in few samples due to previously mentioned reasons. Also, the distance between two groups increased as the discrimination between them increased, except in few samples and that is according to sample dispersion that was mentioned before. The first four fungal combinations of F05-F06, F09-F11, F03-F04 and F01-F05 were the closest to each other with a low discrimination index ranging between 27.18% to 61.79%, then samples started to be well discriminated (Table 8).

Table 8. Similarity test table for group 1 of fungal samples (F1-F11), presenting Euclidian distance, P-value and pattern discrimination index between fungal samples.

Product names	Reference samples	Distances	P Value	Pattern discrimination index (%)
F05D5	F06D5	0.16	9.82	27.18
F09D5	F11D5	0.12	13.65	42.45
F03D5	F04D5	0.16	6.26	50.87
F01D5	F05D5	0.34	5.14	61.79
F01D5	F02D5	0.40	2.82	69.90
F07D5	F09D5	0.29	3.67	73.72
F01D5	F06D5	0.50	1.97	74.08
F02D5	F04D5	0.36	9.47	78.11
F07D5	F11D5	0.34	4.24	78.45
F01D5	F03D5	0.52	11.14	82.40
F08D5	F10D5	0.71	2.38	82.72
F02D5	F03D5	0.44	1.45	83.97
F01D5	F04D5	0.57	5.63	84.92
F02D5	F05D5	0.74	1.91	91.52
F02D5	F06D5	0.90	0.53	92.37
F03D5	F06D5	0.85	0.36	92.83
F03D5	F05D5	0.74	2.43	93.13
F01D5	F11D5	0.95	4.88	94.12
F04D5	F06D5	0.96	1.50	94.44
F04D5	F05D5	0.83	2.09	94.71
F02D5	F07D5	0.90	2.94	94.79
F06D5	F09D5	1.04	1.47	95.44
F06D5	F11D5	1.13	1.59	95.96
F06D5	F07D5	1.23	0.53	96.00
F05D5	F07D5	1.10	1.90	96.22
F05D5	F11D5	1.03	3.65	96.55
F03D5	F09D5	1.31	3.49	98.71
F06D5	F08D5	2.74	4.74	98.81
F03D5	F11D5	1.43	3.49	98.84
F01D5	F08D5	2.96	3.39	98.94
F05D5	F08D5	2.83	3.27	99.07
F06D5	F10D5	3.27	2.68	99.07
F03D5	F08D5	2.67	2.33	99.15
F01D5	F10D5	3.55	2.11	99.18
F04D5	F08D5	2.80	1.98	99.24
F05D5	F10D5	3.37	1.63	99.26
F03D5	F10D5	3.31	1.37	99.35
F02D5	F10D5	3.73	1.54	99.41
F07D5	F08D5	3.88	1.49	99.54
F07D5	F10D5	4.45	1.61	99.60
F09D5	F10D5	4.30	1.77	99.64
F10D5	F11D5	4.39	1.89	99.64

Table 9 lists similarity distance test ordered values for compared group 2 fungal samples (F12-F22) on the fifth day. In which, the pattern discrimination index (%) among samples in this group was relatively larger than it was among samples in group 1. Where, the lowest value started with 70.52% between F14 and F21, and the highest value ended with 99.99% between F13 and F15, revealing the dissimilarity between fungal isolates in this group.

The pattern discrimination index was less than 90% only between F14 and F16 related to F21. Although, the distance between samples having 99% discrimination index and more was less in range (*i.e.* 0.61-3.95) compared to group 1 distance (*i.e.* 2.83-4.39) (Table 8), the discrimination pattern was larger and ranged from 99% among F13 and F18 to 99.99% among F13 and F15. This phenomenon is due to the large variety of fungal genotypes in this group that in contrast revealed low dispersion and high Di power of sensors array. The P-value generally decreased as the discrimination value increased except in few samples (Table 9) due to previously mentioned reasons.

Table 9. Similarity test table for group 2 of fungal samples (F12-F22), presenting Euclidian distance, P-value and pattern discrimination index between fungal samples.

Product names	Reference samples	Distances	P Value	Pattern discrimination index (%)
F14D5	F21D5	0.26	20.52	70.52
F16D5	F21D5	0.37	18.21	79.82
F12D5	F18D5	0.20	9.54	90.80
F12D5	F16D5	0.43	15.16	93.90
F12D5	F21D5	0.68	3.53	94.80
F18D5	F21D5	0.73	7.35	95.13
F20D5	F21D5	0.89	5.15	95.56
F14D5	F20D5	0.63	4.65	96.21
F14D5	F16D5	0.61	4.16	96.30
F13D5	F16D5	0.73	5.31	97.86
F14D5	F19D5	0.45	4.99	97.95
F16D5	F20D5	1.21	8.06	98.45
F12D5	F13D5	0.41	2.78	98.58
F13D5	F18D5	0.61	7.25	99.00
F14D5	F18D5	0.98	4.55	99.36
F18D5	F20D5	1.60	2.56	99.42
F21D5	F22D5	2.27	3.98	99.45
F12D5	F20D5	1.57	0.78	99.45
F12D5	F14D5	0.94	2.13	99.46
F13D5	F20D5	1.64	2.16	99.51
F17D5	F21D5	2.32	0.62	99.53
F16D5	F17D5	2.07	1.97	99.73
F15D5	F21D5	3.30	0.73	99.77
F17D5	F20D5	2.64	0.25	99.81
F15D5	F20D5	3.10	0.57	99.86
F12D5	F19D5	1.28	2.38	99.87
F15D5	F16D5	3.22	1.37	99.88
F13D5	F19D5	1.46	1.64	99.91
F14D5	F17D5	2.40	0.53	99.93
F15D5	F17D5	1.80	1.81	99.94
F14D5	F15D5	3.23	0.61	99.96
F12D5	F17D5	2.28	0.45	99.96
F17D5	F22D5	4.25	1.36	99.97
F15D5	F19D5	2.84	0.68	99.97
F13D5	F17D5	2.68	0.56	99.97
F17D5	F19D5	2.21	0.55	99.97
F15D5	F18D5	3.42	1.59	99.97
F15D5	F22D5	5.53	1.24	99.98
F12D5	F15D5	3.58	0.75	99.98
F13D5	F15D5	3.95	0.58	99.99

The following PCA scores plots Figure 26 and Figure 27 of the fifth day's data for both fungal groups group 1 and group 2 show the relative clustering of fungal samples as it genetically revealed previously by the UPGMA tree in Figure 14 and Figure 15, respectively. In which, the grouping and clustering of PCA and the genetic tree have almost the same pattern rhythm for different fungal isolates.

In group 1 fungal samples (F1-F11) (Figure 26), it has both subgroups A and B as the UPGMA tree clustering in Figure 14. Where subgroup A with its two clusters I and II, having fungal samples F8 and F10 in cluster I and F2, F3 and F4 included in cluster II. And subgroup B with F6, F5 and F1 included in cluster III, and cluster IV consists of F7, F9 and F11.

Meanwhile, group 2 fungal samples (F12-F22) (Figure 27) has group C and D as the UPGMA tree clustering in Figure 15. Where, group D has only F15 and F17 included in cluster IV which are distinct to each other and from other fungal samples. However, group C has the majority of fungal samples categorized in clusters I, II and III. In which, the largest and closest fungal samples in this group are gathered in cluster I (*i.e.* F12, F13, F16, F18 and F21), followed by cluster II having the second closest fungal isolates (*i.e.* F14 and F19), leaving cluster III with relatively close F20 and F22.

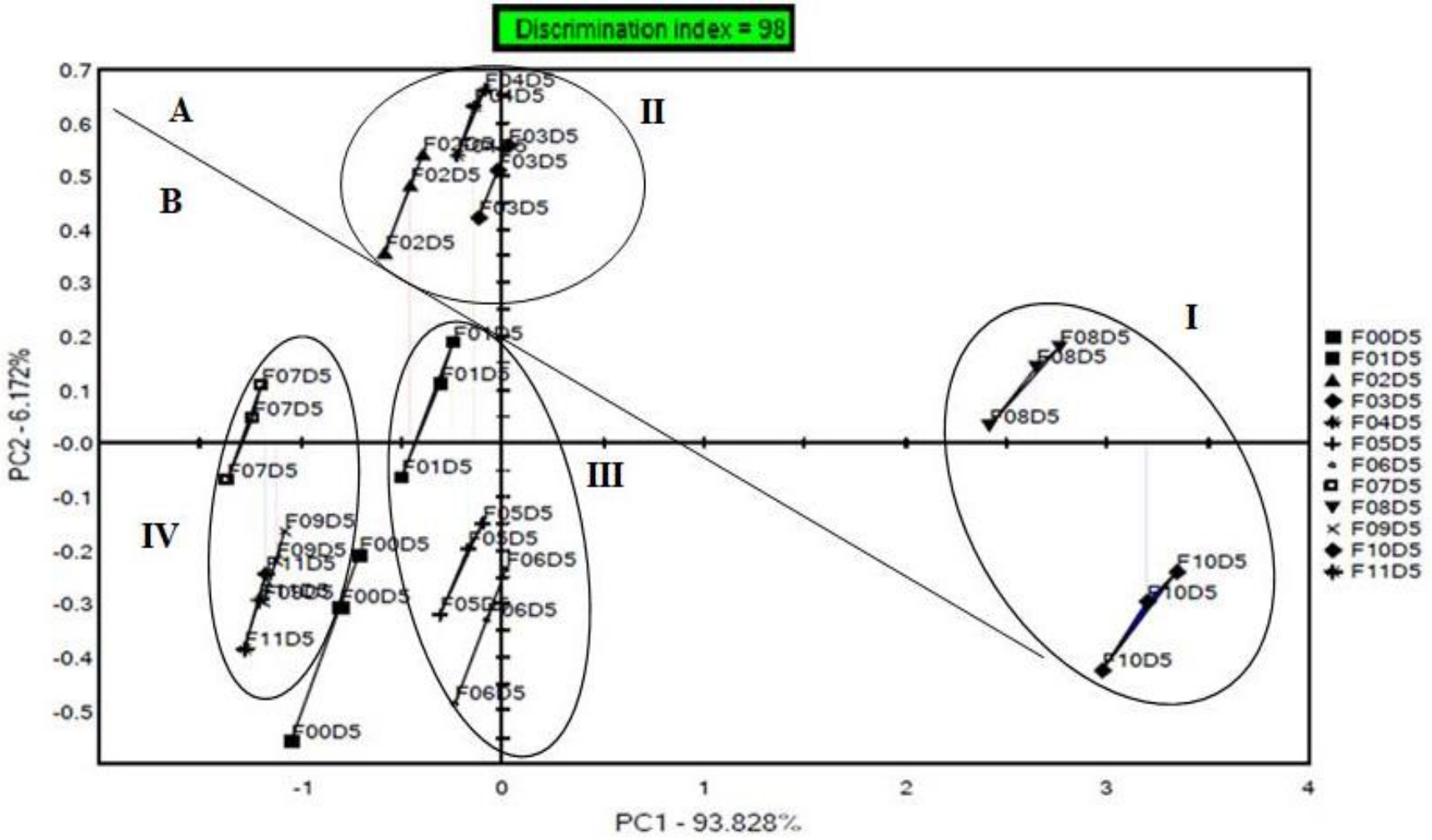

Figure 26. PCA scores plot showing clustering rhythm of group 1 fungal samples (F1-F11) according to UPGMA dendogram (Figure 14). It has a very close clustering similarity.

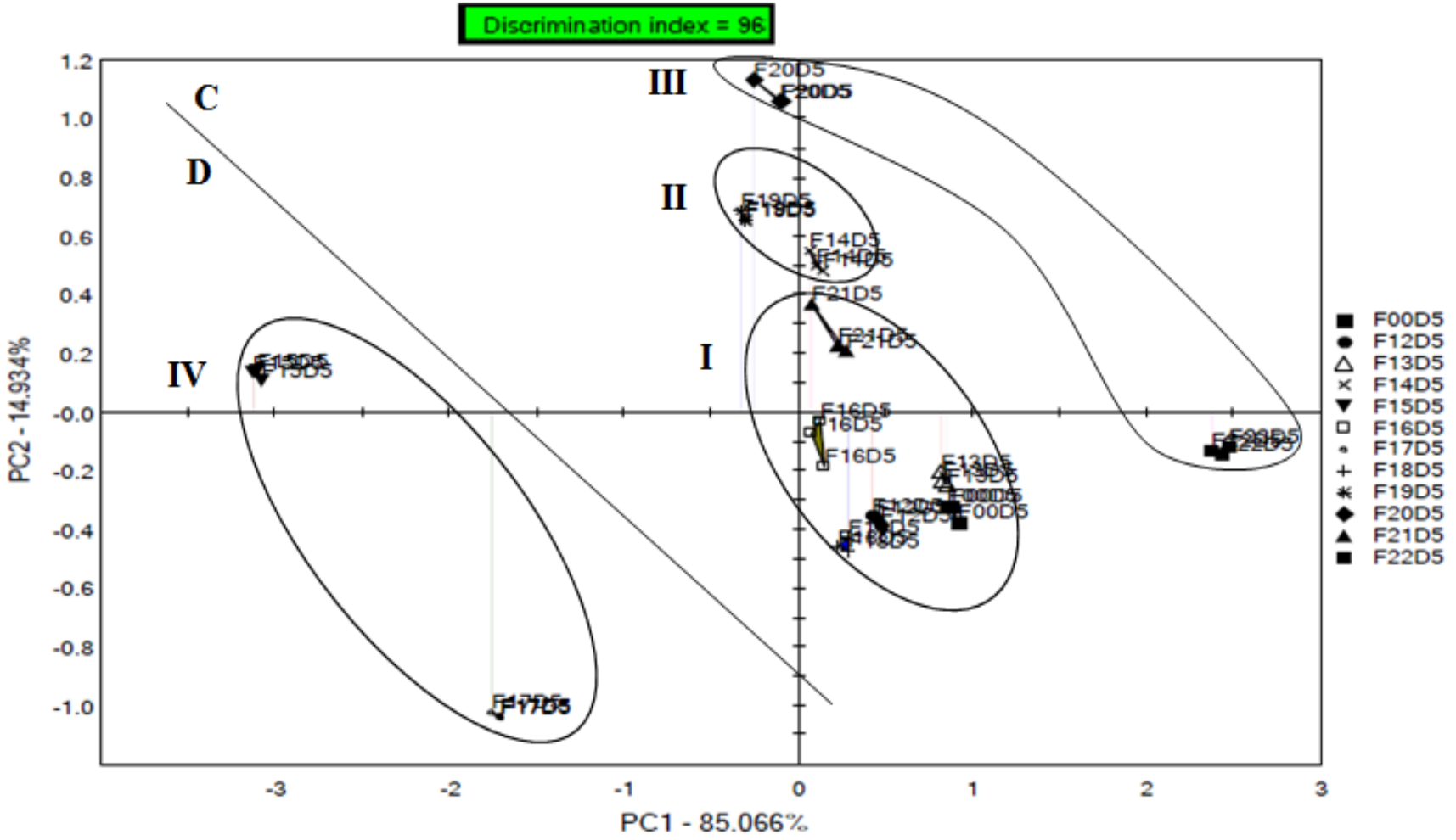

Figure 27. PCA scores plot showing clustering rhythm of group 2 fungal samples (F12-F22) according to UPGMA dendogram (Figure 15). It has a very close clustering similarity.

4.2 Bacterial experiment results

4.2.1 Bacterial DNA data analysis

Total DNA extraction of 22 different bacteria using TRI reagent method are shown in Figure 28, which was grouped into two groups according to ET's auto-sampler's capacity (Table 2).

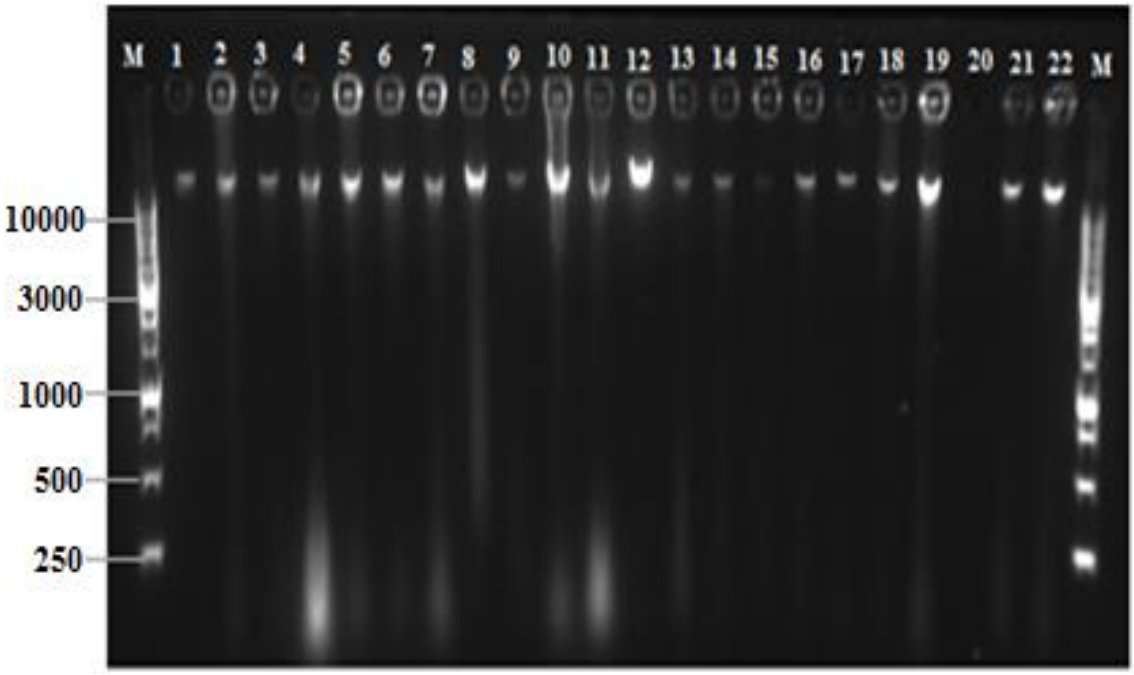

Figure 28. Gel electrophoresis documented photo of total DNA isolated from 22 different bacterial isolates using TRI reagent method for genomic isolation. Lanes from 1-22 represents bacterial isolates from 1-22. M= 1 Kbp ladder as a molecular size marker.

The four used combinations of universal 16S rRNA coded bacterial primers (Table 3) showed well identifiable bands in Figure 29 A-D.

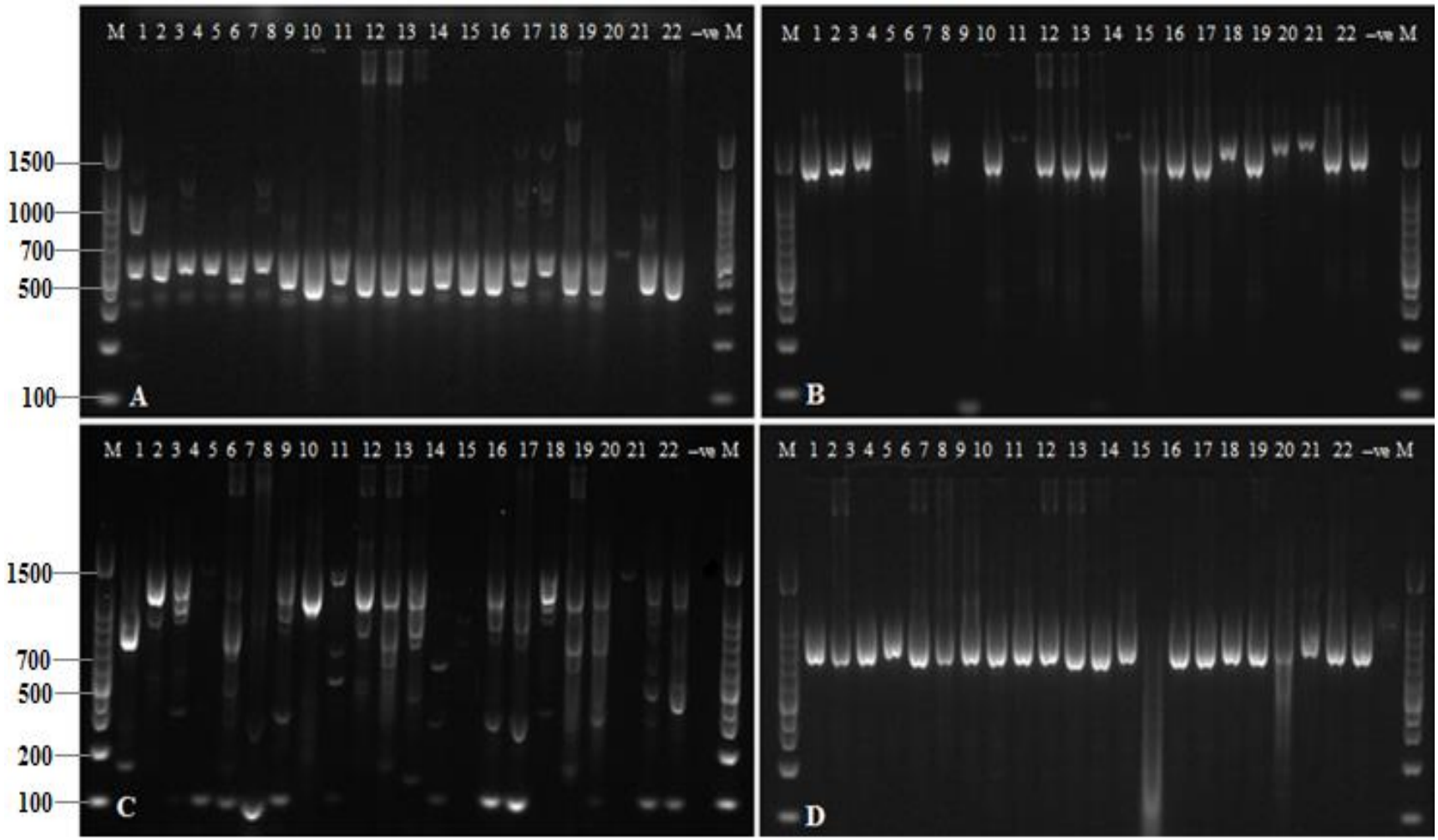

Figure 29. Gel electrophoresis documented photos for 16S rRNA amplification in 22 different bacterial isolates using four different 16S rRNA universal primers' combinations. A: primers 341F and 805R, B: primers 27F and 1492R, C: primers 341F and 1492R and D: primers 27F and 805R. M= 100 bp ladder as a molecular size marker. –ve= represents negative control sample.

Table 10. The 16S rRNA bands' profiles scores of 22 different bacterial genotypes using 16S rRNA3 coded primer for each marker. Where, 1= present band and 0= absent band.

Primer	**Sample No.**																					
16S rRNA3 (bp)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1010	0	1	1	0	1	0	0	0	1	0	1	0	0	1	0	0	1	0	1	1	1	1
950	0	0	1	1	0	0	1	1	0	1	1	1	0	0	1	1	0	0	0	0	1	0
900	0	1	0	0	1	0	1	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0
780	1	0	0	0	0	1	0	0	0	1	1	1	0	0	1	1	0	0	1	0	1	1
710	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0
650	0	1	1	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0
550	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	1	1	1	0	0
510	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0
430	0	0	1	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0
400	0	0	0	1	1	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0
350	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
300	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	1	0	1	0
250	0	0	0	1	1	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0
150	1	0	0	0	0	1	0	0	0	0	1	1	0	0	1	1	0	0	1	0	0	1
100	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0
Total bands	2	3	5	5	6	3	4	2	4	3	7	6	3	3	5	5	4	4	5	3	7	3

The primer coded 16S rRNA3 (with forward 341F and reversed 1492R primers combination) in Figure 29 C resulted in polymorphic profiles that were used for UPGMA data analysis.

The numbers of scored bands produced by this primer were 15, including 11 polymorphic markers and only 4 monomorphic markers, scoring a percentage of 73.33% polymorphism, which indicated a high level of genetic variation among the examined bacterial genotypes (Table 11).

The photo illustrating bands fingerprinting of the selected 16S rRNA3 primers for the 22 bacterial genotypes is shown in Figure 29 (C). Where the largest produced fragment was approximately 200 bp and the smallest recognized produced fragment was approximately 1.10 Kbp (Table 11).

Table 11. Base sequence of the 16S rRNA3 coded primer which produced polymorphic finger-printing in 22 different bacterial genotypes, number of total alleles, number of amplified monomorphic, polymorphic bands as well as the percentage of polymorphism.

Primer code	Annealing temperature (℃)	Size range (bp)	Total No. of alleles	No. of Monomorphic bands	No. of Polymorphic bands	% Polymorphism
16S RNA3	50	200-1010	15	4	11	73.33

The pair-wise genetic similarity estimates, for 16S rRNA3 profiles based on Dice similarity coefficient for group 1 of bacterial isolates (B1-B11), are given in Table 12. The similarity coefficient ranged from 0.032 to 0.999. The highest similarity range of 0.999-0.995 was observed between bacterial genotypes numbered 3, 4, 5, 6 and 7. The second highest similarity (*i.e.* 0.990) was between genotypes numbered 9 and 10. While third highest value (*i.e.* 0.987) was between the two genotypes numbered 2 and 10 and the lowest one (*i.e.* 0.032) was between isolates 1 and 9 (Table 10).

For group 2 of bacterial isolates (B12-B22) (Table 13), the similarity coefficient ranged from 0 to 1. Where the highest value of similarity (*i.e.* 1) was recorded between genotypes numbered 13 and 22, followed by a range of 0.999- 0.995 between isolate numbered 13, 15, 16, 17, 19, 21 and 22. The third highest value of 0.988 was between 15 and 17 numbered samples. The other genetic similarity values ranged from 0 to 0.913 among other isolates in this group.

Table 12. Similarity matrix, based on Dice coefficient distance, for group 1 bacterial isolates (B1-B11) based on 16S rRNA3 marker variation

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**M**
1	1.000											
2	0.058	1.000										
3	0.444	0.547	1.000									
4	0.233	0.893	0.996	1.000								
5	0.195	0.957	0.996	0.999	1.000							
6	0.421	0.573	0.998	0.998	0.999	1.000						
7	0.214	0.936	0.995	0.998	0.997	0.997	1.000					
8	0.056	0.351	0.092	0.204	0.251	0.098	0.197	1.000				
9	0.032	0.980	0.474	0.783	0.897	0.491	0.841	0.541	1.000			
10	0.143	0.987	0.872	0.993	0.891	0.908	0.993	0.232	0.990	1.000		
11	0.208	0.948	0.983	0.980	0.981	0.982	0.985	0.154	0.868	0.978	1.000	
M	0.113	0.461	0.478	0.482	0.479	0.479	0.481	0.008	0.400	0.486	0.472	1.000

Table 13. Similarity matrix, based on Dice coefficient distance, for group 2 bacterial isolates (B12-B22) based on 16S rRNA3 marker variation.

	12	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**M**
12	1.000											
13	0.000	1.000										
14	0.000	0.909	1.000									
15	0.000	0.998	0.986	1.000								
16	0.058	0.999	0.985	0.999	1.000							
17	0.000	0.996	0.889	0.988	0.999	1.000						
18	0.000	0.968	0.946	0.970	0.971	0.972	1.000					
19	0.000	0.998	0.959	0.999	0.999	0.998	0.969	1.000				
20	0.048	0.483	0.314	0.382	0.586	0.725	0.603	0.635	1.000			
21	0.000	0.995	0.843	0.954	0.996	0.995	0.963	0.996	0.803	1.000		
22	0.000	1.000	0.869	0.974	0.999	0.997	0.968	0.998	0.776	0.998	1.000	
M	0.000	0.137	0.328	0.465	0.047	0.118	0.000	0.383	0.086	0.087	0.632	1.000

The cluster analysis using UPGMA method, for group 1 bacterial isolates (B1-B11) (Figure 30) showed two main subgroups A and B with a distance of 19.5 according to distance scale, which indicates the dissimilarity between the two groups.

In subgroup A, the isolate numbered 8 (included in cluster III) was clearly different from cluster I and II in a distance of 22. Meanwhile, cluster I and II are closer to each other in a branch distance of 84. Cluster I composed of six isolates, where five of them (samples numbered 3, 4, 5, 6 and 7) with the closest Dice similarity value around 0.999 and distance of 99. In which, the six sample numbered 11 was similar to the above five grouped samples in a distance of 98. In cluster II there are three isolates (numbered 2, 9 and 10) with a 0.999 coefficient value of similarity between 9 and 10 ranged to 0.98 with the branched numbered 2 (Table 12). Group B composed of only one leaf presenting isolate numbered 1 that was different from all other tested isolates (Figure 30).

In group 2 bacterial isolates (B12-B22), the UPGMA genetic tree also revealed two main subgroups C and D (Figure 31). The subgroup D consists of only one leaf sample (numbered B12) with a similarity coefficient range of 0.048-0.058 (Table 13) that indicates the heterogeneity between this sample and all other isolates.

Subgroup C has the majority of bacterial isolates that were clustered into two branched clusters I and II in a distance of approximately 58 according to the distance scale. Where, cluster II composed of only one leaf of genotype numbered B20. On the other hand, cluster I composed of all 9 other bacterial genotypes that were tested in this subgroup. This cluster is divided to four enter groups labeled a, b, c and d (Figure 31). In which, group a contained four isolates with the highest similarity distance of 100 between samples B13 and B22 and a distance of 99 between the other two samples in this group (samples numbered 17 and 21). Group b branched from group a at a distance of 98 and it is composed of three bacterial isolates (numbered 15, 16 and 19) with the same high Dice coefficient value of 0.999 (Table 13). Genotype coded B18, that is included in group c, is at a similarity distance of 97 with the other groups (a and b). While, genotype numbered B14 among cluster I, and grouped as d, is far from all other groups at a distance branch of approximately 94 according to similarity distance scale (Figure 31).

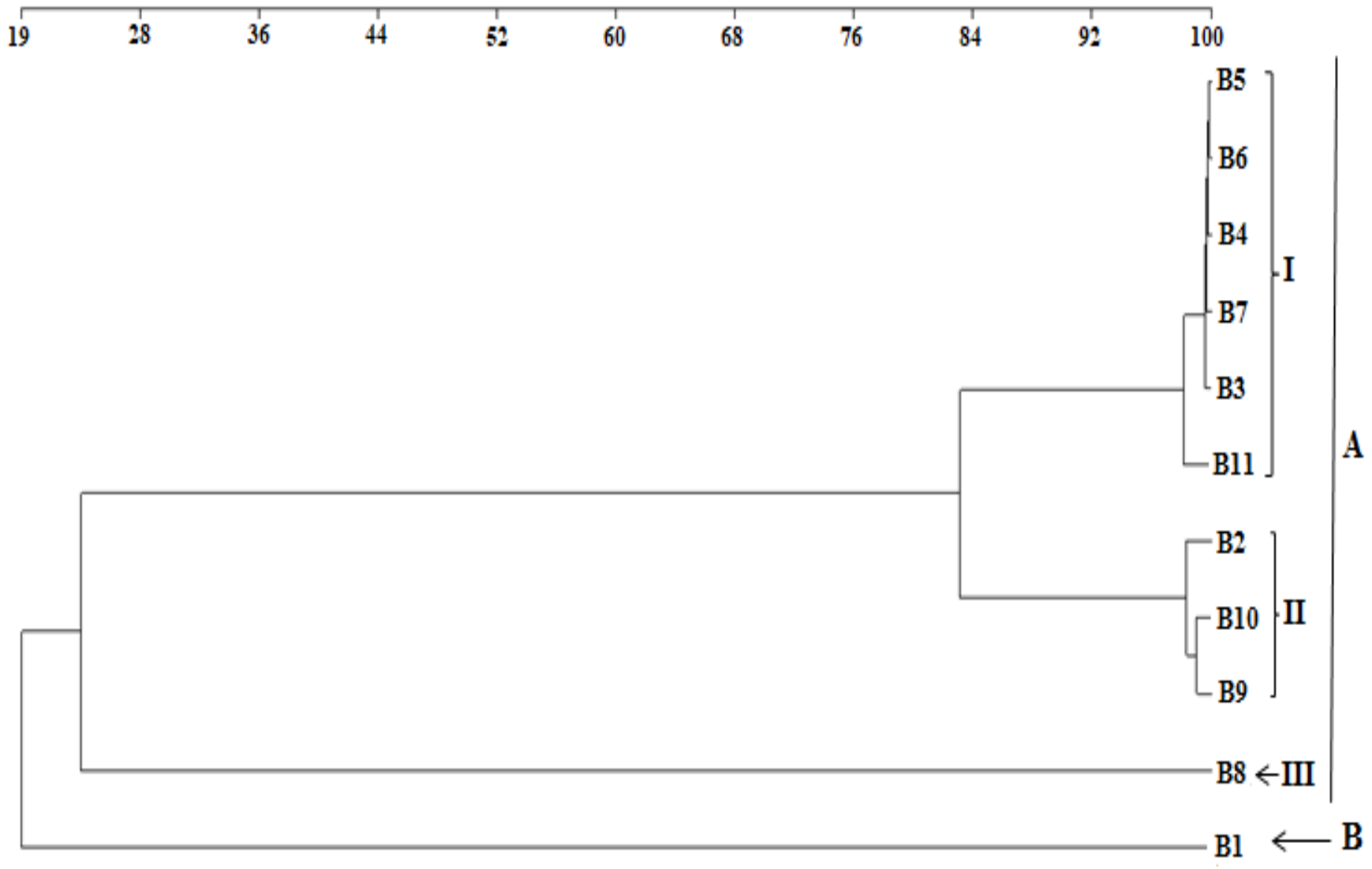

Figure 30. UPGMA tree illustrating the genetic diversity among group 1 bacterial genotypes (B1-B11) based on 16S rRNA3 markers and constructed using the SYNGENE software.

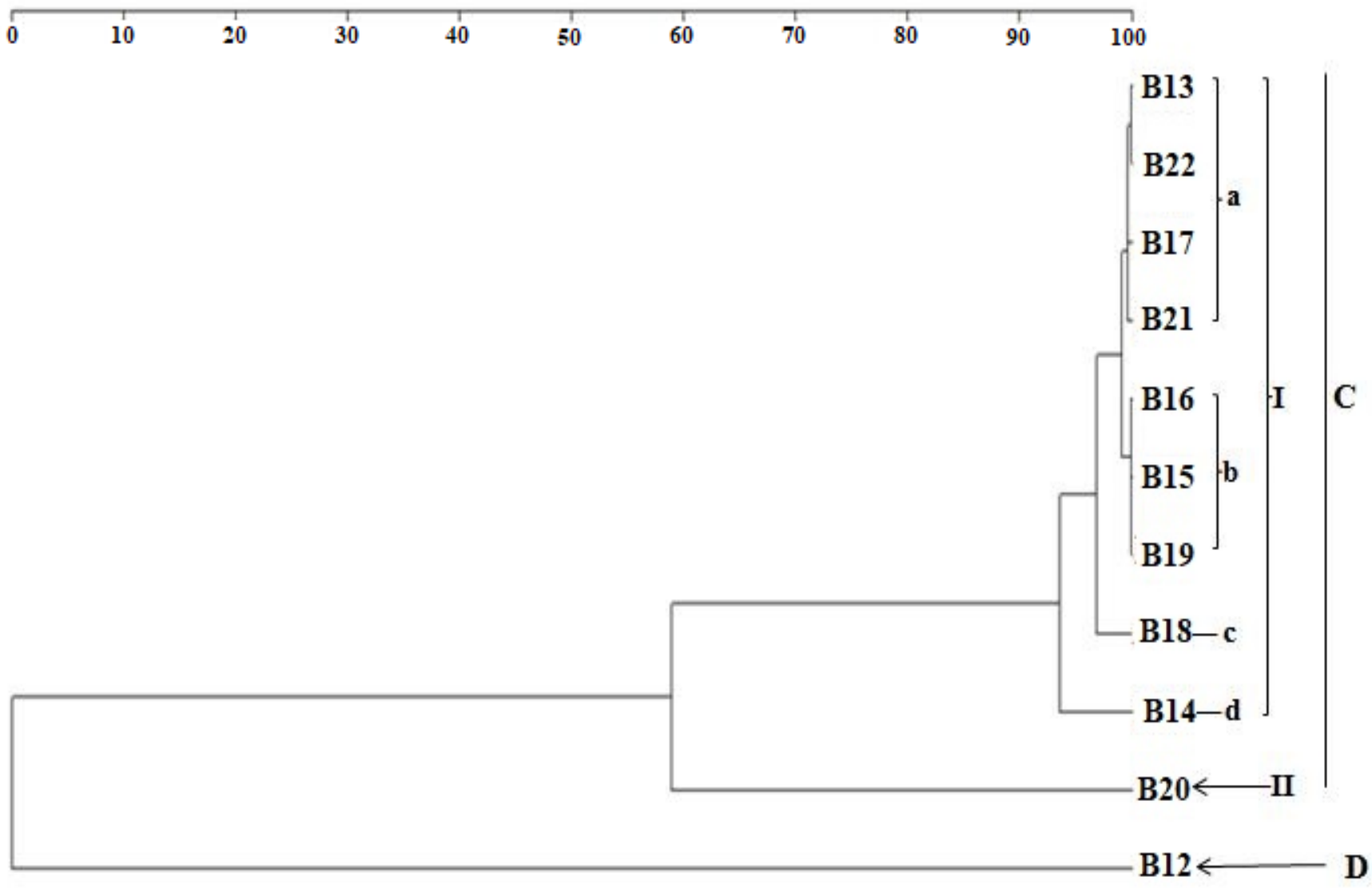

Figure 31. UPGMA tree illustrating the genetic diversity among group 2 bacterial genotypes (B12-B22) based on 16S rRNA3 markers and constructed using the SYNGENE software.

4.2.2 Bacterial ET data analysis

Evaluation of bacterial metabolites by ET revealed a significant discrimination on both grouping levels for each day and between each sample in the same tested period.

The discrimination powers for each sensor for group 1 bacterial samples (B1-B11) are shown in Figure 32.

A

Index	Sensors	Discrimination power
7	JB	0.584
4	CA	0.111
3	BB	0.096
5	GA	0.091
1	ZZ	0.076
2	JE	0.049
6	HA	0.032

B

Index	Sensors	Discrimination power
1	ZZ	0.985
5	GA	0.960
3	BB	0.894
2	JE	0.733
7	JB	0.573
4	CA	0.239
6	HA	0.052

Figure 32. Astree II ETs sensory array discrimination power for group 1 bacterial samples (B1-B11). A: shows the discrimination power for each sensor of NB media inoculated with bacteria at the day of inoculation. B: shows the discrimination power for each sensor of NB after 96 h of bacterial growth.

In which, the discrimination power for this group according to tasted NB media at the inoculation day (Figure 32- A) ranged from 0.032 for HA

sensor to 0.584 for JB in the same test day, which considered as a low power. Also, it shows how the discrimination power changed after 96 h of inoculation (Day 4) (Figure 32- B), that represent a very close discrimination range of 0.985 to 0.960 for two sensors (ZZ and GA) that are considered the highest discriminative sensors, followed by BB sensor with 0.894 recorded power and leaving HA with the lowest power of 0.052 for at the end of the fourth day (Figure 32).

In the meantime, the discrimination powers for each sensor for group 1 bacterial samples (B12-B22) are shown in Figure 33.

A

Index	Sensors	Discrimination power
4	CA	0.151
3	BB	0.137
5	GA	0.126
1	ZZ	0.096
2	JE	0.079
7	JB	0.069
6	HA	0.044

B

Index	Sensors	Discrimination power
1	ZZ	0.999
3	BB	0.988
5	GA	0.869
4	CA	0.683
7	JB	0.676
2	JE	0.501
6	HA	0.103

Figure 33. Astree II ETs sensory array discrimination power for group 2 bacterial samples (B12-B22). A: shows the discrimination power for each sensor of NB media inoculated with bacteria at the day of inoculation. B: shows the discrimination power for each sensor of NB after 96 h of bacterial growth.

Where, the discrimination power ranged from 0.044 for HA to 0.151 for in the day of inoculation (Figure 33- A). The discrimination power values changed after 96 h of inoculation (Figure 33- B) sensors readings ranged from 0.999 for ZZ sensor to 0.96 for BB sensors to be recorded with the highest power, followed by GA with 0.869 value and the lowest discriminative sensor was HA with 0.103 power value at the end of 96 h of inoculation.

Moreover, the evaluation of the discrimination index (Di) revealed a significant difference between the centers of gravity and dispersion of each subgroup. Where, this index changed each tested day following the change in NB media according to bacterial consumption of the media for their growth or the release of secondary metabolites for their survival. In which, PCA was used to show the change in the discrimination index from the day of inoculation to the fourth day of bacterial growth. This PCA had the ability to present groups' clusters following each day, where two principal components (PC1 and PC2) were able to show approximately 100% of the data variation (the sum of both PCs) of the generated data as shown in Figure 34 to Figure 38.

Furthermore, during the day of inoculation (D0) all samples grouped together with a Di of -456 (Figure 34). Then bacterial groups stared to

separate with slightly Di change to -19 after 24 h of inoculation (D1) (Figure 35). Also, the Di slightly changed during passed 48 and 72 h with a value of -7 and -0.5, respectively. This might be due to low bacterial growth rate of this group isolates. In which, the Di value changed to have a high discriminative power after 96 h of bacterial growth to have a positive value of 95 (Figure 38).

For group 2 of bacterial samples (B12- B22), the PCA and the change in the Di value of each measured day are shown in appendix (B) Figures 49- 53. In which, in this group of bacterial isolates Di value was only negative with a value of -291in the day of inoculation (D0) (Figure 49) indicating the closeness of these samples to each other and to the control NB sample. Where, after 24 h of bacterial growth the Di value between different bacterial isolates changed into high positive value of 95 (Figure 50) that remain constant after 48, 72 and 96 h of bacterial growth as shown in Figures 51, 52 and 53, respectively.

Moreover, it can be observed how the two bacterial groups have different Di value patterns according to the types of bacterial genotypes in each group and their relation to each other. As group 1 bacterial samples have slightly changed over tested periods, group 2 bacterial samples showed high Di change after only 24 h of inoculation (*i.e.* 95) (Figure 50) that remain

constant till the fourth day of growth (Figure 53). Where, This indicates the discriminative variety of bacterial isolates in this group.

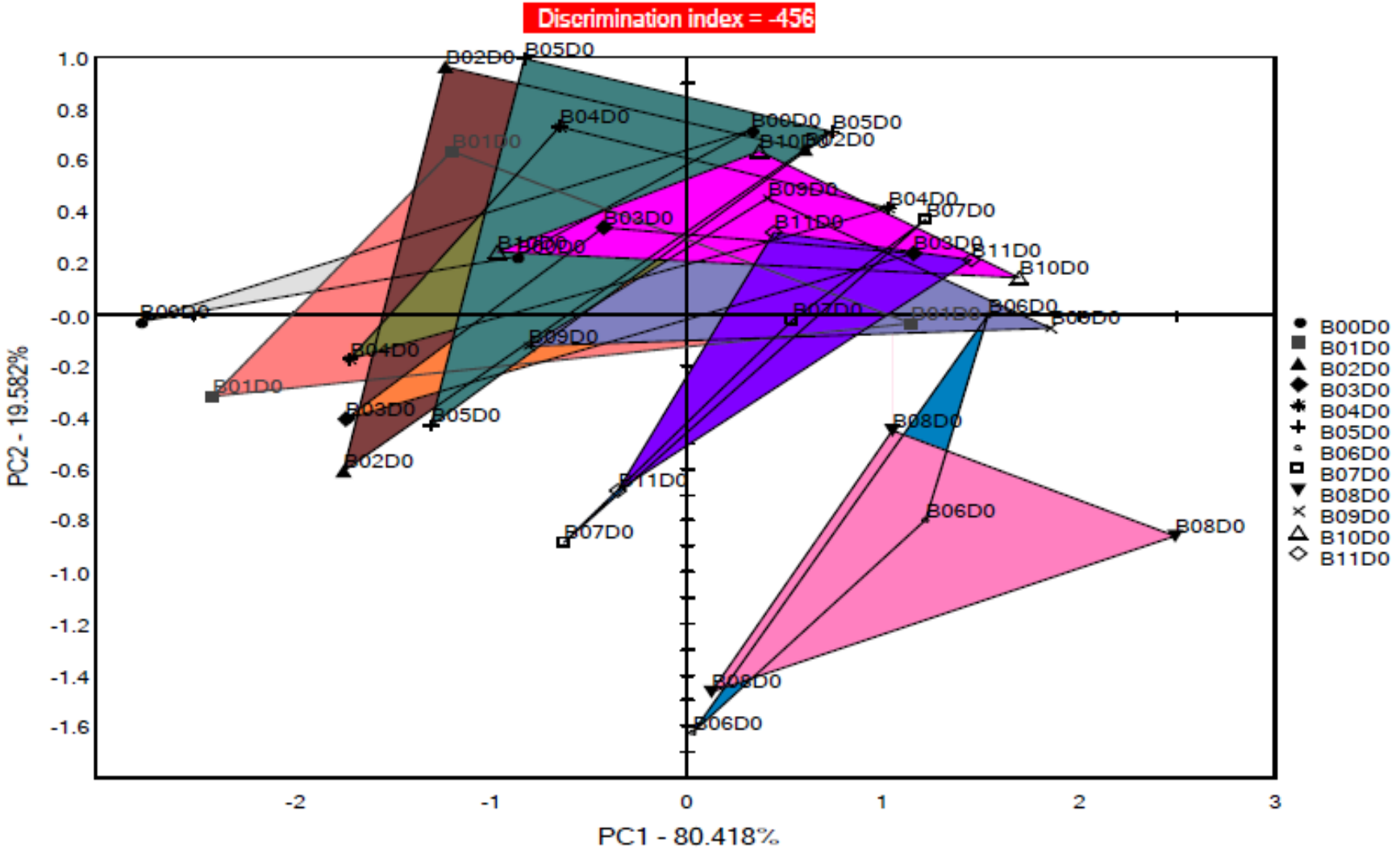

Figure 34. PCA scores plot of group 1 bacterial samples (B1- B11 with B0 as control) on the day of inoculation (D0), showing no discrimination with an index value of -456 between samples. PC1 and PC2 explain 100% of the total variation.

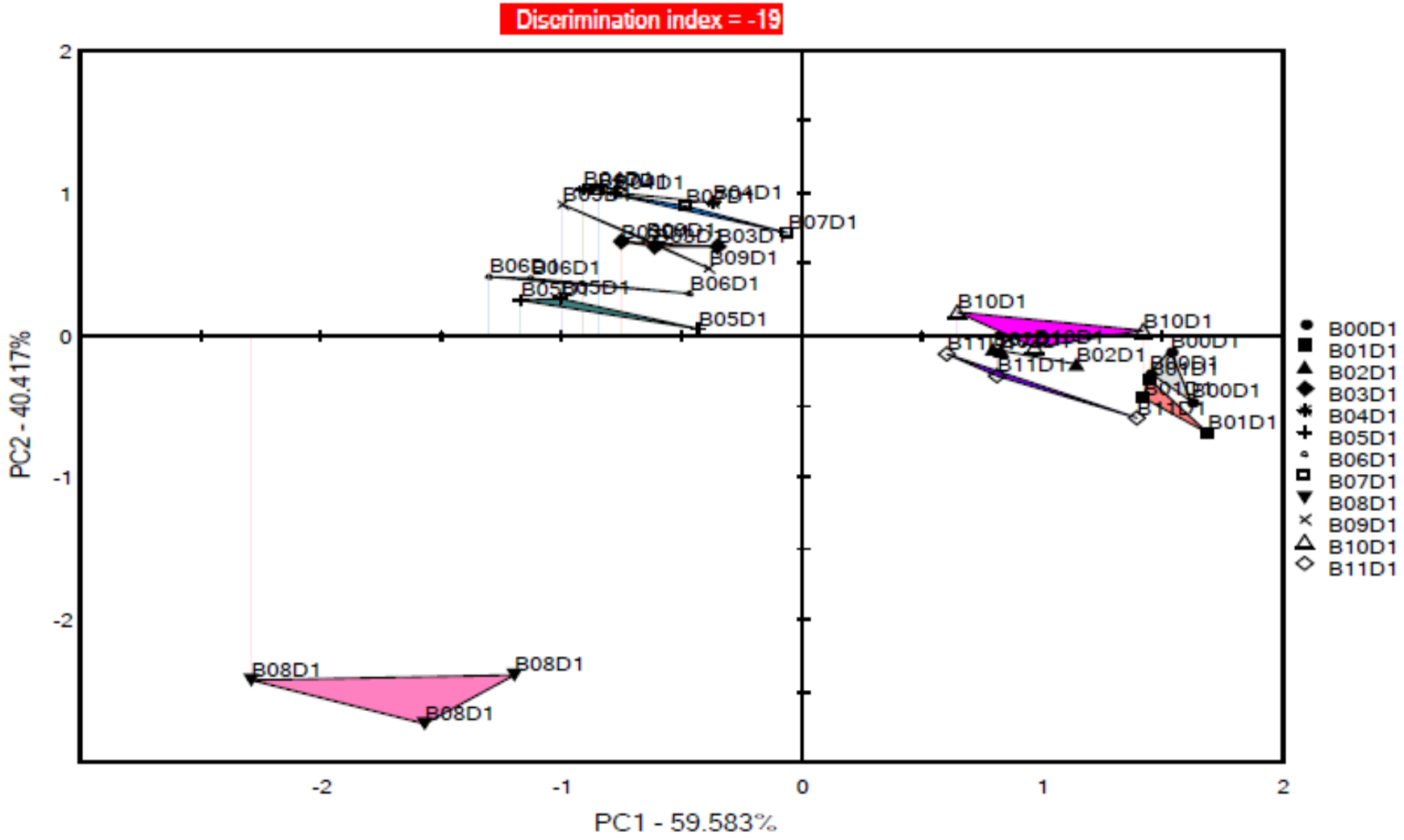

Figure 35. PCA scores plot of group 1 bacterial samples (B1- B11 with B0 as control) after 24 h of inoculation (D1), showing change on the discrimination index value to -19 between samples. PC1 and PC2explain 100% of the total variation.

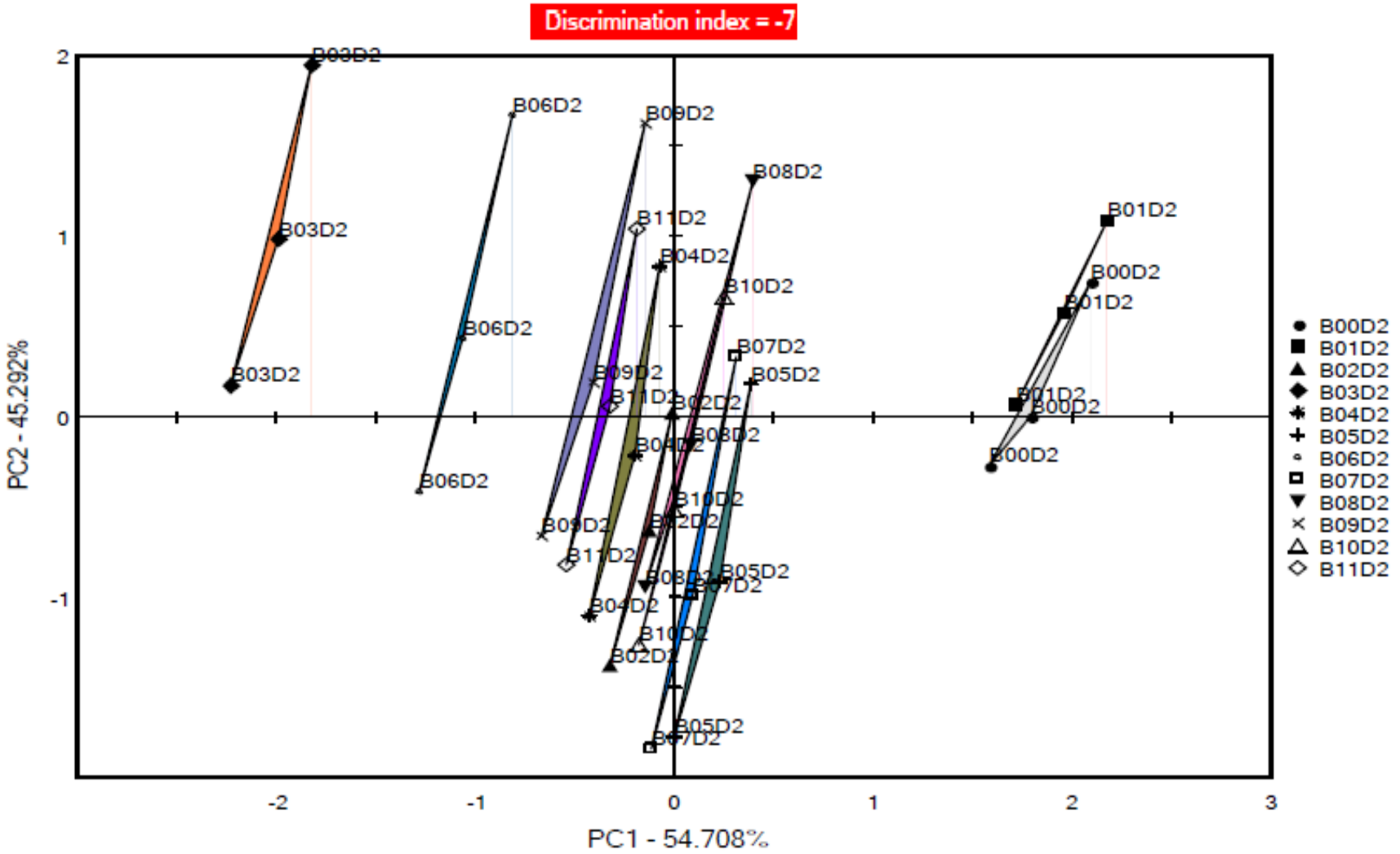

Figure 36. PCA scores plot of group 1 bacterial samples (B1- B11 with B0 as control) after 48 h of inoculation (D2), showing another slightly change on the discrimination index value to -7 between samples. PC1 and PC2 explain almost 100% of the total variation.

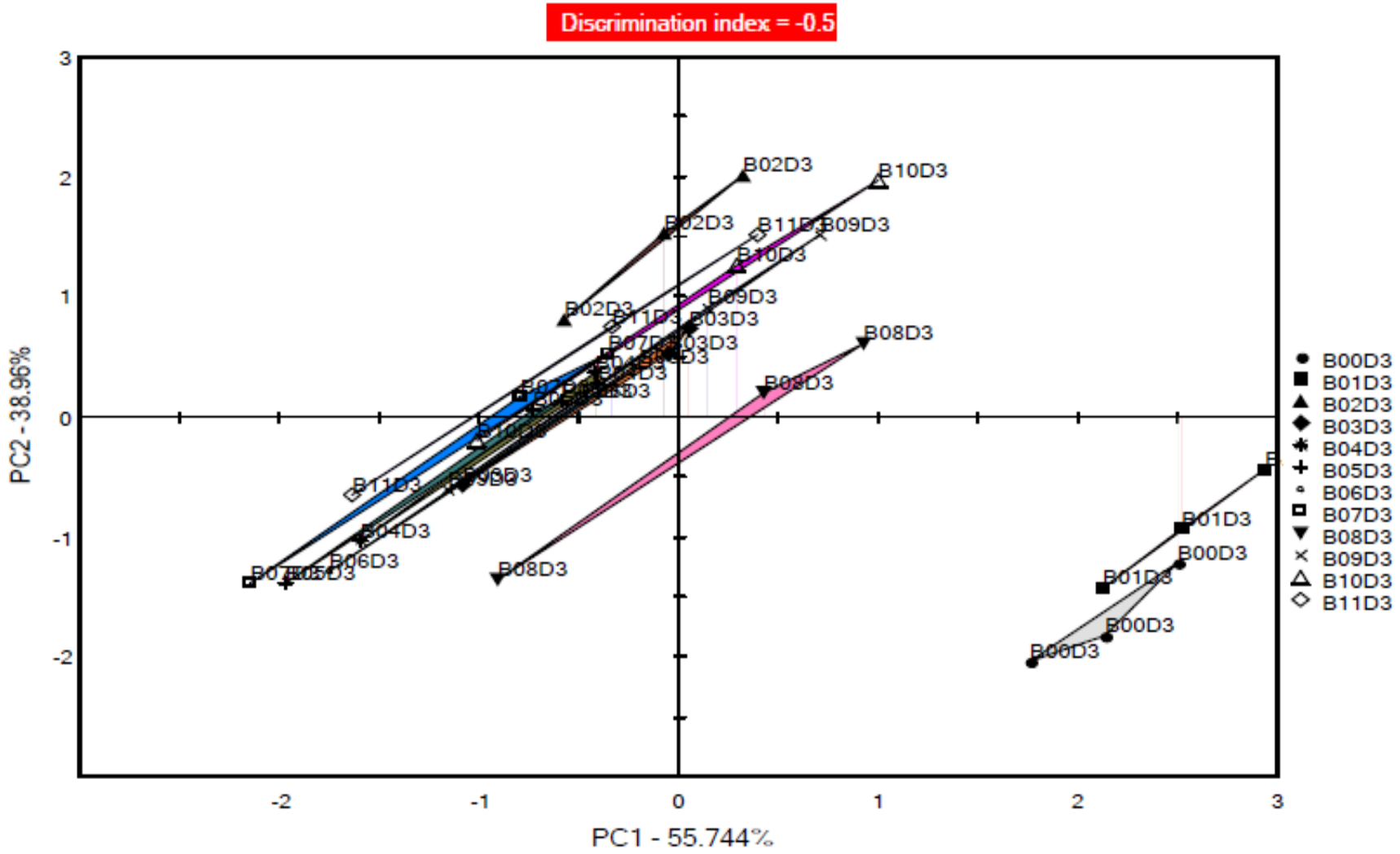

Figure 37. PCA scores plot of group 1 bacterial samples (B1- B11 with B0 as control) after 72 h of inoculation (D3), showing change in the discrimination index value to -0.5 between samples. PC1 and PC2 about 100% of the total variation.

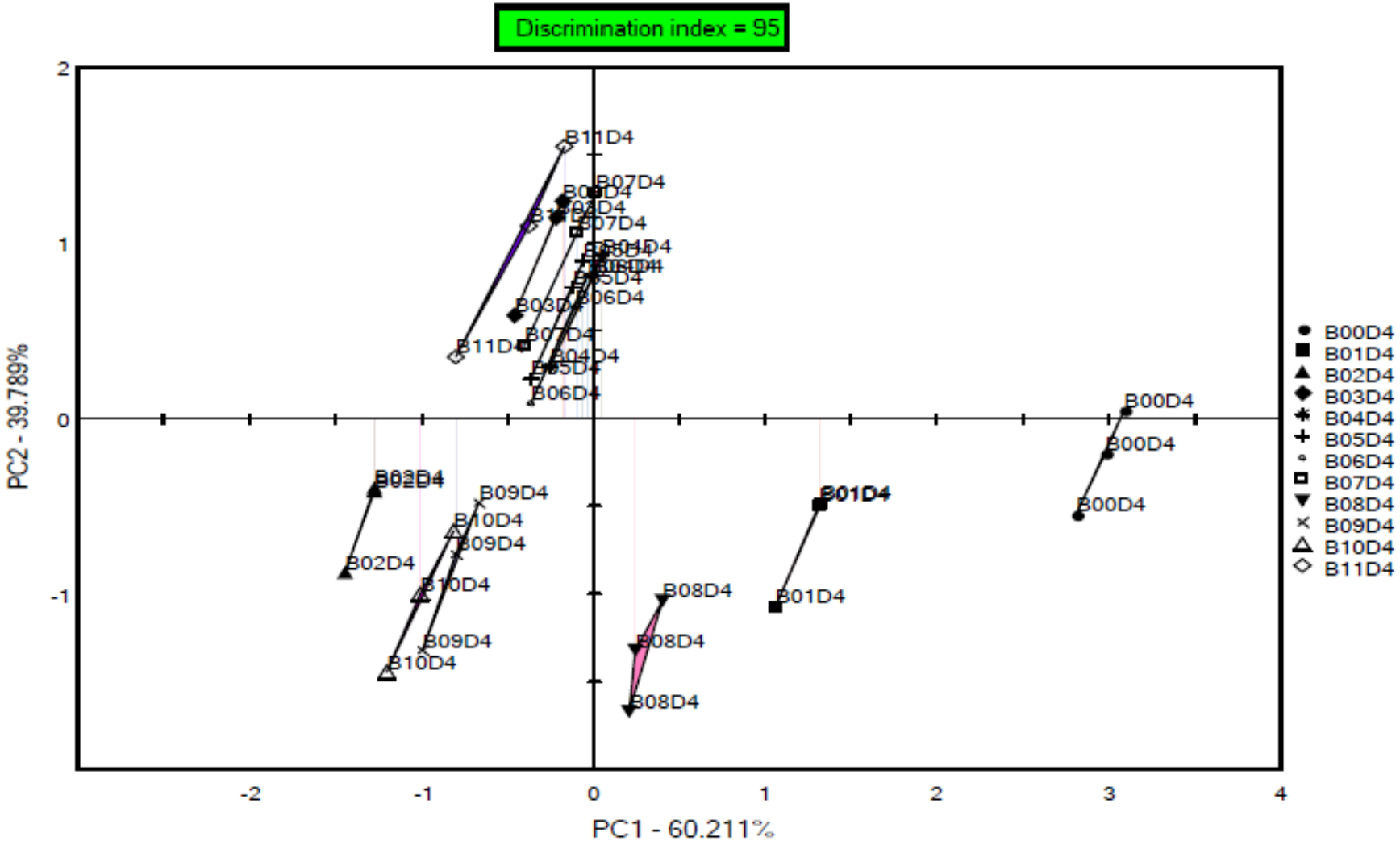

Figure 38. PCA scores plot of group 1 bacterial samples (B1- B11 with B0 as control) after 96 h of inoculation (D4), showing high change on the discrimination index value to reach 95 between samples. PC1 and PC2 100% of the total variation.

For the change in the discrimination value according to tested days individually, a PCA scores plot was created to follow the grouping between different tested days in order to identify were they overlapped.

So far, for group 1 bacterial samples (B1-B11) the Di value according to each tested day was 71 (Figure 39) and for groups 2 bacterial samples (B12-B22) the Di value was 66 (Figure 40), indicating the high discrimination of data variety according to each tested day, also indicating how ET can track the change of each day's properties and that there wasn't overlapping through the tested discriminative days for both bacterial group 1 and 2.

According to previous analysis, NB media with bacterial cultures having at least 48 h of growth rate can be suitable candidate for group clustering directly without being tested each day.

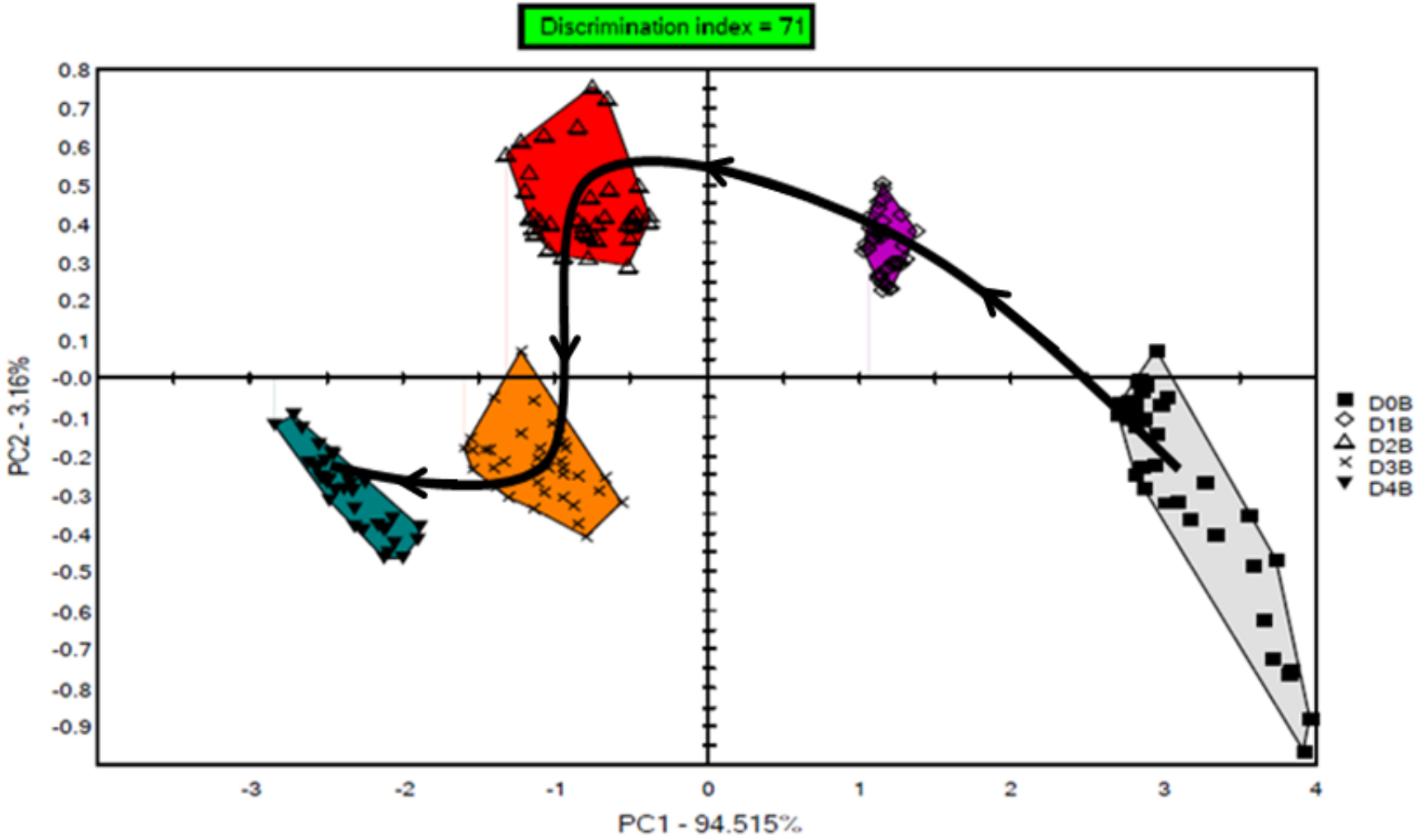

Figure 39. PCA scores plot for gathered data according to tested day for group 1 of bacterial samples (B1-B11). Showing each tested day grouping and data decline after 96 h of bacterial inoculation that shows a high discrimination index value of 71. PC1 and PC2 explain about 100% of the total variation.

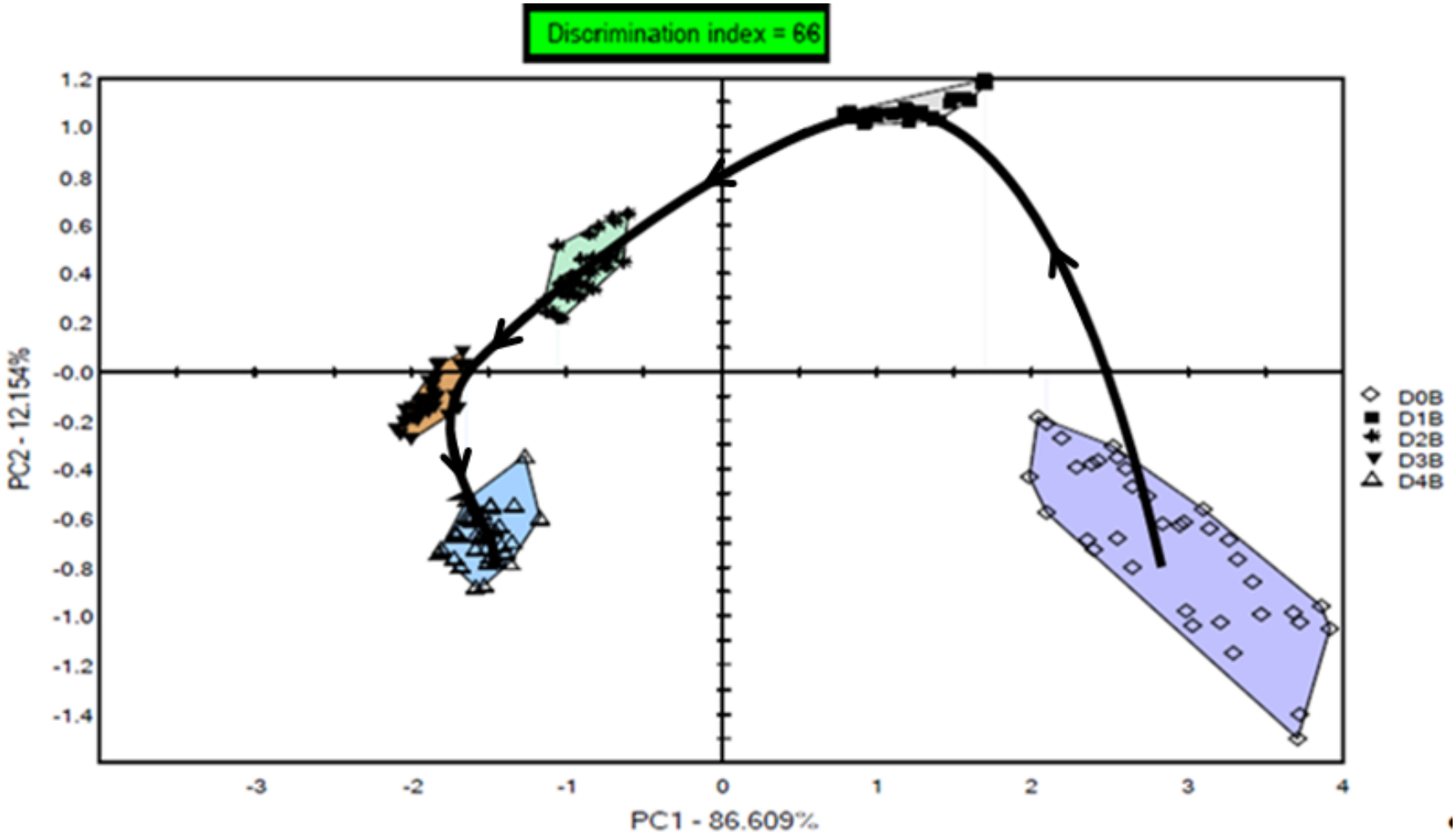

Figure 40. PCA scores plot for gathered data according to tested day for group 2 of bacterial samples (B12-B22). Showing each tested day grouping and data decline after 96 h of bacterial inoculation that shows a high discrimination index value of 66. PC1 and PC2 explain about 100% of the total variation.

In this study, day 4 (D4) collected data for both bacterial groups 1 and 2 were used to build similarity distance test table between the centroids of the defined grouped samples (two by two) and generate the corresponding clustering.

Table 14 shows the results of similarity distance test among different group 1 bacterial samples (B1-B11). In general, in this study the pattern discrimination index (%) of each sample to the other ranged, for group 1 of bacterial samples, from 3.18% between B03 and B11 to 95.68% between B01 and B02 revealing the highest and lowest similar bacterial samples, respectively (Table 14). Where, the P-value generally decreased as the discrimination value increased except in few samples due to previously mentioned reasons (Alpha MOS, 2009). Also, the Euclidian distance between two samples increased as the discrimination between them increased, except in few samples and that is according to sample dispersion that was mentioned before.

The first six bacterial combinations of B03-B11, B05-B06, B03-B07 and B04-B5 have below 10% pattern discrimination index value. Whereas, B03 and B01 bacterial samples corresponded to B08 and B02 combinations have the highest discrimination values of 95% and 94.4% according to other bacterial combinations that ranged from 10.58% to 93.05% (Table 14).

Table 14. Similarity test table for group 1 of bacterial samples (B1-B11), presenting Euclidian distance, P-value and pattern discrimination index between fungal samples.

Product names	Reference samples	Distances	P Value	Pattern discrimination index (%)
B03D4	B11D4	0.16	40.63	3.18
B05D4	B06D4	0.12	29.97	3.24
B03D4	B07D4	0.14	11.86	3.63
B04D4	B05D4	0.12	33.49	3.88
B07D4	B11D4	0.30	9.24	8.45
B04D4	B06D4	0.20	10.78	8.59
B09D4	B10D4	0.25	11.64	10.58
B04D4	B07D4	0.26	4.55	11.98
B05D4	B07D4	0.29	7.85	14.08
B05D4	B11D4	0.46	17.52	20.4
B06D4	B07D4	0.41	5.49	23.36
B04D4	B11D4	0.50	25.07	23.75
B03D4	B05D4	0.38	34.17	26.63
B06D4	B11D4	0.57	6.78	27.13
B03D4	B04D4	0.38	5.28	27.95
B03D4	B06D4	0.49	3.68	36.58
B05D4	B09D4	1.62	4.99	84.65
B07D4	B09D4	1.89	5.66	85.61
B06D4	B10D4	1.75	2.63	86.03
B04D4	B09D4	1.71	3.43	86.30
B01D4	B11D4	2.38	1.53	87.4
B02D4	B06D4	1.58	3.05	87.93
B05D4	B10D4	1.85	2.54	88.07
B07D4	B10D4	2.12	3.07	88.37
B08D4	B11D4	2.45	4.69	88.57
B02D4	B07D4	1.88	5.61	89.03
B01D4	B06D4	1.84	0.46	89.06
B04D4	B10D4	1.95	1.79	89.32
B02D4	B05D4	1.65	3.15	89.86
B01D4	B09D4	2.06	1.13	90.17
B00D4	B01D4	1.78	0.83	90.43
B01D4	B04D4	1.88	0.84	90.44
B06D4	B08D4	1.90	5.14	90.58
B01D4	B05D4	1.93	0.40	90.63
B03D4	B10D4	2.14	3.03	90.89
B02D4	B04D4	1.77	3.84	91.32
B07D4	B08D4	2.31	4.82	91.80
B01D4	B10D4	2.27	0.88	91.90
B02D4	B03D4	1.87	3.81	91.98
B05D4	B08D4	2.02	4.86	92.22
B02D4	B08D4	1.80	2.63	92.56
B04D4	B08D4	2.05	5.55	92.61
B01D4	B03D4	2.26	0.44	93.05
B03D4	B08D4	2.40	3.89	94.40
B01D4	B02D4	2.57	0.68	95.68

Table 15 lists similarity distance test ordered values for compared group 2 bacterial samples (B12-B22) on the fourth day. In which, the pattern discrimination index among samples in this group was relatively larger than it was among samples in group 1 bacterial samples. Where, the lowest value started with 12.91% between B13 and B22, and the highest to 99.8% between B14 and B22.

The pattern discrimination index was less than 50% only between two bacterial combinations of B13, B17 and B22 revealing the similarity between these samples (Table 15).

Although, the distance between samples having 99% discrimination index and more, it was less in distance range (0.84-3.8). The discrimination pattern was larger and ranged from 99.02% among B19 and B21 to 99.8% among B14 and B20. This phenomenon is due to the large variety of bacterial genotypes in this group that revealed a low dispersion and the high Di power of sensors array. The P-value generally decreased as the discrimination value increased except in few samples due to previously mentioned reasons that were mentioned before (Table 15.)

Table 15. Similarity test table for group 1 of bacterial samples (B12-B22), presenting Euclidian distance, P-value and pattern discrimination index between fungal samples.

Product names	Reference samples	Distances	P Value	Pattern discrimination index (%)
B13D4	B22D4	0.08	67.44	12.91
B13D4	B17D4	0.20	35.41	45.29
B17D4	B22D4	0.23	12.71	69.19
B13D4	B15D4	0.39	14.77	78.78
B18D4	B21D4	0.42	13.55	81.66
B15D4	B17D4	0.35	6.00	82.69
B15D4	B22D4	0.34	7.91	86.06
B17D4	B21D4	0.43	12.2	87.23
B13D4	B21D4	0.56	6.93	87.92
B13D4	B16D4	0.57	4.46	90.07
B12D4	B20D4	1.08	5.38	90.72
B16D4	B19D4	0.36	3.34	91.00
B15D4	B16D4	0.39	4.22	91.11
B13D4	B18D4	0.96	9.87	93.89
B17D4	B18D4	0.85	6.50	94.29
B12D4	B18D4	1.52	3.66	94.59
B16D4	B22D4	0.49	3.10	94.79
B21D4	B22D4	0.63	3.19	95.03
B13D4	B19D4	0.89	6.54	95.20
B16D4	B17D4	0.65	3.87	95.57
B14D4	B19D4	0.50	5.42	95.98
B15D4	B21D4	0.75	1.71	96.20
B18D4	B22D4	1.03	1.93	96.61
B15D4	B19D4	0.75	1.07	96.80
B12D4	B21D4	1.93	2.29	97.02
B15D4	B18D4	1.17	6.39	97.23
B19D4	B22D4	0.81	4.07	97.46
B12D4	B17D4	2.34	3.17	97.89
B13D4	B20D4	1.65	3.22	98.29
B12D4	B15D4	2.68	3.58	98.44
B16D4	B21D4	1.08	2.62	98.62
B12D4	B16D4	2.98	2.84	98.8
B18D4	B19D4	1.84	2.66	98.92
B12D4	B19D4	3.29	1.30	98.98
B19D4	B21D4	0.84	1.35	99.02
B14D4	B16D4	1.42	1.50	99.04
B14D4	B15D4	1.21	1.48	99.14
B20D4	B22D4	1.72	1.96	99.16
B15D4	B20D4	1.81	0.85	99.20
B12D4	B14D4	3.01	0.10	99.28
B14D4	B22D4	1.31	0.59	99.36
B16D4	B20D4	2.17	0.83	99.56
B19D4	B20D4	2.52	0.06	99.61
B14D4	B21D4	1.92	0.64	99.62
B14D4	B20D4	3.80	0.59	99.80

The following PCA scores plot (Figure 41and Figure 42) of the fourth day's data for both bacterial groups (group 1 and group 2) shows the relative clustering of bacterial groups as it genetically revealed previously by the UPGMA tree (Figure 30 and Figure 31).

In which, the grouping and clustering of PCA and genetic tree have almost the same pattern for different bacterial isolates. In group 1 bacterial samples (B1-B11) (Figure 41), has both subgroup A and B. Where, subgroup B includes only B01 that is discriminated among all other bacterial samples. Meanwhile, subgroup A have all other bacterial samples contained in three clusters I, II and III. Cluster II consists on B08 bacterial isolate, cluster II composed of three bacteria B02, B09 and B10 with close distance, leaving cluster II with the majority of bacterial samples B03, B04, B05,B06, B07 and B11.

Meanwhile, group 2 bacterial samples (B12-B22) (Figure 42) have subgroup C and D, subgroup D has only B12 that is distinct from other bacterial samples. However, subgroup C has the majority of bacterial samples categorized in clusters I and II. Where, cluster II has only B20 sample, leaving cluster I with all nine bacterial samples grouped in a, b, c and d. In which, group c having B18 and group d having B14 are distinct on the sides of cluster I center. In that center, laying group a including B13, B22, B17

and B21 having the highest similarity leaving group b with B15, B16 and B19 with the second highest similarity among bacterial genotypes (Figure 42).

These results indicate the compatibility of both ET and molecular methods in clustering of different microorganism tested samples.

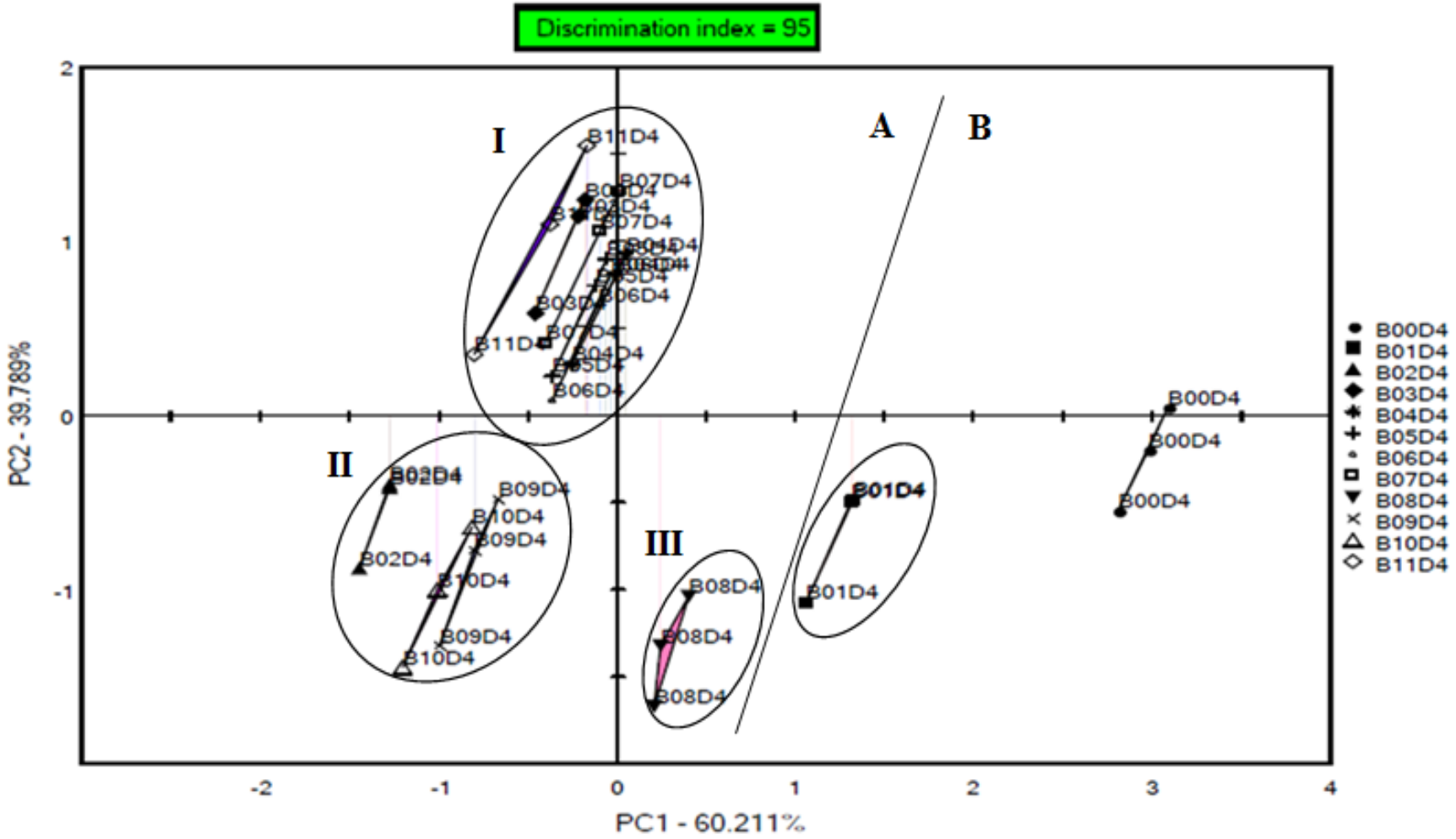

Figure 41. PCA scores plot showing clustering rhythm of group 1 bacterial samples (B1-B11) according to UPGMA dendogram (Figure 30). It has a very close clustering similarity.

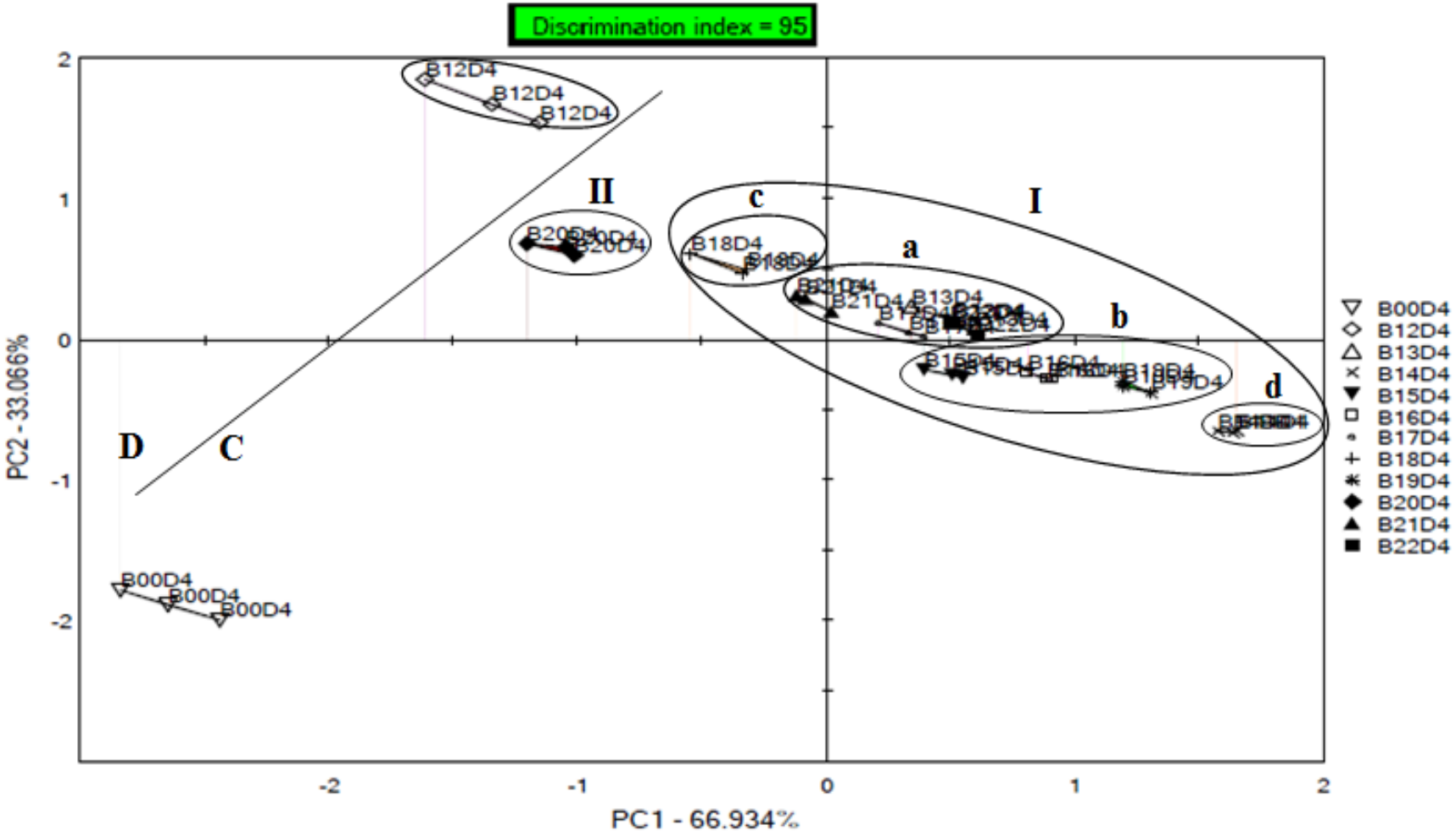

Figure 42. PCA scores plot showing clustering rhythm of group 2 bacterial samples (B12-B22) according to UPGMA dendogram (Figure 31). It has a very close clustering similarity.

5 Conclusion

Electronic tongue can be considered as a promising analytical method for monitoring microbial growth and follow their metabolites production during their growth. This research and results can be used for the next establishing step in distinguishing different fungal and bacterial genotypes. Also, in a long run will open a wide range for using sensors for monitoring microbial activities for fermentable, industrial and categorizing applications.

Moreover, the results confirmed the possibility of using an Astree II ET, a conventional potentiometric applicable technique, as an alternative fast assessment tool to distinguish complex and native state microorganism's bimolecular foot-printing in a liquid media, relying on chemical changes due to microbial primary metabolite production through growth phases and secondary metabolite assembly and differentiation during stationary phase.

Additionally, this highlights the beneficial use of Et as an alternative and in vitro assessment tool to other sophisticated techniques. Also, the fact that combining ET with other detecting technologies can be used for more accurate monitoring and optimizing schemes due to its high sensitivity, low detecting limit of microbial metabolites sensory and safety index.

As recommendations for future researches, further studies must carried out in order to monitor sensors' temperature dependence and charge transfer affect by the adsorption of solution component. Also, to create large and specified foot-printing databases for microbial tasted metabolites' complexes in a liquid media for fast and easy microbial detecting and classification.

Abstract in Arabic (الملخص)

إمكانيّة التَّصنيف الغير مباشر للكائنات الحيّة الدَّقيقة من خلال مُنتجات عملياتِ الأيض لها بواسطة مجس كيماوي (اللّسان الإلكتروني) والتَّحليل المتعدد العوامل للبيانات

الطالبة: باسمة علي أبو رميلة

المشرف: الدكتور نواف أبو خلف

الملخص

أصبح من الشائِع حاليا استخدام أنظمة المجسَّات الكيميائيّة كأداة تحليلية للعديد من المحاليل المعقدة، حيث يعد اللِّسان الالكتروني أحد هذه المجسَّات والذي يعتمد مبدأ عمله على استخدام مجموعة متعددة من المجسَّات ذات صفات حسيِّة عالية وخصائص انتقائية منخفضة. هذا البحث عبارة عن تجربة لدراسة امكانيَّة استخدام جهاز اللِّسان الالكتروني (الذي يعمل على مبدأ قياس فرق الجهد) كوسيلة بديلة وسريعة في عملية تصنيف الكائنات الحية الدقيقة (مثل البكتيريا والفطريات) في حالتها الأصلية والمعقدة، بالاعتماد على استشعار مفرزات عملياتها الأيضية في وسط مائي.

تضمن هذا البحث عزل 44 فطر وبكتيريا مختلفة، حيث تم اكثارها في وسط مائي مناسب لكل نوع منها، واستخدام مرشِّح هذا الوسط ليتم قياسه باستخدام جهاز اللِّسان الالكتروني (من نوع Astree II Alpha MOS) خلال فترة نموها. بعد ذلك تم تحليل البيانات المجمعة بواسطة هذه المجسّات باستخدام طرق التَّحليل المتعدد العوامل للبيانات (وبشكل أساسي باستخدام التحليل الأساسي

للمكونات (PCA))، واستخدام هذه التَّحليلات لتصنيف الكائنات من نفس النوع وفقًا لأوجه التشابه أو الاختلاف بين بعضها البعض، وأيضاً متابعة دورة نموها. في هذه الأثناء تم التحقق من نتائج تصنيف اللِّسان الالكتروني بمقارنتها مع التَّصنيف باستخدام قواعد البيانات الجزيئية (الحمض النَّووي DNA).

كانت نتائج هذه الدِّراسة واعدة، حيث أن المجسّات المستخدمة اظهرت قدرة تصنيفية عالية تراوحت بين 0.927–1 بين العزلات الفطرية و0.960–0.999 بين العزلات البكتيرية في نهاية فترة القياس. وكذلك فإن قيمة مؤشر التصنيف لتمثيل ال PCA كانت 98 و96 بين العزلات الفطرية و 95 بين العزلات البكتيرية. إضافة لذلك فإن فحص التشابه بين العزلات كشف عن التشابه الكبير بين العزلة F5 و F6 بمقدار 27.18% في المجموعة لأولى للفطريات، و بمقدار 70.52% بين العزلة F14 و F21 في المجموعة الثانية للفطريات، وبمقدار 3.18% بين العزلة B3 و B11 في المجموعة البيكتيرية لأولى، وبمقدار 12.91% بين العزلة B13 و B22 في المجموعة البكتيرية الثانية.

وكذلك فالنتائج أوضحت أن أنماط التَّصنيف التي نتجت عن طريق تقنية اللِّسان الالكتروني كانت مطابقة لأنماط التَّصنيف التي نتجت عن طريق التِّقنية الجزيئية، وتمكن الجهاز من تتبُع دورة النُّمو لهذه الكائنات وتِبيان فترات التَّداخل (توقف التَّغير الكيميائي) داخل الوسط المائي.

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Appendix A

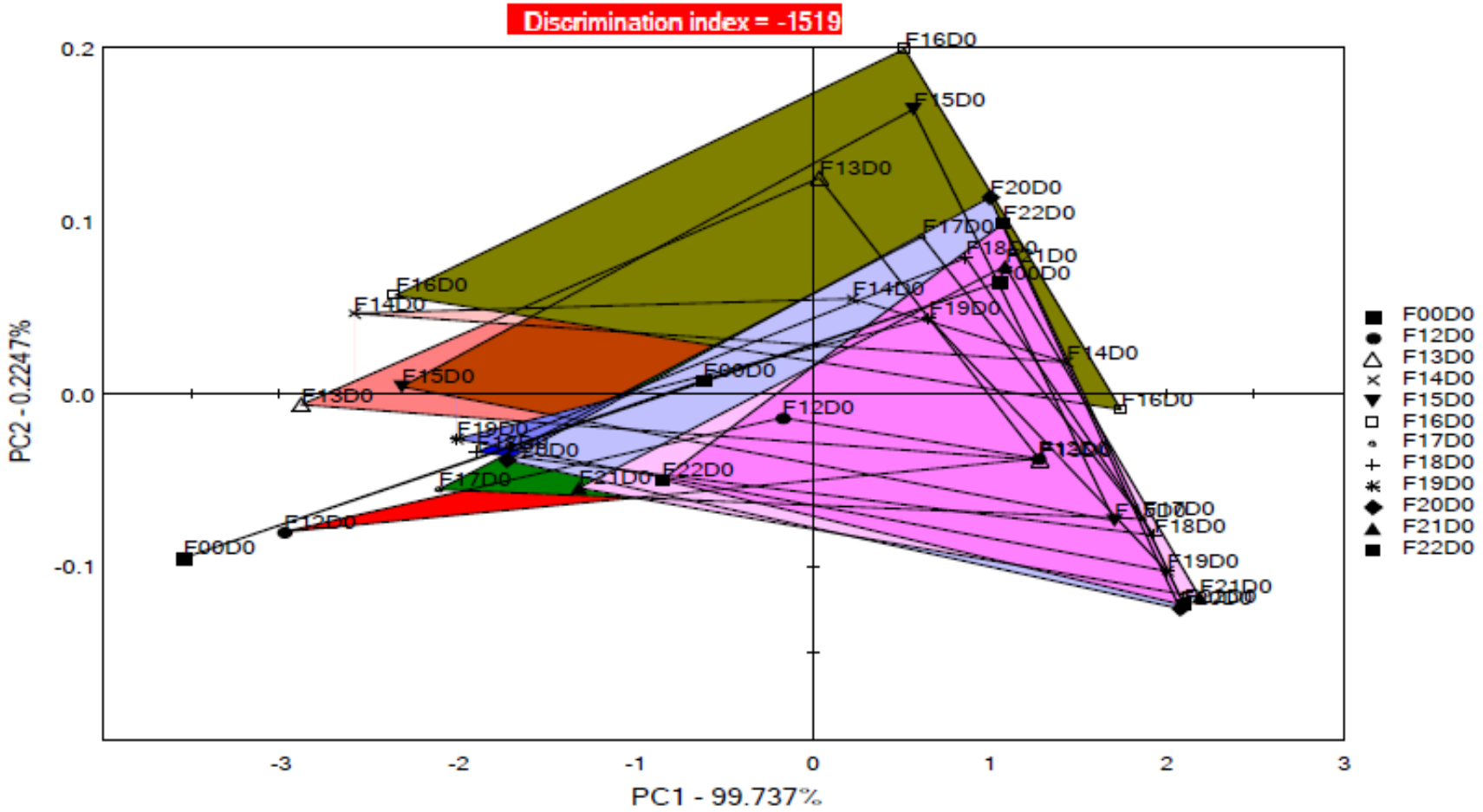

Figure 43. PCA scores plot of group 2 fungal samples (F12- F22 with F0 as control) on the day of inoculation (D0), showing no discrimination with an index value of -1519 between groups. PC1 and PC2 almost 100% of the total variation.

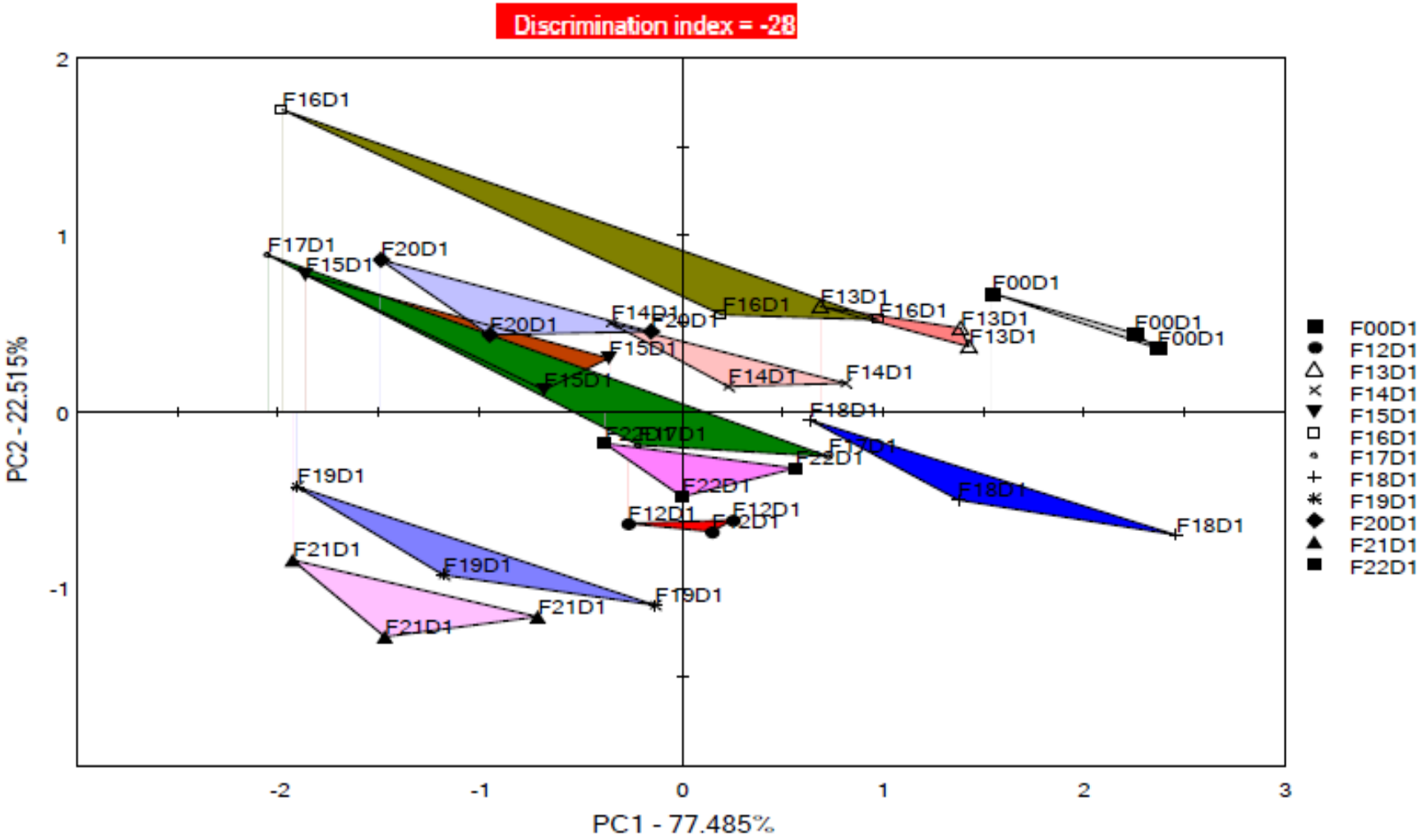

Figure 44. PCA scores plot of group 2 fungal samples (F12- F22 with F0 as control) after 24 h of fungal inoculation (D1), showing changed discrimination index value to -28 between groups. PC1 and PC2 explain 100% of the total variation.

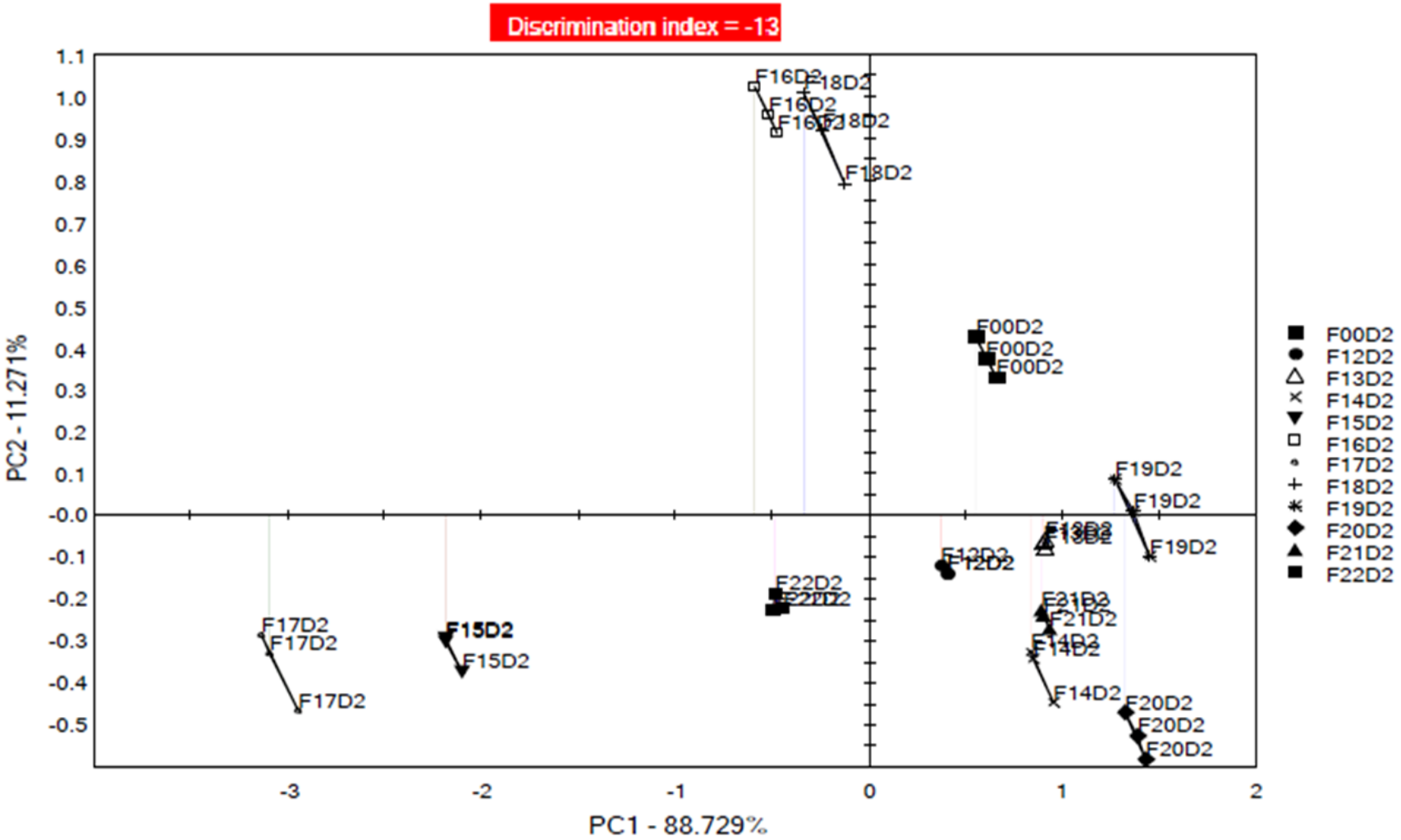

Figure 45. PCA scores plot of group 2 fungal samples (F12- F22 with F0 as control) after 48 h of fungal inoculation (D2), showing changed discrimination index value to -13 between groups. PC1 and PC2 explain 100% of the total variation.

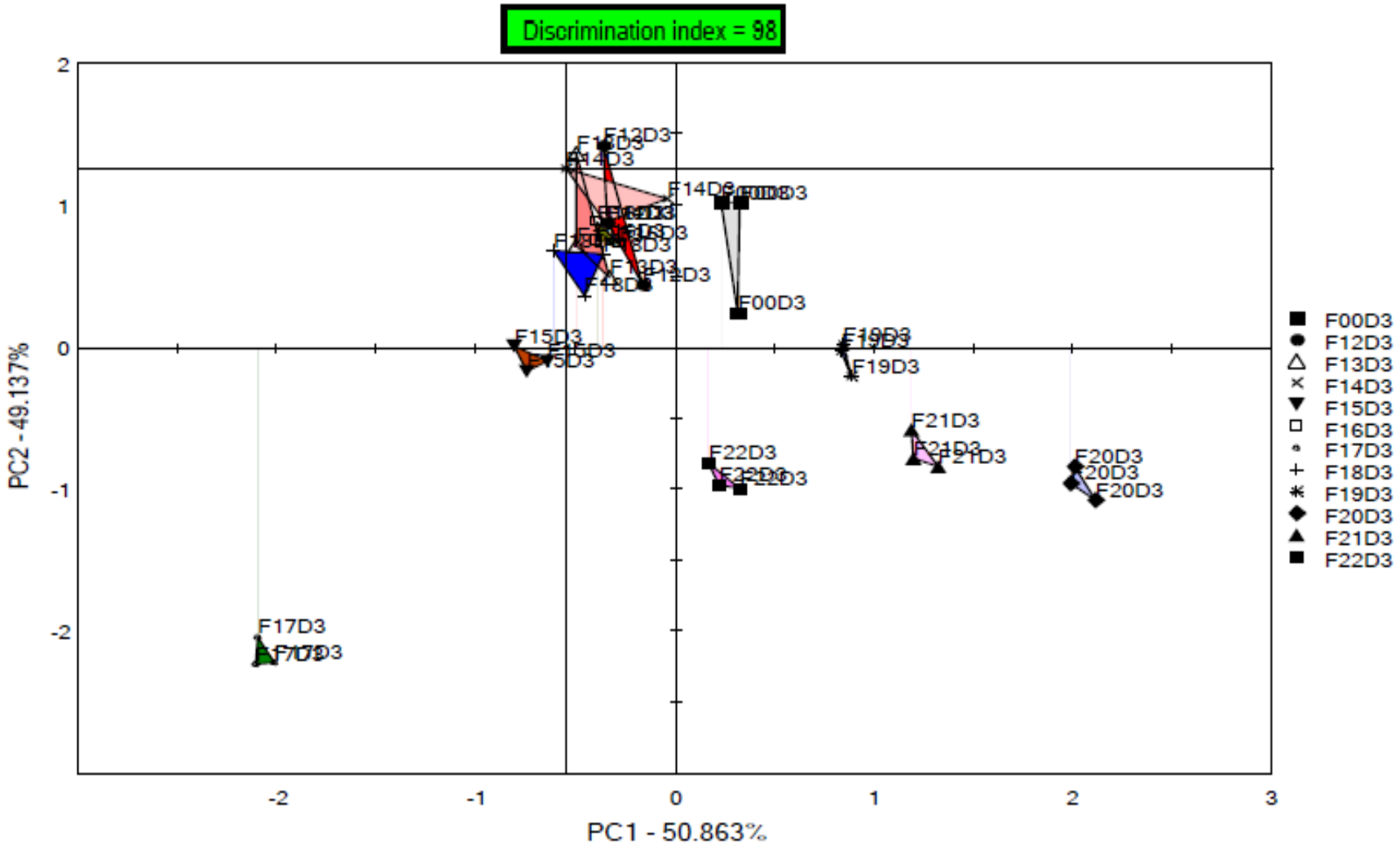

Figure 46. PCA scores plot of group 2 fungal samples (F12- F22 with F0 as control) after 72 h of fungal inoculation (D3), showing high discrimination index value of 98 between groups. PC1 and PC2 explain 100% of the total variation.

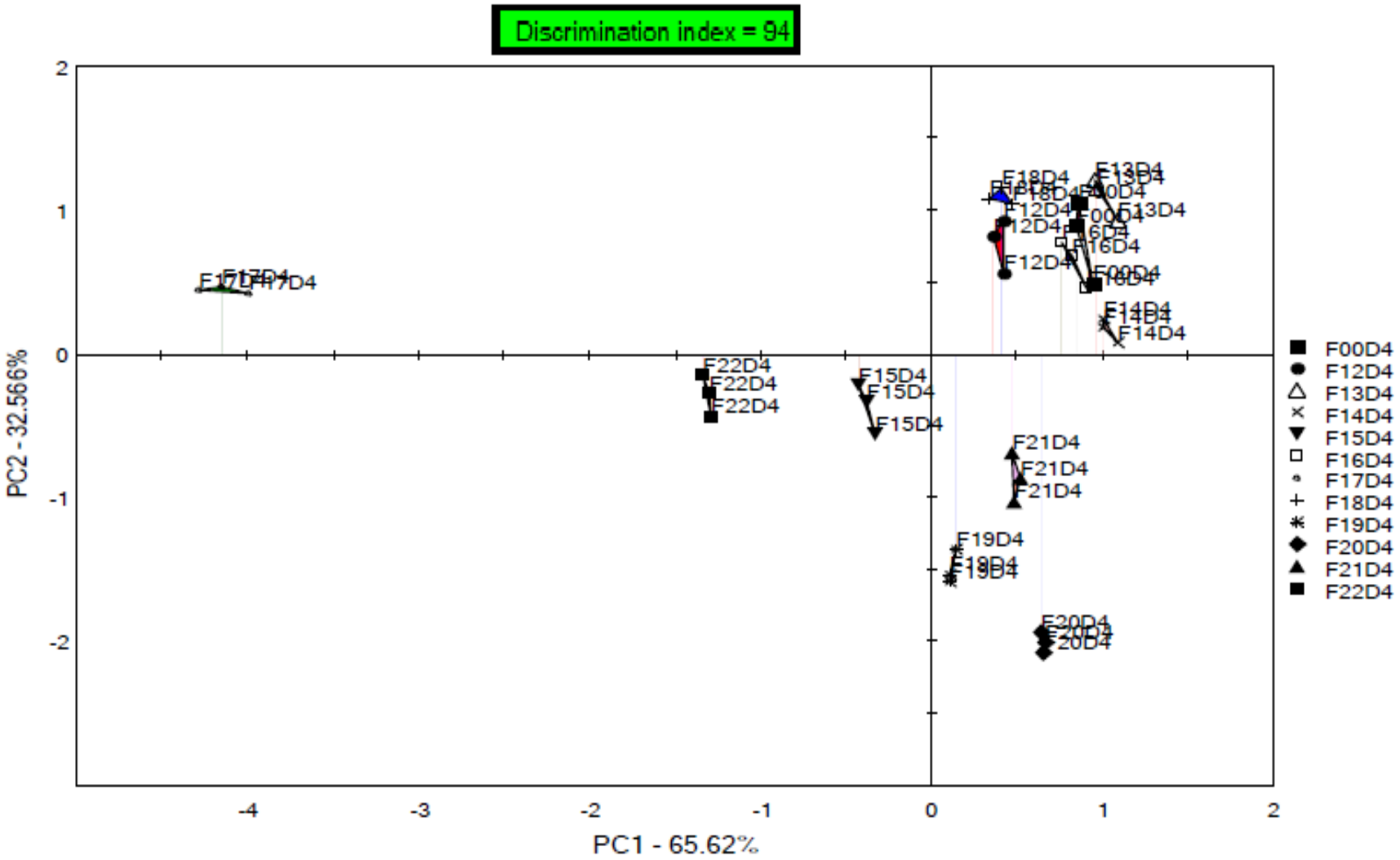

Figure 47. PCA scores plot of group 2 fungal samples (F12- F22 with F0 as control) after 96 h of fungal inoculation (D4), showing high discrimination index value of 94 between groups. PC1 and PC2 explain about 100% of the total variation.

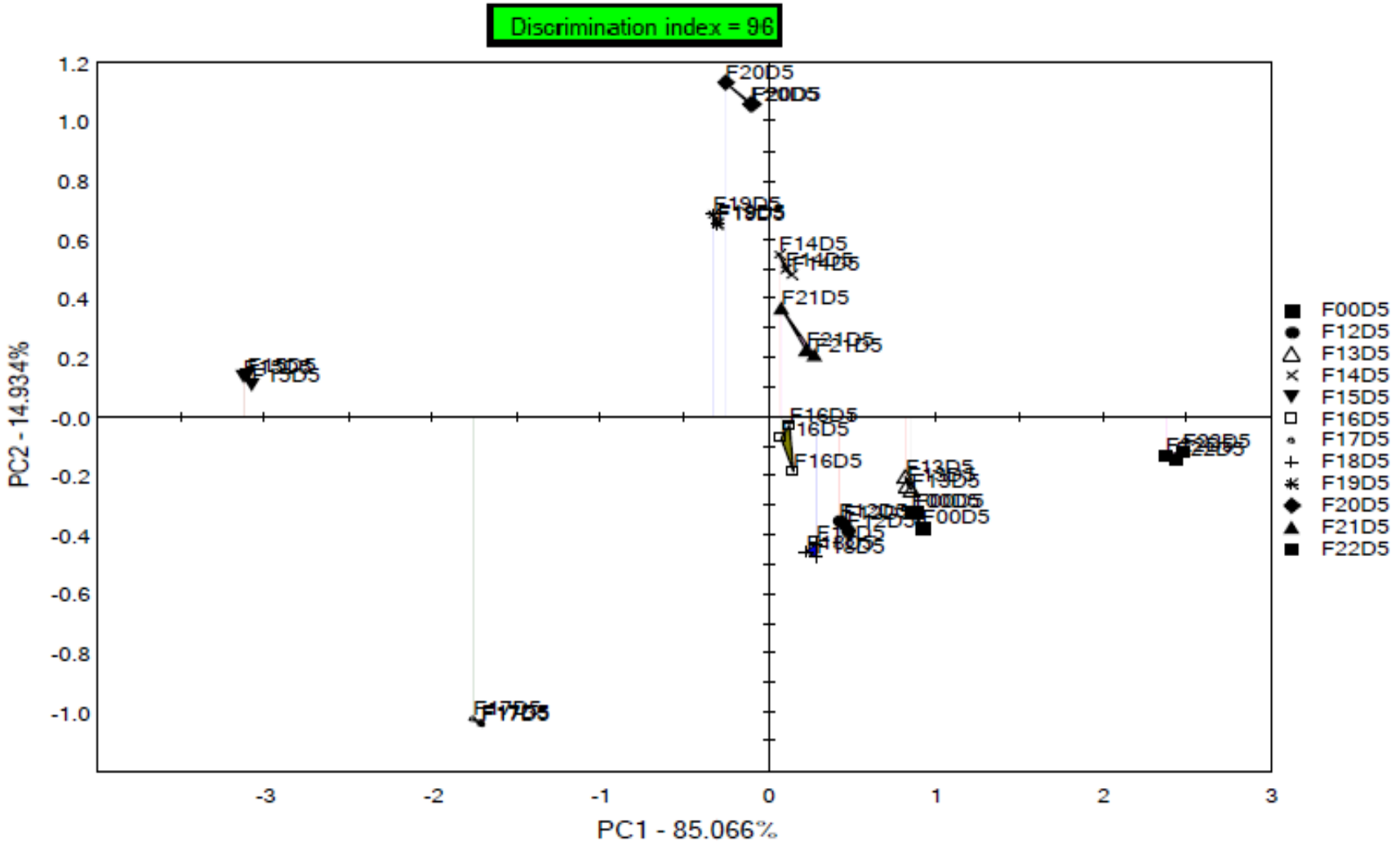

Figure 48. PCA scores plot of group 2 fungal samples (F12- F22 with F0 as control) after 120 h. of fungal inoculation (D5), showing high discrimination index value of 96 between groups. PC1 and PC2 explain 100% of the total variation.

Appendix B

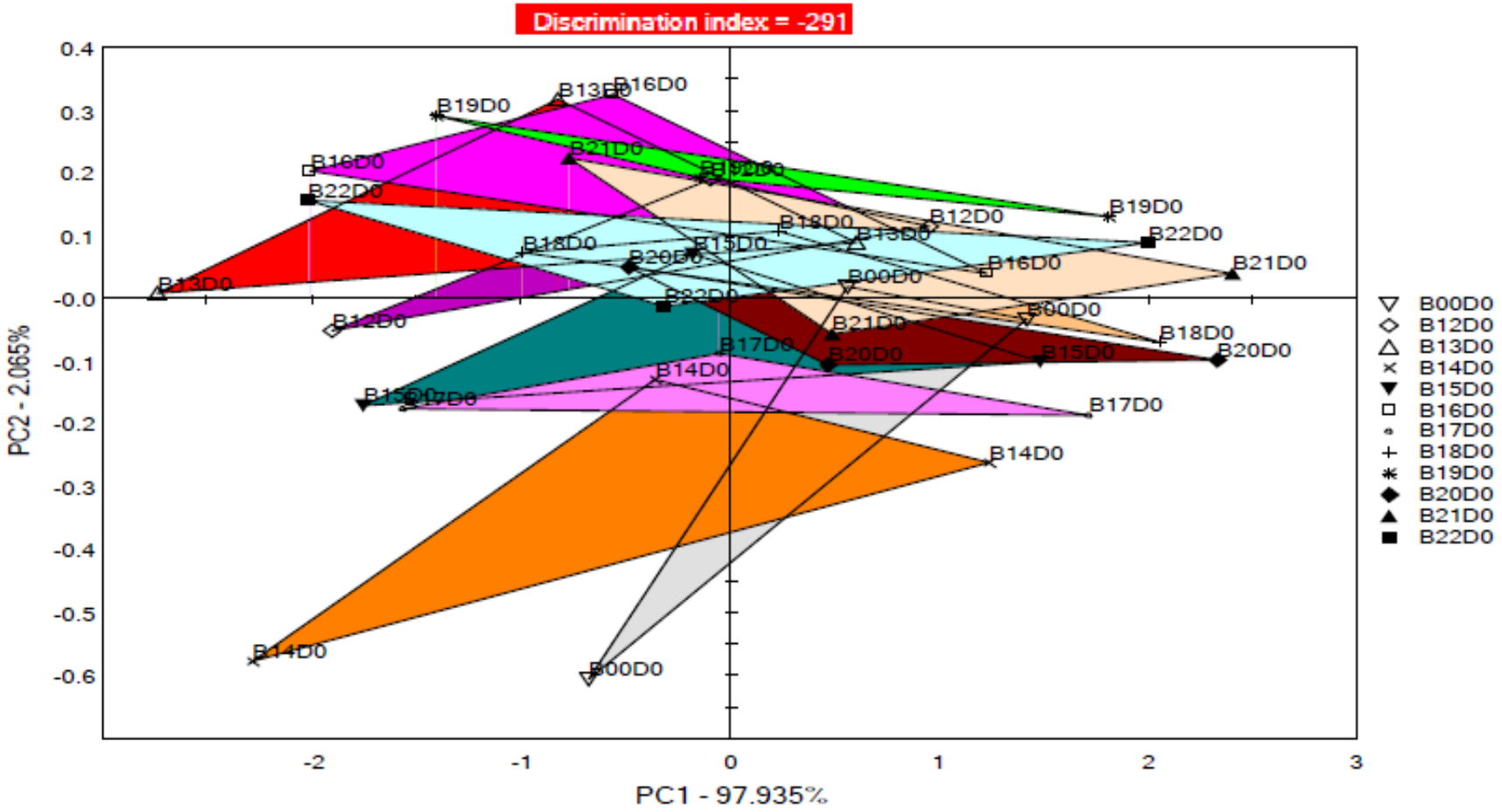

Figure 49. PCA scores of group 2 bacterial samples (B12- B22 with B0 as control) on the day of inoculation (D0), showing no discrimination with an index value of -291 between groups. PC1 and PC2 explain 100% of the total variation.

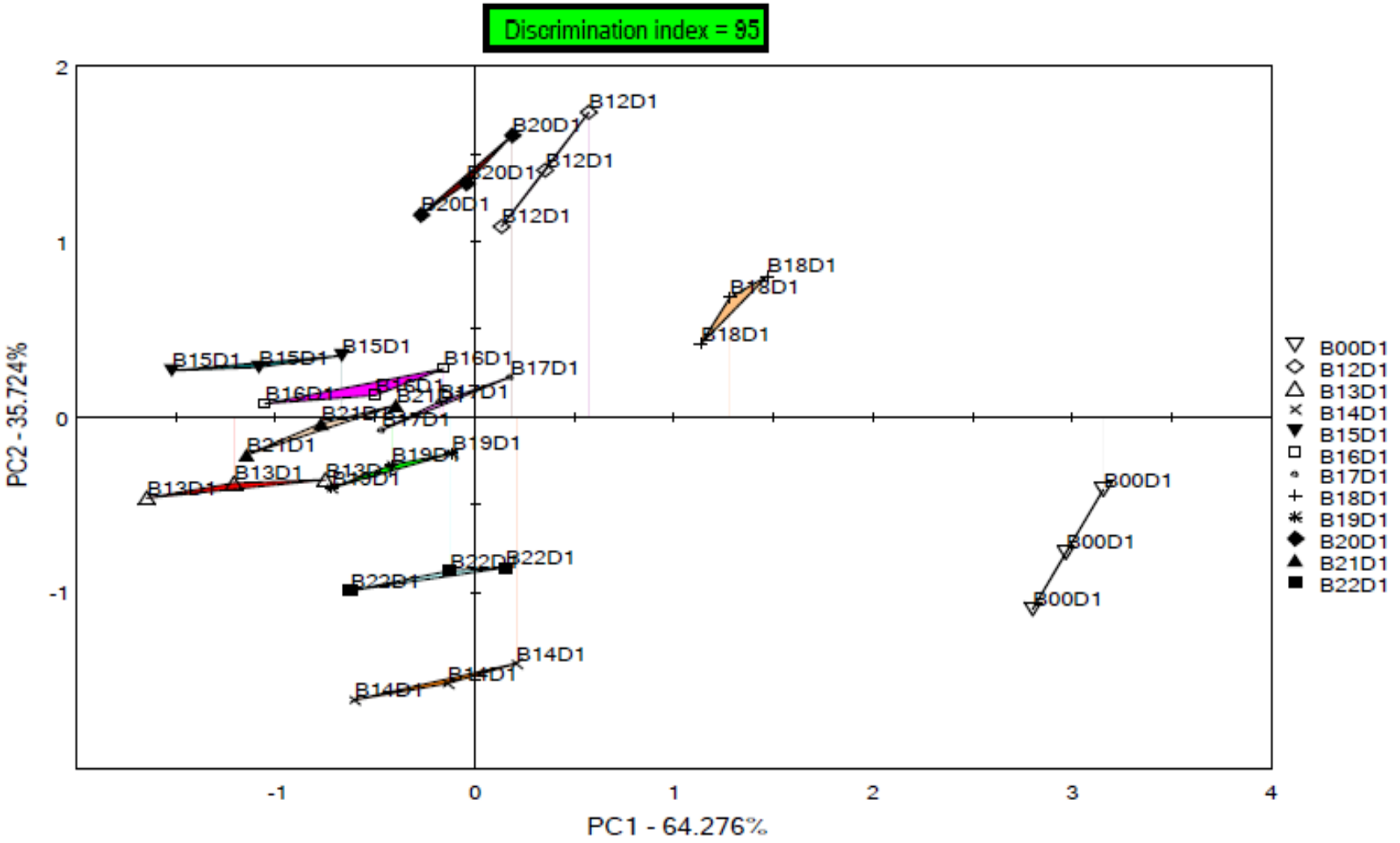

Figure 50. PCA scores plot of group 2 bacterial samples (B12- B22 with B0 as control) after 24 h of inoculation (D1), showing fast and high discrimination index value change to 95 between groups. PC1 and PC2 explain 100% of the total variation.

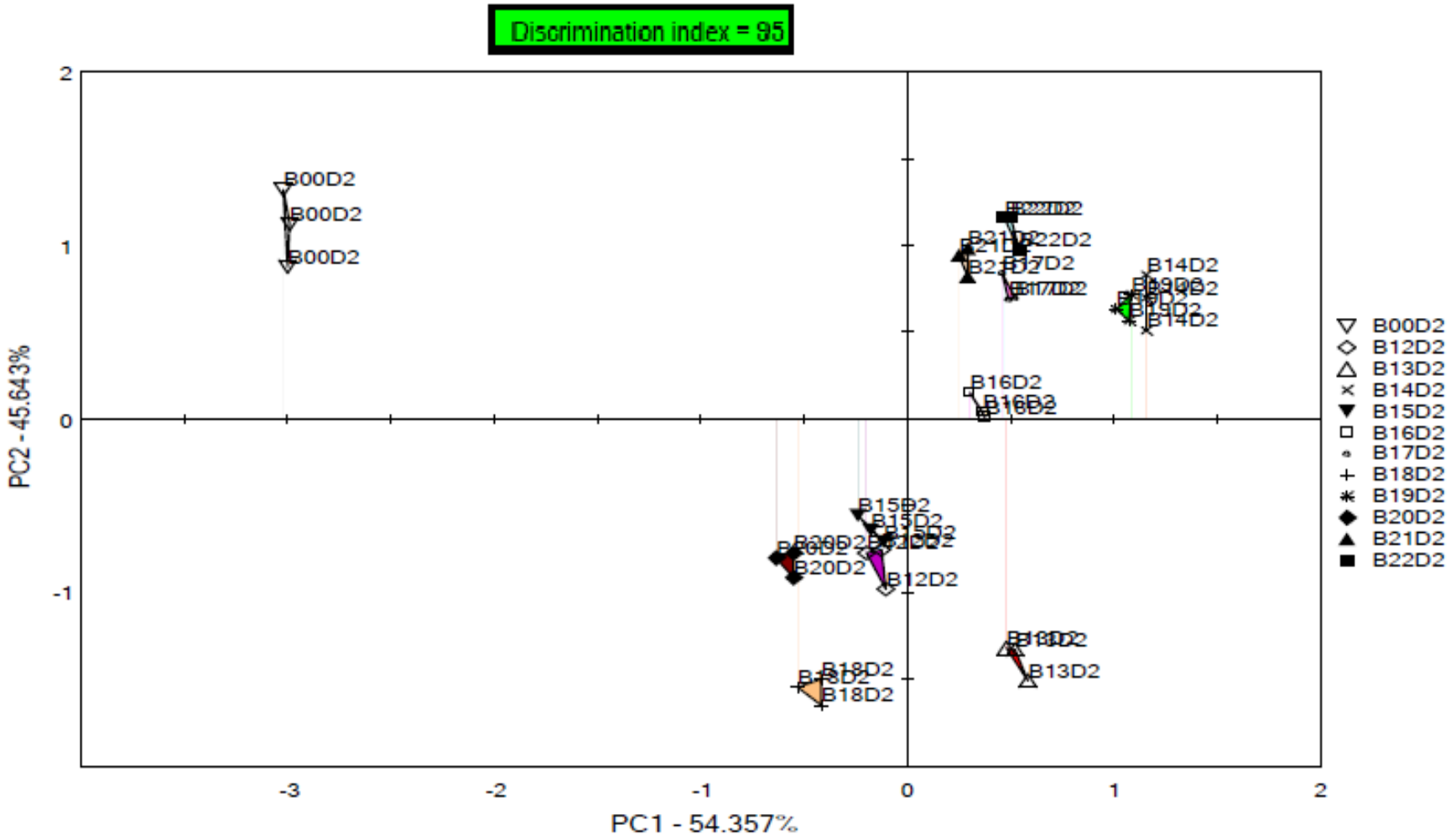

Figure 51. PCA scores plot of group 2 bacterial samples (B12- B22 with B0 as control) after 48 h of inoculation (D2), showing constant and high discrimination index value of 95 between groups. PC1 and PC2 explain 100% of the total variation.

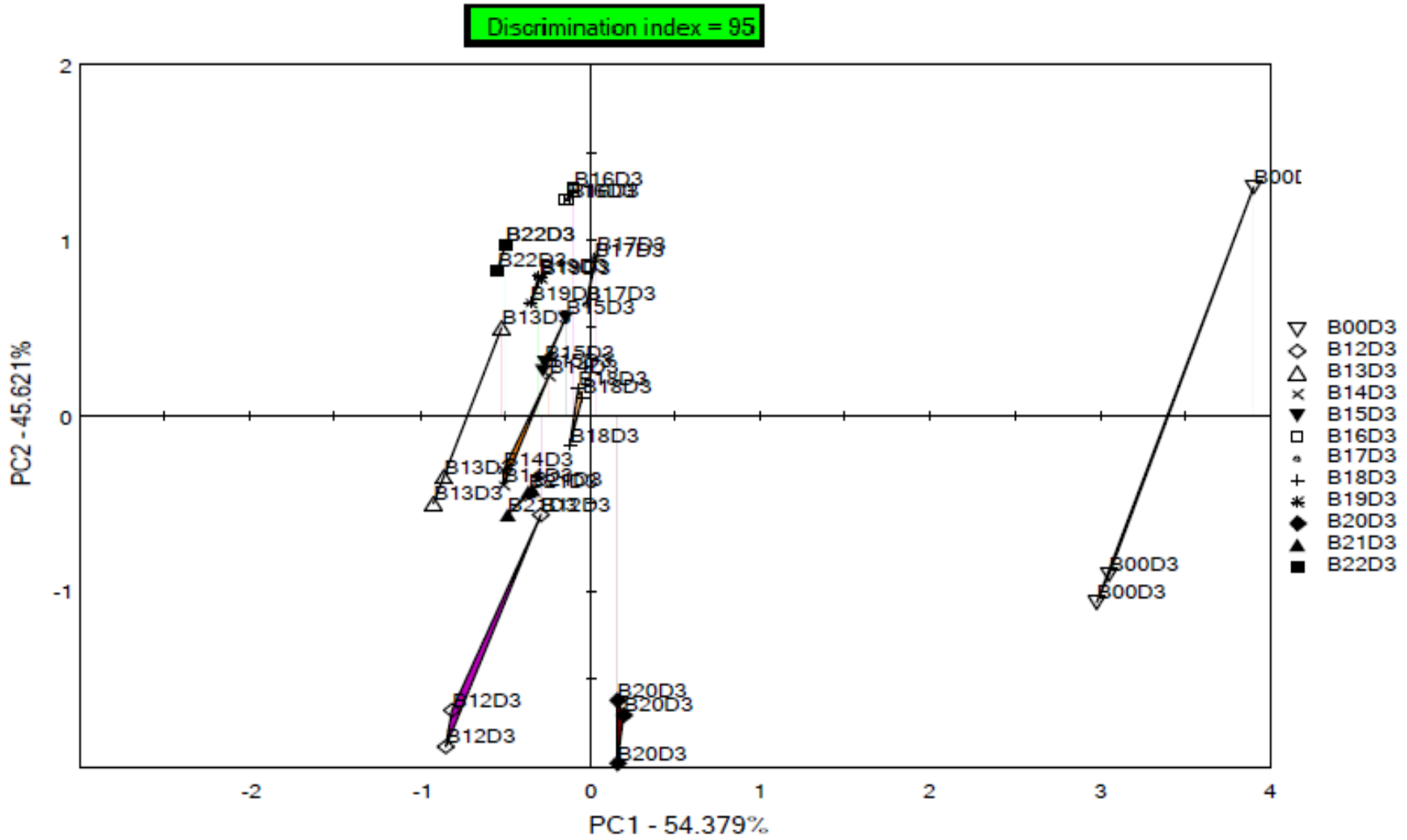

Figure 52. PCA scores plot of group 2 bacterial samples (B12- B22 with B0 as control) after 72 h of inoculation (D3), showing constant high discrimination index value of 95 between groups. PC1 and PC2 explain almost 100% of the total variation.

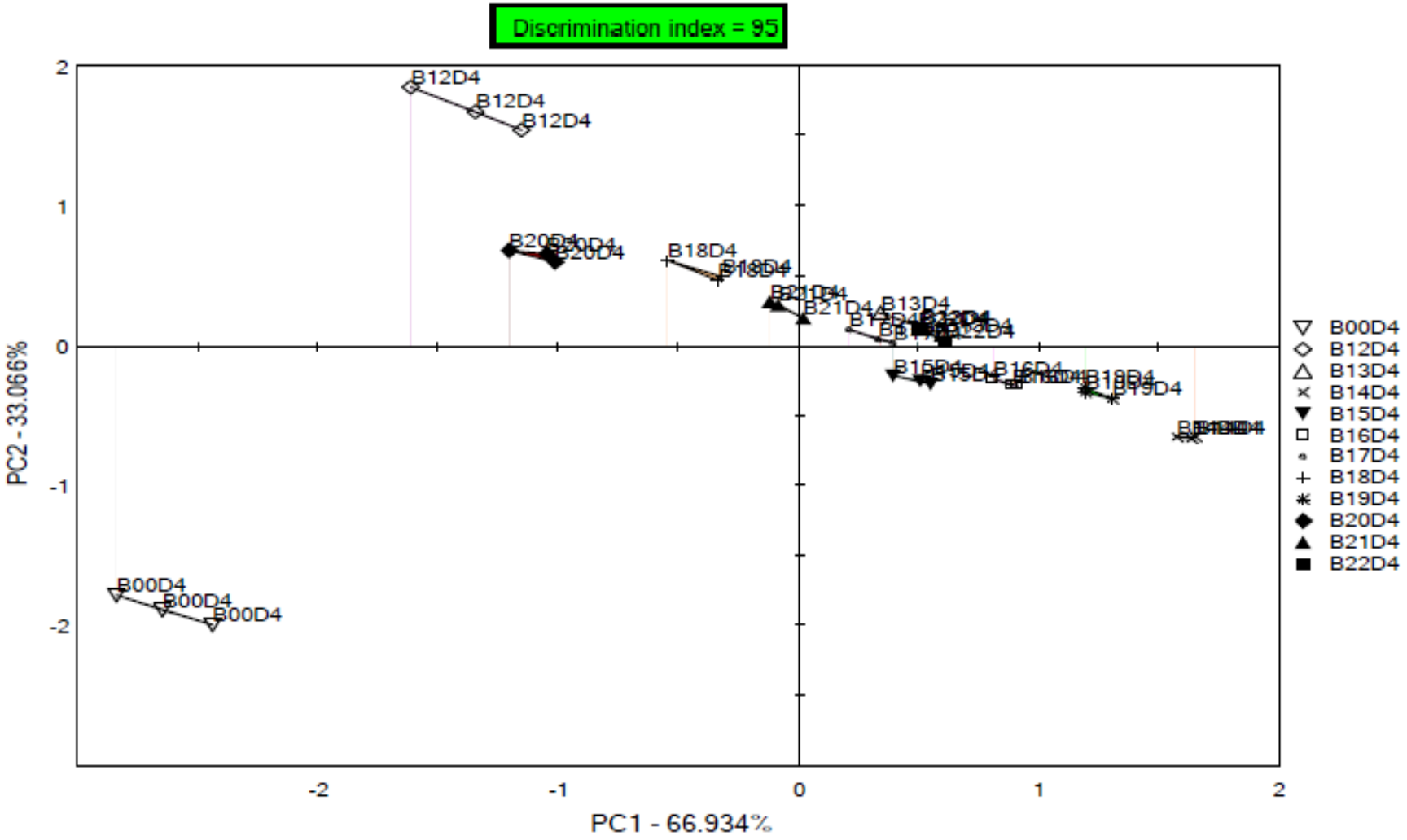

Figure 53. PCA scores plot of group 2 bacterial samples (B12- B22 with B0 as control) after 96 h of inoculation (D4), showing fast and high discrimination index value of 95 between groups. PC1 and PC2 explain 100% of the total variation.

The End