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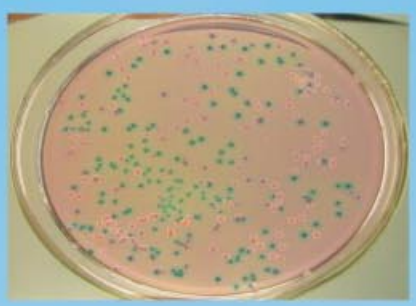



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The Viable but Non-culturable State in *Xanthomonas citri* subsp. *citri* is a Reversible State Induced by Low Nutrient Availability and Copper Stress Conditions

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Abstract: *Xcc* (*Xanthomonas citri* subsp. *citri*) causes citrus bacterial canker, a leaf, stem and fruit spotting disease that affects most commercial citrus species and cultivars. Copper compounds, widely used for management of this pathogen, have been reported as inducers of a VBNC (viable but non-culturable state) in plant pathogenic bacteria. VBNC may be considered as a state preceding bacterial death or as a survival mechanism under adverse conditions. Several experiments were performed to characterize the reversibility and persistence of the VBNC state in *Xcc*. VBNC was induced in low nutrient medium or with amendment of copper at concentrations used for field disease control. The VBNC condition was demonstrated to persist up to 150 days after copper treatment and was reversed after the addition of culture media without copper or amendment with citrus leaf extract. *Xcc* viability was evaluated by recovery of colonies on culture media, confirmed by membrane integrity, respiratory activity and by real-time RT-PCR targeting a sequence from the *gumD* gene. Besides, the colonies recovered were pathogenic on citrus leaves. These results confirm that the VBNC state in *Xcc* is inducible and reversible and therefore may occur in the phyllosphere when *Xcc* is under copper stress or starvation.

Key words: VBNC, resuscitation, *Xanthomonas citri* subsp. *citri*.

1. Introduction

CBC (citrus bacterial canker), caused by *Xcc* (*Xanthomonas citri* subsp. *citri*), is one of the major citrus diseases affecting most commercial citrus species and cultivars, and it is a serious leaf, stem and fruit spotting disease [1]. In areas where CBC is present, integrated control of the disease relies on the use of *Xcc*-free plant material, the use of cultivars with low susceptibility, adequate orchard management and the control of CBC epidemics by employing wind breaks and applying foliar sprays of copper formulations [2, 3].

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Although copper is an essential cofactor of many enzymes involved in bacterial metabolism, it is toxic at high concentrations [4], acting as a respiratory poison or by reacting with many bacterial proteins [5, 6]. Copper resistance in Gram negative bacteria, including *Xcc*, is commonly acquired by horizontal transfer of the resistance genes on plasmids as a consequence of repeated exposure to copper sprays [6-8]. On the other hand, bacterial cells cope with environmental stresses by various survival mechanisms [9, 10]. One of these mechanisms in Gram negative bacteria is to enter into the VBNC (viable but non-culturable) state [11], first demonstrated by Colwell and coworkers in *Vibrio cholerae* [12] and later described in several plant

pathogenic bacteria such as *Agrobacterium tumefaciens* [13], *Xanthomonas campestris* [14], *Ralstonia solanacearum* [15], *Erwinia amylovora* [16] and more recently in *Xcc* [17]. VBNC was described as a state which bacteria enter when facing adverse conditions [18]. Bacteria in the VBNC state remain viable but fail to grow on standard culture media [11, 19-21]. In the VBNC state, bacteria exhibit low metabolic activity [22], and are less susceptible to environmental stresses [23]. Many factors induce this dormant condition, as high or low temperatures, osmotic pressure, oxygen concentration, nutrient limitation, radiation or chemical substances [11, 12, 22].

Since the first description of the existence of the VBNC state as an ecologically relevant survival strategy it has been under debate. Some authors considered it as a simple non reversible state previous to cell death and without any epidemiological significance [11]. On the other hand, molecular studies performed in recent years support the claim that bacterial cells adopt the VBNC state as a survival strategy and for example, overexpression of several genes involved in protein synthesis have been detected in VBNC cells in *Vibrio cholerae* [24]. In order to consider the VBNC condition as a survival mechanism, two main requirements must be met: reversibility, ability of cells to resuscitate, and maintenance of the condition for as long as the stress continues, persistence. Resuscitation and persistence of VBNC cells are essential characteristics to discriminate this condition from a step preceding bacterial death that have already been demonstrated in several bacterial models [16, 25].

Although the VBNC condition has recently been reported in *Xcc* by del Campo et al. [17], recovery of culturability was not demonstrated, leaving the biological significance of this bacterial state unanswered. In addition, the non culturable state described by these authors was induced by short term exposure using a copper concentration higher than occurs as a result of copper spray applications in orchards [17].

Consequently, to assess the reversibility and persistence of VBNC and to provide evidence for metabolic and gene activity for *Xcc* cells in the VBNC state, the aim of this work was to study the effect of nutrient starvation and copper as inducers of VBNC, at concentrations that are encountered in the phyllosphere of citrus trees.

2. Materials and Methods

2.1 Bacterial Strains and Growth Conditions

Strain 306 of *Xcc* from the Collection of Plant Pathogenic Bacteria of IAPAR (Instituto Agronômico do Paraná, Brazil), for which the complete genome sequence is available [26], and strain CFBP 2911, from the Collection Nationale des Bactéries Phytopathogènes (France), were used in all the assays. Both 306 and CFBP 2911 are Asiatic type (A) canker strains, and were isolated from different geographic areas (Brazil and Pakistan, respectively). Bacteria were maintained on Yeast Peptone Glucose Agar (YPGA) medium [27] and grown for inoculum preparation in Luria Bertani (LB) broth. LB cultures were incubated under shaking at 26 ºC for 48 h and concentrations were monitored by optical density at 600 nm.

2.2 Copper and Nutrient Limitation Assay

Batch cultures were prepared in 250 mL flasks with sterilized AB (1 g/L NH_4Cl, 0.3 g/L $MgSO_4$, 0.15 g/L KCl, 0.01 g/L $CaCl_2$, 2.5 mg/L $FeSO_4$, 3 g/L K_2HPO_4 and 1 g/L NaH_2PO_4) and supplemented with $CuSO_4$ at 0.5, 5, 10 and 50 μM. Medium not supplemented with copper was used to determine the starvation effect on the VBNC condition. AB medium was used because of its minimal ability to bind copper ions [16]. An exponentially growing suspension of 10^7 CFU/mL of *Xcc* strain, 306 or CFBP 2911, was added to each batch culture and kept at 26 ± 2 °C for 5 months in the dark. Two replicates were performed for each strain.

2.3 Culturability of Xcc Cells in Batch Cultures

Culturability of *Xcc* in batch cultures was determined by plating and recovery of colonies on YPGA medium. An aliquot of 1 mL of each bacterial suspension was regularly pelleted from each treatment during a five-month period, resuspended in 1 mL of PBS at pH 7 and 100 µL plated in triplicate on the medium. CFU (colony forming units) were counted from 2 to 7 days after incubation at 26 °C.

2.4 Membrane Integrity and Respiratory Activity of Xcc in Batch Cultures

To determine membrane integrity, bacterial cells were harvested and stained with BacLight Live/Dead kit (Molecular Probes, Inc., Eugene, OR, USA) as follows: 500 µL of *Xcc* from the batches were mixed with 500 µL of a mixture of 300 µL of SYTO-9 and 200 µL propidium iodide, and incubated in the dark for 15 min. The suspension was then filtered through a 0.22 µm pore filter, and green and red cells were counted and classified as live or dead using an epifluorescence microscope (Nikon ECLIPSE E800, Tokyo, Japan) under 100× magnification [28]. Twenty microscopic fields were evaluated for each sample and the average of viable and non-viable cells calculated for comparative purposes.

The alamarblue fluorometric cell viability assay (Invitrogen, Rockeville, MD), was applied for evaluation of respiratory activity as previously described [29]. Microtiter plates were amended with 100 µL alamarblue reagent, inoculated with 100 µL of bacterial suspension from the batches and incubated overnight at room temperature in the dark before color evaluation. Bacterial suspensions with active or viable cells turned pink while those with non active or non viable cells turned blue. Ten replicates per treatment were performed. Wells without amended bacteria were used as negative controls, whereas the bacterial suspensions at 10^5 CFU/mL without copper and with nutrient deprivation were used as positive controls. All the experiments were independently repeated.

2.5 Detection of mRNA from gumD in Batch Cultures

Viability of the bacterial population in batch cultures was also evaluated by rt-RT-PCR detection of mRNA from *gumD* gene. Presence of a 445 bp transcript from *gumD* gene was monitored by real time-RT-PCR as previously described [30, 31], before and after bacteria were treated to induce the VBNC state. Total RNA extractions were performed from bacteria in the batch cultures using the RNeasy mini kit (Qiagen, California, USA) and treated with Turbo DNase free following the manufacturer's instructions (Ambion, USA). Purified RNA samples were used for reverse transcriptase rt-RT-PCR reaction or stored at -80 °C until further use.

Amplifications were carried out by adding five µL of the extracted RNA at 0.1 µg/µL concentration to a reaction mixture containing 12.5 µL of 2× Quanti probe master mix (Qiagen), 0.25 µL Quanti RT-mix, one µL of 10 µM of *MG-GumD1F* (CAT TGC AGT GGG CGT CAA GT) and *MG-GumD1R* (TCG ACC AAC GGC GGA TGT AGT) primers, and 0.5 µL of 10 µM of the *J-GumD TaqMan* probe (FAM-AAT GGT TTC CGT GGC GAG ACG–TAMRA) in a total volume of 25 µL. Control RNA samples were not supplemented with reverse transcriptase to check for DNA contamination. Amplifications were run in a SmartCycler (Cepheid, USA) with an initial reverse transcription step of 30 min at 50 °C, an activation step of 15 min at 95 ºC followed by 40 cycles at 94 °C for 15 s and 60 °C for 1 min.

To study the evolution of a *Xcc* population in VBNC condition, total RNA was extracted from 1 mL suspensions from the batch cultures at three stages: immediately after bacterial addition to the batches, when 100% of the bacterial population was confirmed as non culturable (t = 0), and after 10 days (t = 10). Average Ct was obtained from each RNA extraction and statistically analysed to determine differences among exposure times and copper concentrations.

2.6 Resuscitation Assays

Ability of different compounds such as glutamate,

citrate, asparagine, fructose, glucose, EDTA and phosphate buffer, all at 100 μM concentration, or three culture media (Wilbrink, WB [32], YPG or LB), as well as citrus leaf extract, was evaluated for complexing copper ions using Microquant copper kit following manufacturer's instructions (Merck, Darmstadt, Germany). To obtain citrus leaf extract, 100 mg of grapefruit leaf *cv*. Duncan (*Citrus paradisi*), previously washed with sterile water and disinfected with 70% ethanol, were grounded in 5 mL of PBS and thereafter filter sterilized.

The compounds with the highest copper chelating activity were evaluated to reverse the VBNC condition in the batch cultures in which 100% of the bacterial population was confirmed as non cultivable. 1 mL from these batch cultures was added to 9 mL of each compound at 100 μM concentration or culture media or grapefruit leaf extract and incubated at room temperature for one week under shaking at 1.000 g/min, as shown in Table 1.

After seven days, CFU were determined by colony counting on YPGA plates as described above. To demonstrate that the resuscitation was not the result of a regrowth from a few undetected culturable cells, dilution series were performed according to the method described in a previous work [33]. VBNC and resuscitated cells were tested for pathogenicity in assays conducted by injection-infiltration of detached grapefruit leaves as described in Ref. [34]. Negative controls consisted of AB medium without bacteria, whereas positive controls included a suspension of strain 306 of *Xcc* in PBS at 10^7 CFU/mL.

To evaluate the period of time that *Xcc* could persist in a reversible VBNC condition, a time course resuscitation experiment was performed from the batch culture at 5 μM copper sulfate. Cultivability recovery was attempted, as described above, adding as chelating agents LB, YPG or WB culture media, glutamate, citrate or asparagine as chemical compounds or citrus leaf extract from grapefruit.

Cultivability was evaluated 90 days after bacterial batch inoculation, when the entire population was firstly considered in a non cultivable condition and no chelating agent was added, and after adding the chelating agents 3, 5, 10, 20, 30, 60 and 90 days later.

2.7 Statistical Analyses

In all assays data of total, viable and culturable cell counts were transformed to log. Mean comparisons in each point were done to determine significant differences ($P < 0.05$) among viable, culturable and total cells in the batch cultures with different copper sulfate concentrations and for the Ct average obtained after *gumD* amplification from RNA of bacterial suspensions at different time periods. Data from the samples were subjected to analysis of variance and the means separated by Student-Newman-Keuls procedure. All statistical analyses were performed using Statgraphics Plus for Windows 4.1 (Statistical Graphics, Rockville, MD, USA).

3. Results

3.1 Induction of VBNC in Xcc

To examine the induction of the VBNC condition,

Table 1 Resuscitation of VBNC cells with chelating agents in different copper concentrations after seven days incubation.

Chelating compounds	Copper concentration (μM)			
	0.5	5	10	50
LB medium	+	+	-	-
YPG medium [27]	+	+	-	-
WB medium [32]	+	+	-	-
EDTA[a]	+	-	-	-
Sodium glutamate	+	+	+	-
Sodium citrate	+	+	+	-
Asparagine	+	+	+	-
Grapefruit leaf extract (1/100)	+	+	+	+
Phosphate buffer	+	-	-	-

+: Appearance of one or more colonies on YPGA plates; -: No colonies on YPGA plates; [a]Ethylene diamine tetraacetic acid.

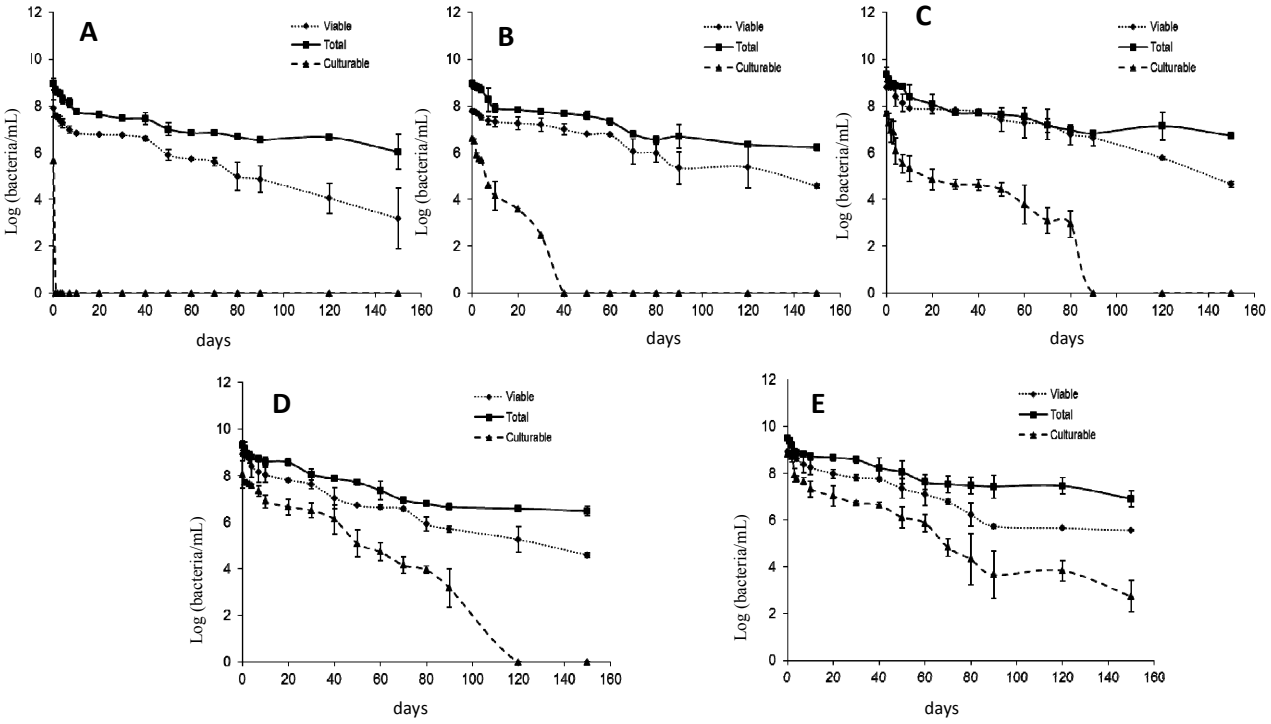

Fig. 1 Five-month time-course of *X. citri* subsp. *citri* population in AB medium with copper sulfate at 50 (A), 10 (B), 5 (C), 0.5 μM (D) concentrations or without copper (E). For culturable cells graphs show the average of the three replicates for each dilution and time. For total and viable cells, each point was the average of the counts of cells in 20 microscopic fields. Error bars represent standard deviation of the means. Data of each time were subjected to analysis of variance to determine significant differences ($P < 0.05$) among viable, culturable and total bacterial populations.

culturable, viable and total bacterial populations were evaluated in batch cultures of two *Xcc* strains grown under different copper concentrations in time course experiments lasting 150 days, as shown in Fig. 1. Since no significant differences were obtained ($P > 0.05$) between the two *Xcc* strains, data for each strain were combined for analysis.

From an initial concentration of 10^8 bacteria/mL of viable *Xcc* cells added to the batch, around 10^2 CFU/mL became unculturable immediately after exposure to 50 μM of copper sulfate. No culturable cells were detected 24 h later, although green fluorescent bacteria, and therefore putative VBNC cells at 10^7 cells/mL concentration, were observed under fluorescence microscopy (Fig. 1A). After 150 days at 50 μM of copper sulfate, VBNC cells were still detected at a concentration of around 10^3 cells/mL. In the entire batch cultures amended with different copper sulfate concentrations at different time periods 100% of no culturable cells were detected. However, viability of bacteria was confirmed by membrane integrity and respiratory activity from all the batch cultures after 150 days (Fig. 1). Moreover, culturable bacteria were found from the cultures treated with copper sulfate at concentrations of 10, 5 and 0.5 μM and from the control without copper sulfate after 30, 80, 90 and 150 days, respectively (Figs. 1B-1E). Therefore, viable bacterial populations, at those time periods and copper sulfate concentrations, were encompassed by culturable and VBNC cells in variable proportions. In addition, at 50, 10 and 5 μM of copper sulfate, significant differences ($P < 0.05$) were observed between the average number of viable and culturable cells immediately after inoculation, demonstrating rapid induction of VBNC in a fraction of the bacterial population (Figs. 1A-1C). At the lowest concentration (0.5 μM copper sulfate), the

appearance of VBNC cells was observed after 24 hours (Fig. 1D) and even without copper sulfate in the medium, the presence of VBNC cells was observed after 72 h (Fig. 1E).

3.2 Resuscitation of VBNC Xcc Cells

Low nutrient supply or copper ions acted as VBNC inducers; consequently, the addition of nutrients as well as reducing the copper concentration or its removal might reverse the VBNC state to a culturable condition. To test this hypothesis, different chelating compounds and various culture media were tested in order to induce resuscitation of VBNC cells.

First, the potential of several compounds as chelating agents to complex copper ions was evaluated using the Microquant kit. All compounds analyzed were able to complex copper, reducing the amount of this ion in solution. Citrus leaf extract and several culture media were able to bind 100% of the soluble copper in concentrations from 0.5 μM to 50 μM. Percentage of copper capture was variable for other compounds: glutamate was able to remove from 20% to 70% of copper, fructose and glucose around 20%, and phosphate buffer was able to bind 50% of soluble copper, as shown in Fig. 2.

Culture media and those compounds that showed higher chelating capacity were selected to recover *Xcc* cells from the VBNC condition. In addition, other chelating agents such as EDTA, asparagine and citrate, previously used in other studies on *E. amylovora* resuscitation [16], were also evaluated. Cell resuscitation was always completed when total bacterial population in the batches was established to be in a VBNC state by colony counting on culture plates and viability confirmed by membrane integrity and respiratory assays. Consequently, samples from each batch containing different copper concentrations were analyzed at different time periods, as shown in Table 1.

Resuscitation from batch cultures amended with copper sulfate at concentrations of 0.5 μM and 5 μM was observed using LB, YPG and WB culture media. Using EDTA, culturable bacterial cells were recovered only from 0.5 μM copper sulfate batch cultures. Glutamate, citrate and asparagine resuscitated *Xcc* from batches with copper sulfate at 0.5, 5 and 10 μM concentration. Interestingly, grapefruit leaf extract was the most effective for reversing VBNC condition since culturable cells could be recovered from batches treated even with 50 μM of copper sulfate.

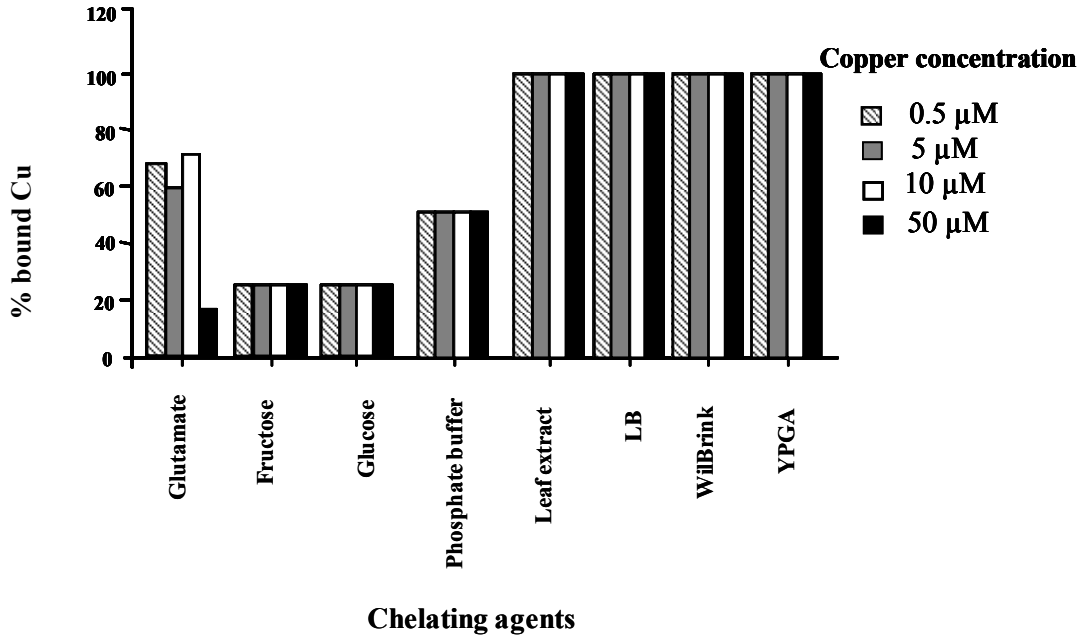

Fig. 2 Copper complexing ability of several compounds at 0.5, 5, 10 and 50 μM copper concentrations in AB medium.

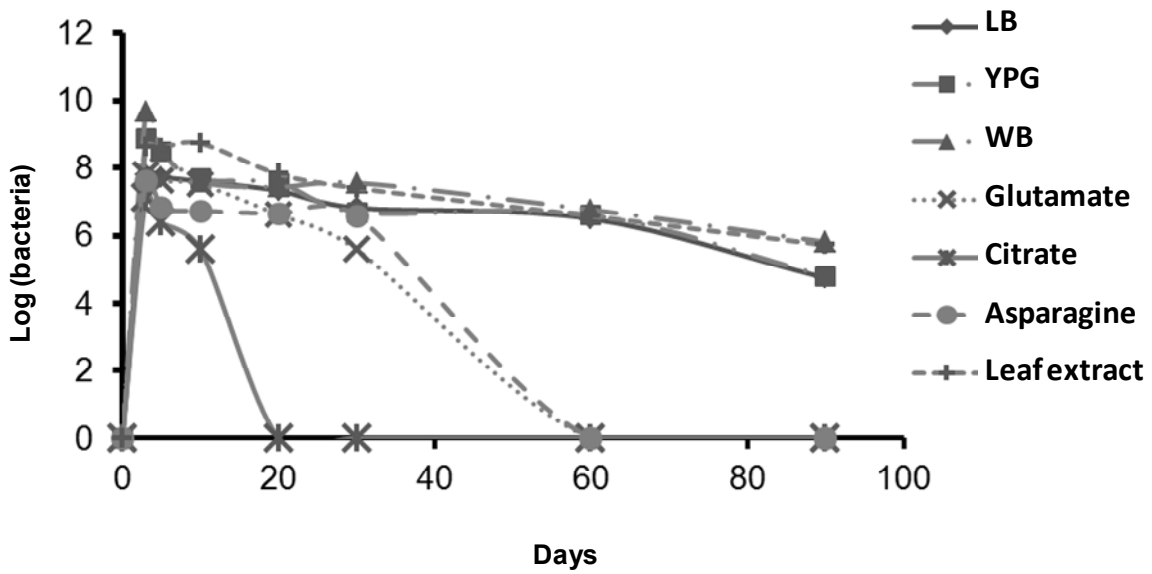

Fig. 3 Recovery of cultivability from *X. citri* subsp. *citri* VBNC cells treated with copper sulfate at 5 μM concentration after the addition of several chelating agents and culture media. At time 0 the bacterial population was 100% non-culturable and no chelating agents were added.

In all assays, identification of the resuscitated bacteria was confirmed by recovery of colonies on culture media and detection of the 445 bp *gumD* fragment by real-time PCR [31]. In addition, pathogenicity of resuscitated cells was confirmed in detached grapefruit leaves. Moreover, *Xcc* cells from batches at 0.5, 5 and 10 μM copper sulfate concentrations and in the VBNC state were able to induce symptoms in plant leaves after addition of chelating agents.

As shown in Fig. 3, cultivable cells were recovered after the three months experiment when culture media or leaf extract were used for resuscitation, although a significant reduction in recovery of cultivable bacteria was shown through the time. Using citrate and asparagine for resuscitation, cultivability could be recovered only 30 days after VBNC induction and with glutamate just after 10 days (Fig. 3).

3.3 Determination of Bacterial Population Viability by Detection of a GumD RNA Fragment by rt-RT-PCR

The *gumD* fragment of 445 bp from the mRNA transcript was always detected in batch cultures in which the presence of VBNC cells had been onfirmed by membrane integrity and respiratory assays (Fig. 4). Variation in threshold cycles (Ct), and therefore in viable bacteria concentration, was related to the period of time exposed to copper as well as to the copper concentration in the batch. Cts obtained by rt-RT-PCR from bacterial RNA in batch cultures were always lower at the initial stage of the assay, when culturable bacteria were still present, revealing a higher concentration of viable cells (Fig. 4). Significantly higher Ct average ($P < 0.05$) and therefore, a lower concentration of viable bacteria was shown in all the batches when culturable cells were not found. No significant differences were found between Cts obtained from the sample at t = 0 (first time 100% bacterial population in the batch was identified as non cultivable) and t = 10 days from the batches without copper or with 0.5 μM and 5 μM copper concentration, indicating stabilization of the *Xcc* population in the VBNC condition. However, differences in Ct averages were observed between t = 0 and t = 10 days when bacteria were exposed to 10 μM and 50 μM of copper sulfate, suggesting mortality of a proportion of the bacterial population under these copper concentrations (Fig. 4).

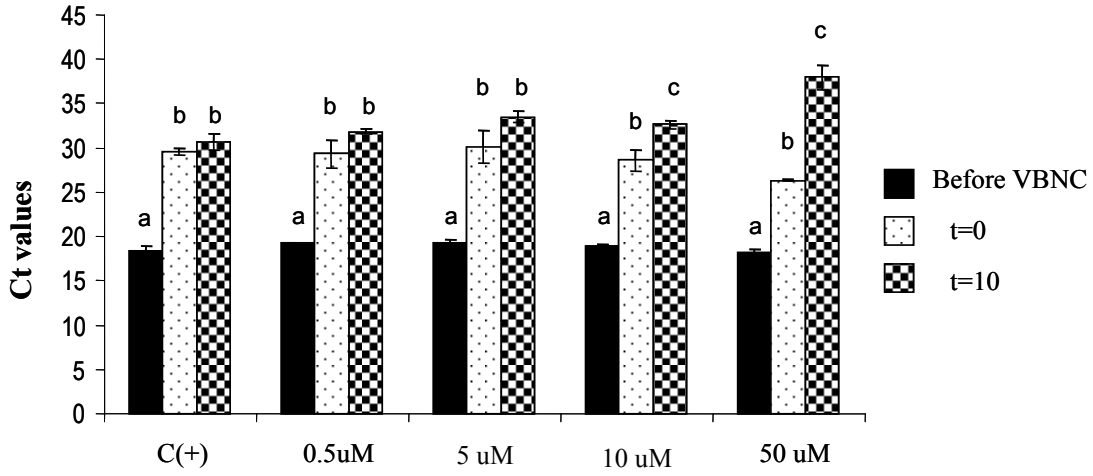

Fig. 4 Average threshold cycles (Ct) resulting after rt-RT-PCR from *gumD* mRNA obtained from *X. citri* subsp. *citri* batches with copper at different concentrations. rt-RT-PCR was performed from RNA extracted immediately after bacteria addition to the batch (before VBNC), when the total population was first considered non cuturable (t = 0) and 10 days after this stage (t = 10). The graph shows the average of at least two PCR reactions for each sample and error bars display standard deviation. Means with the same letter within a sample do not differ significantly according to the Student-Newman-Keuls multiple range test ($P < 0.05$).

4. Discussion

Many factors have been described to induce the VBNC condition in bacteria [11, 12, 22]. Exposure to chemical substances, such as copper, commonly used to control citrus bacterial diseases [3, 35], has been reported to induce VBNC in several Gram negative plant pathogenic bacteria [14, 16] including *Xcc* [17]. However, in this bacterium VBNC has been described after short-term treatments with copper at high concentrations, equivalent to three times the free soluble copper concentration applied in regular field treatments [17]. Moreover, reversibility of the putative VBNC condition in *Xcc* was not assessed in the aforementioned study. Hence the two most important assumptions to consider VBNC as a survival strategy were not fulfilled in the CBC pathogen. By contrast, our work demonstrates that the non culturable state of *Xcc* cells, induced by starvation or low copper exposure, endured while the adverse conditions continued but could be reversed when the environmental stress stopped. The ability of copper sulfate to induce the VBNC condition in *Xcc* was demonstrated and monitored for a five-month period and the reversion of this state demonstrated after 90 days, when the stressful conditions were removed by the addition of chelating components or supplementary nutrients. Furthermore, in the absence of copper, low nutrient availability alone was enough to induce the VBNC state in *Xcc*, although copper clearly sped up the metabolic adjustment to this condition. The higher the concentration of copper in the medium, the faster *Xcc* entered the VBNC state and slighter the culturability recovery. This effect of copper combined with starvation was confirmed for *Xcc* as previously described for *X. campestris* pv. *campestris* [14].

It is important to point out that the studies on VBNC condition were performed with copper concentrations more likely to be encountered by *Xcc* in the field than those concentrations previously employed by del Campo et al. [17]. Moreover, and in order to avoid overestimation of VBNC population, cultivability was assayed on media plates incubated for more than four days, considering that *Xcc* colonies

under stress conditions may need longer to develop as compared to bacteria without environmental pressure. It is also remarkable that, in this study, viability of bacterial cells in the batches was evaluated by three independent methodology approaches: membrane integrity, respiratory assays [28, 29] and detection of an mRNA fragment from *gumD* gene, recently described as a viability marker for *Xcc* [31]. The results with *gumD* also showed that part of the putative VBNC population had to be considered as simply a prior step to cell death, as described in other models. The proportion of cells in actual VBNC state or in a prior step to cell death was influenced by copper concentration.

As the resuscitation of a proportion of non-culturable cells in the batch cultures was achieved by the addition of nutrients or removal of copper with several compounds and leaf extracts, the existence of an authentic VBNC condition for *Xcc* was confirmed. The reversal of the VBNC state was apparently due to sequestration of copper ion. Both citrus leaf extract and culture media were able to facilitate viable bacteria to recover culturability after the total population entered into the VBNC. As described in *E. amylovora* [16] this result may be due, at least partially, to a combined effect of the complexing activity and nutrient availability of the plant extract or culture media to the bacteria. Herein this synergistic effect was demonstrated by the fact that some compounds with lower complexion ability, such as glutamate, were more effective than others like EDTA with a higher copper-ion capturing efficiency [16]. In addition, not all the substances were able to chelate 100% yet showed the same effect on recovery from VBNC; for example the culture media tested did not produce the same effect as citrus leaf extracts, despite having the same copper-ion capturing ability or being a clear source of bacterial nutrients. The results indicate that reversion of the VBNC condition in *Xcc* is complex and based on the combined effect of complexing of copper and nutrient availability. On the other hand, the ability of citrus leaf extract to act as a strong copper chelating agent is interesting because it suggests that *Xcc* and other bacteria may be exposed to lower amounts of copper ions than initially expected after plant or fruit control treatments employed in the citrus industry.

5. Conclusion

Copper compounds in concentrations similar to those employed in the field, as well as low nutrient conditions, have proven to be inducers of a long term VBNC state in *Xcc*. The VBNC state has been confirmed as a reversible survival strategy that can last for months, giving the pathogen a powerful tool to overcome stressful conditions. The reversibility and persistence of the VBNC condition has been demonstrated in this work as a first step, although the epidemiological significance of this state in *Xcc* in an ecologically relevant context, ie., in situ, on plant surfaces, or even in survival structures such as biofilms, described for *Xcc* [36], need to be evaluated further. Such information will help to establish effective preventive measures to avoid the introduction of *Xcc* in CBC-free areas, and must be considered for new control strategies in areas where the disease is endemic.

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Chemical Characterization and Insecticidal Activity of Ethyl Acetate and Dichloromethane Extracts of *Drypetes gossweileri* against *Sitophilus zeamais*, *Tribolium castaneum* and *Rhyzopertha dominica*

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Abstract: The use of botanical pesticides in pest management during storage against insects is often encouraged because synthetic insecticides produce undesired effects. Bark powder of *Drypetes gossweileri* is used by farmers in pest management in Central Africa Republic. Hexane, dichloromethane, ethyl acetate and methanol were used for extraction bark powder by maceration. Insecticidal activity of the extracts was tested on *Sitophilus zeamais*, *Tribolium castaneum* and *Rhyzopertha dominica*. The mortality rate, the number of surviving insects and the number of emerged insects were measured variables. Calculated mortality was obtained by applying Aboth's formula. The variables are submitted to variance analysis, model with four factors (plants, insects, doses and time) in Minitab 14. The results of analysis showed that the mortality rate has a very highly significant variation following extracts, doses, insects and time considered ($P < 0.001$). Extracts with ethyl acetate and dichloromethane of *D. gossweileri* are effective against *S. zeamais* and *R. dominica* at high doses (1, 0.5, 0.250 g/10 mL of solvent). These effective extracts were characterized by gas chromatography coupled with mass spectrometry. Molecules such as 1-isothiocyanatomethyl, thiocyanic acid, phenylmethyl ester and other were characterizated. These results may consolidate traditional use of *D. gossweileri* in pest management.

Key words: Control, stored grains, pests' management, *Drypetes gossweileri*.

1. Introduction

Food security has remained the primary agenda in Africa food policies, and Central Africa Republic (CAR) is of no exception. Post-harvest losses are recognized to be one of the critical constraints upon food security among resource poor farmers in Africa. In Africa, cereals (maize, millet and sorghum) and legumes (cowpeas, beans and groundnuts) are the main crops and the basics of food self-sufficiency objective defined in most countries [1]. In sub-saharan Africa, the post-harvest losses of grain are estimated from 10% to 20% and represent about $4 billion of total cereal production in these countries [2]. Laboratory

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evaluation showed that when maize was unprotected, damages and losses due to *Sitophilus zeamais* (Motschulsky) reached 20% and 40% respectively in 4 and 8 months of storage [3]. The control of insect pest of stored products (mainly *Sitophilus zeamais*, *Tribolium castaneum* and *Rhyzopertha dominica*) was almost exclusively based on the use of fumigants (methyl bromide and phosphine) and insecticide dust [3]. The numerous cases of poisoning and environmental hazards and insufficient financial resources of African farmers have drawn attention safety means of control pest [4]. Inferences from indigenous traditional practices have uncovered plant chemicals that are useful in pest management, for example, azadirachtin from *Azadirachta indica*, rotenone from *Derris elliptica*, pyrethrum from *Chrysanthemum cinerariaefolium* and nicotine from *Nicotiana tabacum* [5-7]. The anti-pests plants are not fully documented despite their wide use by farmers in Africa. In CAR, crude extracts of natural plants are used in small stores by many farmers to fight against grain insects [8].

Traditionally, African farmers would use various forms of cultural practices and herbal products in pest management and local communities still continue to use an array of insecticidal plants for the control of specific pests [9, 10]. Many studies have been conducted on the basis of use of plants or extracts. *Drypetes gossweileri* (*D. gossweileri*) has been used in traditional medicine for treating fever, malaria, intestinal worms in Central Africa [11, 12]. It was found that the extract of *D. gossweileri* leaves has multiple biological activities, inhibiting the activity of Acetylcholinesterase [13]. Antifungal activity and phytochemical analysis of *D. gossweileri* were studied [14]. *D. gossweileri* is often used in Boukoko (Central African Republic) during postharvest to protect food against pests but there is no study on the pest management of *D. gossweileri* in relation with *Sitophilus zeamais*, *Tribolium castaneum* and *Rhyzopertha dominica*. The aim of the present study is to evaluate the chemical and insecticidal activities of the extract of bark of *D. gossweileri* in a laboratory to verify the traditional use of this indigenous plant in pest management.

2. Material and Methods

2.1 Plant Material, Solvents and Method of Extraction

2.1.1 Plant Material

The bark of *D. gossweileri* was collected from Boukoko in CAR in 2012 and authenticated by the researchers of the forestry Ministry of CAR and botanical Professors of University of Bangui (CAR) respectively. The plant is located in Forafrica site to number 436 [15]. *D. gossweileri* bark was dried in the shade and grounder to powder after.

2.1.2 Solvents and Method of Extraction

n-Hexane (Reag.USP, Ph.Eur.) PA-ACS; Mininum assay (G.C.): 99.0%; Identity: IR p/t.; Density at 20/20: 0.659-0.663; Refractive index n20/D: 1.375-1.376; Distillation range (> 95% dist.): 67-69 °C;

Dichloromethan stabilized with amylene PA-ACS-ISO; Mininum assay (G.C.): 99.5%; Identity: IR p/t.; Density at 20/4: 1.323-1.325;

Ethyl Acetate PA-ACS-ISO; Mininum assay (G.C.): 99.5%; Identity: IR p/t.; Density at 20/4: 0.9000-0.902;

Mathanol (Reag.USP, Ph.Eur.) PA-ACS-ISO; Mininum assay (G.C.): 99.8%; Identity: IR p/t.; Density at 20/4: 0.791-0.792.

100 g of powder of *D. gossweileri* has been dissolved in 500 mL of Hexan for 5 days in the laboratory. The final hexane extract was recovered after concentration on evaporator at 30 °C. The spent residue of hexane has been dried in air for 2 days and macerated in dichloromethane. Similarly, the exhausted residue of dichloromethane has been macerated in ethyl acetate whereas the spent residue in ethyl acetate has been macerated in methanol [16, 17].

2.2 Insects Rearing

Adults of *S. zeamais*, *T. castaneum* and *R. dominica* were respectively collected from corn, millet and

sorghum in farms and were reared respectively on 100 g of corn, millet and sorghum in laboratory at 27 ± 2 °C and 10 ± 70 r.h.. 7 days after the spawning, adults are eliminated and eggs have evolved to give the first generation of adults from 40 to 45 days after infestation. These adults of the first generation were reared in the same conditions above. 7 days after the spawning adults are eliminated and eggs have evolved to give the second generation adults from 40 to 45 days after infestation. These adults of the second generation (both sexes) used in bioassays were all 1 to 2 days old [18, 19].

2.3 Bioassays

Bioassays tests were conducted at the Regional Centre for Ecotoxicology Studies and Environment Security (CERES-Locustox) in Senegal. The aim of bioassays is to identify effective extracts of *D. gossweileri* against *S. zeamais*, *T. castaneum* and *R. dominica*. Every extract has six doses (1, 0.5, 0.250, 0.125, 0.62 and 0.03 g/10 mL of solvent) and every dose had been tested with five repetitions. Theses doses were obtained by cascading dilution [20]. Experimental units were Petrie glass of 90 cm of diameter containing 20 g of grain (maize, millet or sorghum), infested with 25 insects and applied 1 mL of different doses. Insects are put in contact with treaded grains after having evaporated the solvent for 5 min from the hexane and dichloromethane extracts and 25 min from ethyl acetate and methanol extracts. Experimental units were randomly divided into four groups according to the treatments [21]. Insects group of control were exposed to pure solvent in the same laboratory conditions at 27 ± 2 °C and 10 ± 70 r.h.. Dead insects were removed using forceps every 5 days during 35 days. Corrected mortality (CM) is obtained by Abbott formula [21]: CM = (TM - CM)/(100 - CMt) × 100 (TM = treated mortality, CM = control mortality).

2. 4 Statistical Analysis

Variable mortality is submitted to variance analysis, assigned model with four factors (plants, insects, doses and time) [22]. Variable mortality was transformed to sin arc (x = mortality rate, n = size of population, n = 2519) to normalize the population and stabilize the variance. General Linear Model in Minitab 14 was used for statistical analysis.

2.5 Chemical Analysis

2.5.1 Material and Conditions

- Silica Gel, spe 7086-06,
- Colonne HP5MS: 30 mm × 0.250 mm sur 0.25 µL,
- Detector (190-400 nm),
- Elution solvent: Ethyl acetate,
- Temperature: 325 °C,
- Gaz: Heluim,
-Volume d'injection: 5 µL,
- GCMS 5973 Agilent.

2.5.2 Method

Gas chromatography coupled with a mass spectrometer was used in chemical characterization [23]. 0.5 g of each sample was dissolved in 25 mL of the solvent of extraction. The mixture was purified with a silica column and then recovered and evaporated with rotavaporator. The concentrate was recovered with 2 mL of Ethyl acetate in chromatographic tubes. 5 µL of the extract was vaporized into an injection chamber in the column heading. The column is placed in an oven whose programmed, temperature influences the retention time, it allows to further separate the mixture of compounds. At the end of the column, the compounds are identified by an electron impact detector, coupled with a mass spectrometer. The mass spectral library and based Agilent 5973 GCMS data were used to characterize moleculars.

3. Results

3.1 Results of Bioassays Tests

The results of analysis of variance on the insecticidal effect of extracts of *D. gossweileri* against *S. zeamais*, *T. castaneum* and *R. dominica* showed that the mortality rate was a very highly significant variation according to the dose, the insect and the time on the one part and according to the plant, insect and dose on the

other part ($P < 0.001$). Moreover, it's only the effect of the interaction plant insects dose was not significant ($P > 0.05$), this implies that the insecticidal effect depends on the nature of the plant, the dose and the insect, as shown in Table 1.

Fig. 1 shows the evolution of treatment based extracts, dose, time and insects. Ethyl acetate and dichloromethane extracts of *D. gossweileri* are effective against *S. zeamais* and *R. dominica* at high doses. The extracted with (1, 0.5, 0.250 g/10 mL of solvent). The mortality curve over time shows that T5, T10 and T15 the mortality rate is high, it implies that the efficacy of the extracts with Ethyl acetate and dichloromethane is limited in time.

Table 1 Results of variance analysis with extracts of *Drypetes gossweileri*.

Source of variation	Statistical result		
	DL	F	*P*
Extracts	3	18.08	0.000
Doses	5	81.42	0.000
Insects	2	97.76	0.000
Times	6	88.36	0.000
Extracts doses	15	1.67	**0.051**
Extracts insects	6	21.75	0.000
Extracts times	18	11.44	0.000
Doses insects	10	4.39	0.000
Doses times	30	4.71	0.000
Insects times	12	58.77	0.000
Extracts doses insects	30	1.19	**0.217**
Doses insects times	36	17.21	0.000
Extracts insects times	60	3.62	0.000
Error	2,286		
Total	2,519		

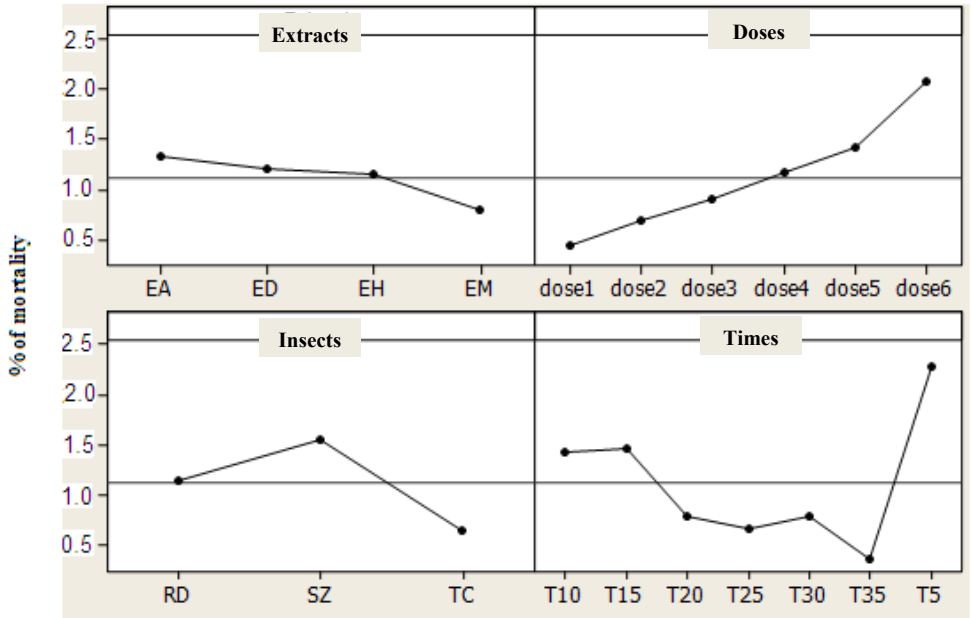

Fig. 1 Main effects plot (fitted means) for rate of mortality with extracts of *Drypetes gossweileri*.

EH = hexane extract; ED = dichloromethane extract; EA = acetyl acetate extract; EM = methanol extract; RD = *Rhyzopertha dominica*; SZ = *Sitophilus zeamais*; TC = *Tribolium castaneum*; T5 = 5 days after treatment; T10 = 10 days after treatment; T15 = 15 days after treatment; T20 = 20 days after treatment; T25 = 25 days after treatment; T30 = 30 days after treatment; T35 = 35 days after treatment.

3.2 Results of Chemical Analysis

Chemical compounds were characterized using Agilent database (5973) in the range of 50 m/z to 550 m/z (Table 2). Dichloromethane extract showed the presence of large peaks, additioned to other minor peaks. The signals for the main peak about 2.727 min (Fig. 2) match the library mass spectra to benzylamine.

GC-MS profil of the chemical compounds contained in dichloromethane extract showed the presence of large peaks 4 to 5 including other minor peaks. The signal for the main peak about 2.116 minutes in Fig. 3 match to the library mass spectra to 1,2,4-Thiadiazole, 5-amino-. The second main peak about 32.636 min match to the library mass spectra to Tricyclooctabenzene, le Benzo [4, 5] imidazo [1, 2-a] pyrimidine-3-carboxylic acid; 4-methyl-2-(thiophen -2-yl)-1,2-dihydro-, methyllamide et Oxazolo [2,3-f] purine-2,4 (1H,3H) -dione, 1,3,6-trimethyl-7-(4-methylphenyl), as shown in Fig. 3.

Table 2 Chemical characterization of dichloromethane and ethyle acetate extracts of *Drypetes gossweileri*.

Rt (min)	Chemical compounds
	Dichloromethane extract
2.110	Propane, 1-Ethoxy- Methyl-; propanamide, N-ethyl
2.367	Chloromethyl thiocyanate; 1,3-Butadiene, 2-fluoro
2.727	Benzaldehyde
3.156	Benzylamine
3.339	Benzyl alcohol
4.053	Phenylethyl alcohol; Spiro [2.4] hepta-4,6-diene
4.448	Benzene, 1-isocyano-2-methyl-; Benzyl nitrile
4.665	Phenylmethyl ester
4.945	Benzoic acid; heptanediamide, N, N'-di-benzoyloxy
6.099	Benzeneacetic acid; propanedioic acid, phenyl-Benzeneacetic acid
6.791	Tridecane
8.283	Benzene, (isothiocyanatomethyl)-; thiocyanic acid, phenylmethy ester
8.763	Tetradecane
8.934	Pentadecyl pentafluoropropionate; hexadecyl heptafluorobutyrate; heptadecyl heptafluorobutyrate
9.368	2-Octenal, 2-butyl-, 6-Octenal, 3,7-dimethyl-; (2,2,6,-Trimethyl-bicyclo [4.1.0] hept-1-yl)-methanol
9.574	2H-1-Benzopyran-2-one
10.037	Sulfurous acid, butylheptadecyl ester; octadecane, 1-chloro-; 1-Octadecanesulphonyl chloride
11.809	4-Hydroxy-3-methoxyphenylacetonitrile; 3-Ethxy-4- methoxyphenylacetonitrile
12.981	Hexanedecane
13.312	Phenol, 3,4,5,-trimethoxy-; 2,4,6(1H,3H,5H)-Pyrimidinetrione,5-ethyl-1,3-dimethyl-5-(1- methylpropyl)
13.524	2(3H)-Furonone, dihydro-5-5-dimethyl-4-(3-oxobuthyl)-; 7-Octenal, 3,7-dimethyl- Decane, 1-(ethenyloxy)
14.198	Tetrapentacontane, 1,54-dibromo-; 2-Butenedioic acid (Z)-, bis (2-methylpropyl) ester; 1-Decanol, 2-hexyl
15.101	Heptadecane; tridecane, 7-hexyl-; Tridecace, 6-propyl
15.975	Benzenemethanamine, N-(phenylmethylene)
16.415	Benzyl Benzoate; heptacosane
17.158	Octadecane
17.341	Hexadecane; hexadecane, 2,6,10,14-tetramethy-; oxalic acid, 3,5-difluorophenyl tetradecyl ester
18.399	2H-Pyran-2-one, 6-[2-E-(3-tolyl) ethenyl]-4-methoxy-2-Methyl-Z-4-tetradecene; 5-Phenyl-piperonylic acid
19.690	Hexadecanoic acid, methyl ester; pentadecanoic acid, 14-methyl-, methyl ester
20.931	n-Hexadecanoic acid; tetradecanoic acid
21.376	Cyclic octaatomic sulfur; ethane, 2-bromo-1,1-difluoro
21.536	N-Benzylbenzamide
22.839	9,12-Octadecadieoic acid (Z,Z)-, methyl ester; methyl 10-trans, 12-cis-octadecadinoate

Table 2 continued

Rt (min)	Chemical compounds
	Dichloromethane extract
22.942	9-octadecadieoic acid, methyl ester(E)-; cis-13-octadecadieoic acid, methyl ester; trans-13-octadecadieoic acid, methyl
23.417	Methyl 16-methyl-heptadecanoate; octadecanoic acid, methyl ester
23.680	9,12-octadecadienoic acid (Z,Z); 1,11-dodecadiene
24.171	9,12-octadecadienoic acid (Z,Z); 9,17-octadecadienal, (Z)-; 9-eicosyne
25.446	Bicyclo [3.1.1] hepan-3-ol, 2,6,6,-trimethyl-, 1.alpha., 2.beta., 3.alpha.,5.alpha.)-; 2H-pyran-2,4(3H)-dione, dihydro-5-ethyl-3,3-dimethyl-6-(2-fluorophenyl)
26.212	Octatriaconthy pentafluoropropionate hexatriaconthy pentafluoropropionate tetratrioacontyl pentafluoropropionate
26.754	Thiourea, (phenylmethyl)-; carbamimidothiooic acid, phenylmethyl ester; acetic acid, 2-methyphenoxy)
27.143	1-Nonadecene; tetrapentacontane, 1,54-dibromo-octatriaconthy pentafluoropropionate
27.589	Urea, N,N'-bis(phenylmethyl)-; benzenemethanamine, alpha.-methyl-, (S)-; benzenamine, N-butyl-
28.372	2,2,4-trimethyl-4-(4'-methoxyphenyl) chromane; 5-[cyano-(3,4-dimethyl-5-oxo-1,5-dihydro-pyrrol-2-ylidene)-methyl] -2,3,3-trimethyl-3,4-dihydro-2H-pyrrole-2-carbonitrile
28.801	Pyrimidine, 2-(4-butylphenyl)-5-hexyl-.±.-2, 3,8-Trioxocephalotaxane; 1-(3,4-Dimethoxy-benzylidene)-6-methyl-1H, 5H-furo [3,4-c]pyridine-3,4-dione
29.058	Benzenamine, N, N-diethyl-4-[2-(4-nitrophenyl) ethenyl]-; 3H-3a-Azacyclopenta [a] indene-2-carbonitrile, 3-oxo-1-(piperidin-1-yl)-4,5,6,7-tetrahydro-
29.178	3-acetoxy-16-pregnenolone; N-(3-Acetylamino-1-4-naphthoquinolin-2)-dihydro-1,4-oxazine; 3-methoxy-D-homoestra -1,3,5(10)-trien-17a-one (8.alpha., alpha., 14.alpha.)
30.024	Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)-17-pentatriacontene; cis-vaccenic acid
30.309	1,2-benzenedicarboxylic acid, mono (2-ethyhexyl) ester; 1,2-benzenedicarboxylic acid, disioocthy ester; bis (2-ethylhexyl) phthalate
30.744	Dodecanoic acid, phenylmethyl ester; octanoic acid, 1,2 phenylmethyl ester
31.104	Benzenz, 1,4-bis [2-(2-methylphenyl) ethenyl]- 3-(4-Methyl-biphenyl-2-yloxy)-phthalonitrile; acetamide, N-[4-[2-(2-pyridinylamino)-4-thiazolyl] phenyl]-
31.424	Thiourea, N, N'-bis (phenylmethyl)-1,3, 5-Triayine, 2-benzylamino-4-chloro-6-phenylamino-, urea, N, N-bis (phenylmethyl)-
31.590	Thiourea, N, N'-bis (phenylmethyl)-o-xylene; benzeneethanol, .alpha. .beta.-dimethyl-
31.841	2-thiazolamine, 4-(4-methoxyphenyl)-N-(4- methylphenyl)-; N, N'-bis (2-hydroxy-alpha-methylbenzylidene) ethylenediamine; p-Iodophenyl phenyl ether
32.207	Butanoic acid, 3-methyl-, 2-phenyl ethyl ester 4-Nitrobenzoylmethyl-.beta.-phenyl propionate oxalic acid, monoamide, N-(2-phenylethl)-, butyl ester
32.361	[1,2] Dithiolo [1,5-b] [1,2] oxathiole-7-SIV, 2-methyl-3-5- diphenyl-; 2-Propanone, 1-phenyl-1-(5-3H-1,2-dithiol -3-ylidene; 2,4,7-trimethyl-8-[2-[2-methylphenyl] ethenyl]-
32.681	Aspidodispermine, O-methyl-, acetate (ester); Tricyclooctabenzene; (1H)benzimidazole, 5-fluoro-2-(4-methylphenyl) -6-(4-methypiperazinl-yl)-
33.144	3-benzylidene, 2-3-dihydro-7-methyl-5phenyl-1H-1,4-bendiazepin-2-0; 2-[2,4-dimethoxybenzylidene], 2H-thiazolo [3,2-a]benzimidazol-3-one
33.304	4-pentadecyne, 15-chloro-; z, z-10,12-hexadecadien-1-ol acetat; 9, 12-octadecadienoic acid (z,z)-phenylmethyl ester
33.693	Glycine, N-benzyloxycarbonyl-, isobuthyl ester pentanoic acid, 4-methyl-, phenylmethyl ester Octanoic acid, phenylmethyl ester
34.304	Squalene; 2,6,10,14,18,22-tetracosahexane, 2,6,10,15, 9,23-hexamethyl-, (all-E)-
34.642	1,3-benzodioxole-6-carboxamide, N-(2'-benzoyphenyl)-; Phthalic acid, hex-2-yn-4-yl undecyl ester; 1H-S-Triazolo [1,5-a]pyridin-4-ium, 2-hydroxy-1-methyl-, hydroxide, (exo)-
34.796	2-phosphabicyclo [3.1.0.] hex-3-ene, 6,6-dimethyl-2,3,4-triphenyl-, (exo)-
	Ethyle acetate extract
1.653	Methanethioamide, N,N-dimethyl-; 2,3-dihydrothiophene, 1,1-dioxide
1.744	Butane, 1-ethoxy-
2.116	Propane, 1-ethoxy-2-methyl-; butane, 1-ethoxy-1,2,4-thiadiazole, 5-amino-
2.733	Benzaldehyde
3.282	Benzyl alcohol
3.825	Pentanoic acid, 3-hydroxy-methyl ester; cis-4-hepten-1-al diethyl acetal
4.048	Phenylethyl alcohol
4.156	Benzyl isocyanate; phthalimidine

Table 2 continued

Rt (min)	Chemical compounds
	Ethyle acetate extract
4.391	Benzyl nitrile; benzonitrile, 2-methyl-
4.682	Acetic acide, phenylmethyl ester
4.848	Benzoic acide; methanol, oxo-, benzoate
5.859	Thiomorpholine; 1,2,3-propanetriol, monoacetate
6.065	Benzene, (2-methoxyethyl)-; benzeneacetic acid
7.568	Benzamide
7.648	2-tert-butyl-pyrroline, 4,9-decadienoic acid, 2-nitrohyl ester, benzene, (propylthio)-
7.763	Benzenemethanol, 4-hydroxy-; benzenemethanol, 3-hydroxy-
8.163	Benzene, (isothiocyanatomethyl)-; thiocyanic acid, phenylmethyl ester
8.814	Benzeneacetamide; toluène
9.591	Acetamide, N-(phenylmethyl)-; dimethyl (1E)-N-hydroxyethanimidoyl; phosphonate
9.963	2-cyclohexen-1-one semicarbazone; ctahydro-2(1H)-quinolinone; 2-isopropoxyphenol
10.397	Benzeneacetonitrile, 4-hydroxy-; phthalimidine
11.112	Phenol, 2,4-bis (1,1-dimethylethyl); phenol, 2,6-bis (1,1-dimethylethyl)
12.969	Hexadecane
13.284	Phenol, 3,4,5-trimethoxy-; 3,4-dimethoxy-6-methylpyrocatechol; 3-thiopheneacetic acid, 5-acethyl-
13.706	Benzenemethanesulfonamide; phenylmethanesulfonylacetic acid; bibenzyl
14.804	Methanone, (1-hydroxycyclohexyl) phenyl-1,4-dioxaspiro [4.5] decan-6-ol
15.627	Tridecanoic acid, 12-methyl-, methyl ester; methyl 12-methyl-tridecanoate; methyl tetradecanoate
16.416	Benzyl benzoate
17.336	2-Piperidinone, N-[4-bromo-n-buthyl]-; 6-octadecenoic acid, methyl ester, (Z)-; 9-hexadecenoic acid, phenylmethyl ester, (Z)-
18.010	Caffeine; 2-methyl-z, z-3,13-octadecadienol; 2-piperidinone, N-[4-bromo-n-buthyl]-
18.519	Phthalic acid, isobuthyl nonyl ester; phthalic acid, isobuthyl octyl ester; phthalic acid, buthyl isohexyl ester
18.993	7,10-hexadecadienoic acid, methyl ester; methyl 7,10-hexadecadienoate;2-(1H)-naphthalenone, octahydro-4a-methyl-7-(1-methylethyl)-, (4a.alpha. 7. beta., 8a.beta.)-
19.702	Hexadecanoic acid, methyl ester; pentadecanoic acid, 14-methyl-, methyl ester
20.096	Carbamic acid, N-(3-oxo-4-isoxazolidinyl)-; benzyl ester; benzenemethanethiol
20.685	n-Hexadecanoic acid; tetradecanoic acid
20.988	Hexadecanoic acid, ethyl ester; tetradecanoic acid, ethyl ester
21.308	Cyclic octaatomic sulfur; 7-Amino-7H-S-triazolo [5,1-c]-S-triazole-3-thiol; 4-(4-Chlorophenylamino) pyrido [3,2-c] pyridazine
21.571	Hexadecanoic acid, 14-methyl-methyl ester; heptadecanoic acid, methyl ester; hexadecanoic acid, 15-methyl-methyl ester
22.051	1-naphthalenepropanol, .alpha.-ethyldecahydro-.alpha., 5, 5,8a-tetramethyl-2-methylene-, [1-S-[1.alpha. (S*), 4a. beta., 8a.alpha.]]-
22.880	9,12-octadecadienoic acid (z, z)-, methyl ester; 8,11-octadecadienoic acid, methyl ester; 9,12-octadecadienoic acid (E, E)-, methyl ester
22.977	9-octadecanoic acid (z)-, methyl ester; 8-octadecanoic acid, methyl ester; 11-octadecanoic acid, methyl ester
23.417	Octadecanoic acid, methyl ester
23.845	9,12-octadecadienoic acid (z, z)-; 9,12-octadecadienoic acid, methyl ester; 2-chloroethyl linoleate
24.034	Linoleic acid ethyl ester; 9,12-octadecadienoic acid, ethyl ester; 9,12-octadecadienoic acid (Z, Z)-
24.137	2-methyl-z, z-3,13-octadecadienol; Ethyl oleate cyclododecyne
24.606	Octadecanoic acid, ethyl ester; methyl 17-methyl-octadecanoate; octadecanoic acid, ethyl ester
24.926	Methyl 9,10-methyllene-octadecanoate; 1,2-benzisothiazole, 3-(hexahydro-1H-azepin-1-yl)-, 1, 1-dioxide; oleic acid
25.400	.gamma.-sitosterol; beta.-pregnane-3,20-dione, 17,21-dihydroxy-, cyclic 1-; butaneboronate.beta.-sitosterol
25.611	17-(1,5-dimethylhexyl)-10,13-dimethyl-4-vinylhexadecahydrocyclopenta [a] phenanthren-3-ol. gamma.- sitosterol. beta.-sitosterol
26.703	7-isopropyl-7-methyl-nona-3,5-diene-2,8-dione carbamimidothioic acid, phenylmethyl ester; acetic acid, (2-methylphenoxy)-
26.829	Methyl 18-methylnonadecanoate; eicosanoic acid, methyl ester; methyl 10-methyl-hexadecanoate

Table 2 continued

Rt (min)	Chemical compounds
	Ethyle acetate extract
26.983	9,12-octadecadienoic acid (Z, Z)-; cyclopropaneoctanal, 2-octyl
27.435	Urea, N, N'-bis (phenylmethyl)-; acetamide, N- (phenylmethyl)-; benzenamine, 4-butyl
27.972	Tricosane; tetracosane; behenyl chloride
28.395	Pyrène, hexadecaydro- .beta.-amyrin .alpha.-amyrin
28.766	1H-benzo [f] cyclopenta [c]quinoline, 2,3-dihydro-4-(2-furyl)-.alpha.1-sitosterol; pyridine-3-carbonitrile, 1,2-dihydro-4-[4-(1,1-dimethylethyl) phenyl]-6-phenyl-2-oxo
29.012	Benzenamine, N,N-diethyl-4-[2-(4-nitrophenyl) ethyl]-; 3H-3a-azacyclopenta[a]indene-2-carbonitrile, 3-oxo-1-(piperidin -1-yl)-4, 5, 6,7-tetrahydro
29.675	9,12-octadecadienoic acid (Z, Z)-; cis-11,14- eicosadienoic acid, methyl ester; 1,2-15,16-diepoxyhexadecane
30.018	Methyl 20-methyl-heneicosanoate; docosanoic acid, methyl ester
30.287	1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester; 1,2-benzenedicarboxylic acid, diisoothyl ester; phthalic acid, neopenthyl 2-pentyl ester
30.675	Dodecanoic acid, phenylmethyl ester; undecanoic acid, phenylmethyl ester
31.064	1,4,4-Trimethyl-2,6-diphenyl-1,4-ihydropyridine-3,5-dicarbonitrile; acetamide, N-[4-[2-(2-pyridinylamino)-4-thiazolyl] phenyl]

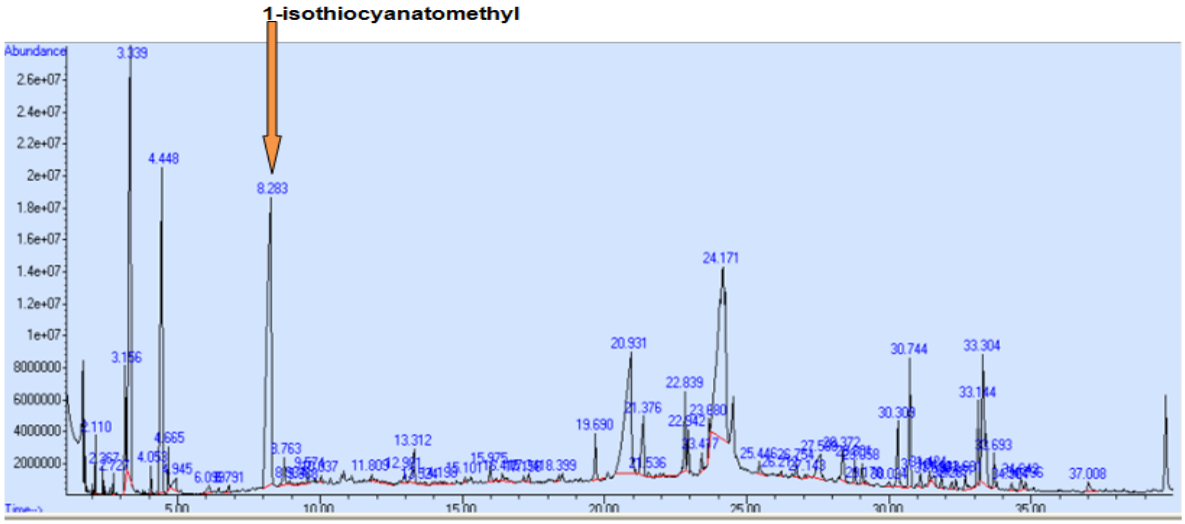

Fig. 2 Chromatographic profil GC-MS of chemical compounds, dichloromethane extract of *Drypetes gossweileri*.

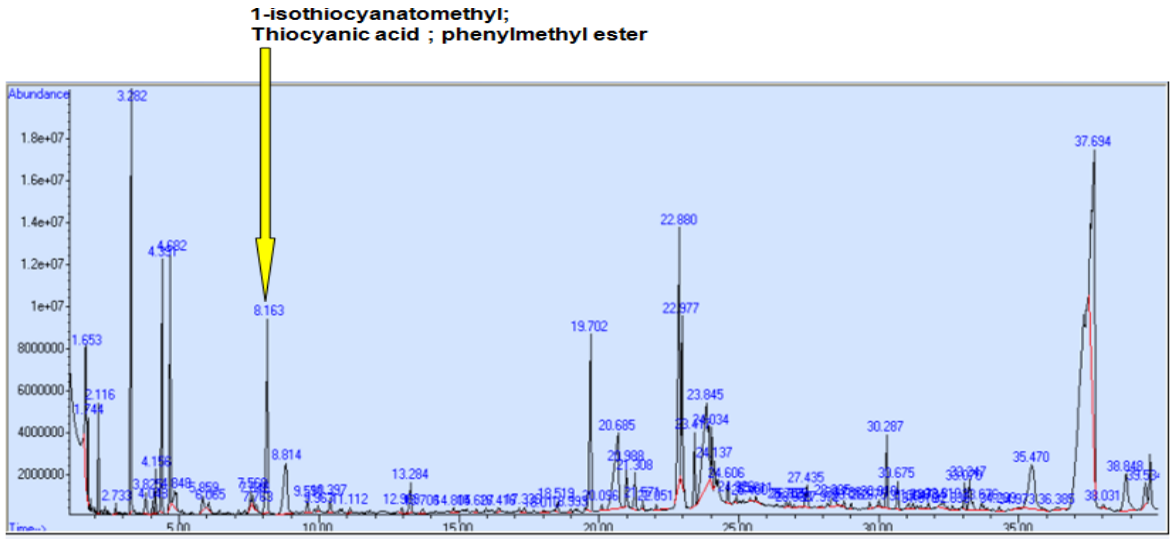

Fig. 3 Chromatographic profil GC-MS of chemical compounds, ethyl acetate extract of *Drypetes gossweileri*.

4. Discussion

Insecticidal activity of four extracts was tested on *S. zeamais*, *T. castaneum* and *R. dominica*. Analysis of variance showed that the mortality rate has a very highly significant variation following extracts, doses, insects and time considered ($P < 0.001$). Dichloromethane and ethyl acetate extracts of *D. gossweileri* are effective against *S. zeamais* and *R. dominica*.

Many authors report that *D. gossweileri* contains alkaloids, phenols, flavonoids, saponins, anthocyanins, anthraquinones, sterols, lipids and essential oils [24]. Alkaloids have repellent or anti appetizing properties against insect pests [25, 26]. *D. gossweileri* is a Euphorbiaceae. The latex of the Euphorbiaceae family contains alkaloids, diterpenes and triterpenes [27]. The results revelated from dichloromethane extract of *D. gossweileri* some molecules as benzene, 1-isocyano-2-methyl-benzyl nitrile, 1-isothiocyanatomethyl, thiocyanic acid, phenylmethyl ester, 1,2 phenylmethyl ester, 3-benzylidene, 2-3-dihydro-7- methyl-5phenyl-1H-1, 4-bendiazepin-2-0, 2-[2,4-dimethoxybenzylidene], 2H -thiazolo [3,2-a] benzimidazol-3-one, benzenamine, 4,4'-(2-phenyl-4,6 -pyrimidinediyl ester, benzylamine, 2-3-dihydro-7-methyl-5phenyl-1H-1,4-bendiazepin-2-0, 2-[2,4-dimethoxybenzylidene], etc.. Thoses chemical compounds belong to three families of secondary metabolites (alkaloids, tannins and terpenes) often used by plants to defend themselves against external aggression [28, 29]. Recent studies [14] proved N-β-glucopyranosyl-p-hydroxy phenyl acetamide, p-hydroxy phenyl acetic acid and p-hydroxyphenyl acetonitrile from *D. gossweileri*. These compounds inhibit the activity of acetylcholinesterase [28, 30]. The acetylcholinesterase protein is best known as a target of organophosphates, carbamates [31, 32] and some natural products. It is likely that the chemical compounds which have been characterized in this study can possess capacity to inhibit the activity of acetylcholinesterase.

Dichloromethane and ethyl acetate extracts of *D. gossweileri* reveal some molecules such as benzyl isocyanate and chloromethyl thiocyanate. Thoses molecular are part of glucosinolates. Glucosinolates are present in all families [33]. They play a role in the resistance of Brassica pests and pathogens [34]. Sinalbine, aliphatic glucosinolate, protects the cotyledons of seedlings of white mustard (*Sinapis alba*) of the beetle attack *Phyllotreta cruciferae* [35]. The role of two indole glucosinolates, sinigrin and glucobrassicin, and their derivatives in the resistance of the cauliflower (*Brassica oleracea*) to *Peronospora parasitica* (downy mildew Brassicaceae) [36] is proved. Among these products, isothiocyanates have the most effective biocidal properties, the compounds of this family come from elsewhere in the composition of trade fumigants (MethylITC, Metam sodium) [37]. *D. gossweileri* could be a source for the synthesis of 1,2,4-thiadiazole, 5-amino-, effective herbicide [38].

D. gossweileri is a higher plant. Higher plants produce much of tannins [39]. The tannin concentrations vary widely depending on the plant species, and within the same species, on the age of the plant; the growth site characteristics and the season [40]. Tannins have a direct toxic against insect species [41]. Tannins have an influence on the growth, development and thev fecundity of insects [25, 41]. It is possible that, as this work has shown subsequent assays of individual components rather than natural mixtures are required to give more insight into the potential toxicity of these natural products.

5. Conclusion

The chromatographic profiles of the different extracts may justify effective insecticidal activity against *S. zeamais* and *R. dominica*. These results may consolidate the traditional use of *D. gossweileri* in pest management.

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Detection of Aflatoxin from Some *Aspergillus* sp. Isolated from Wheat Seeds

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Abstract: Ten seed borne fungi (*Alternaria* sp., *Aspergillus* sp., *Aureobasidium* sp., *Cladosporium* sp., *Dreschslera* sp., *Penicillium* sp., *Rhizoctonia* sp., *Stemphylium* sp., *Mucor* sp. and *Rhizopus* sp.) were isolated and identified from two wheat varieties, the highest frequency of seed borne fungi was observed on wheat cultivar site Mol14 *Alternaria* sp.. Their mean and standard deviation was (5.5 ± 1.69) while the lowest frequency fungal isolated was *Dreschslera* sp. and *Rhizopus* sp.. Their mean and standard deviation was (0.1 ± 0.64). The aflatoxin-producing isolates appeared as gray or black colonies in the UV photographs, whereas nonproducing isolates appeared as white colonies, the plate five colony four (P5CO4) showed the positive results which means the presence of aflatoxin as compaired to the control which showed the ngative results. Ammonium Hydroxide Vapor-Induced Color Change method used which the dish was inverted and 1 or 2 drops of concentrated ammonium hydroxide solution are placed on the inside of the lid. The undersides of aflatoxin-producing colonies quickly turn plum-red after the bottom of the Petri dish has been inverted over the lid containing the ammonium hydroxide aspositive result in (P5CO4) and (P7CO4) observed. Essentially no color change occurs on the undersides of colonies that are not producing aflatoxinsthis indicate to the negative results (control). The main objective of this study is to isolation, identification and rapid detection of aflatoxin from wheat seed borne fungi.

Key words: Aflatoxin, *Apergillus* sp., wheat seeds.

1. Introduction

Seed are regarded as highly effective means for transporting plant pathogens over long distances. Numerous examples exist in agriculture literature for the international spread of plant diseases as a result of the importation of seeds that were infected or contaminated with pathogens [1].

Seed-borne diseases have been found to affect the growth and productivity of crop plants [2-4]. A seed borne pathologen present externally, internally or associated with the seed as contaminant may cause seed abortion, seed rot, seed necrosis, reduction or elimination of germination capacity as well as seedling damage resulting in development of disease at later stages of plant growth by systemic or local infection [5, 6].

Wheat is one of the first cereals known to have been domesticated and wheat's ability to self-pollinate greatly facilitated the section of many distinct domesticated varieties. Wheat is one of the main staple foods of man and is grown in almost all the temperature and subtropical regions of the world. Seed-borne mycoflora of wheat reported recently included *Alternaria alternata*, *Drechslera sorokiniana*, *Fusarium moniliforme*, *F. avenaceum*, *F. graminearum*, *F. nivale*, *F. culmorum*, *F. equiseti*, *F. sporotrichioides*, *Cladosporium herbarum*, *Stemphylium botryosum* [7-9]. The effects of such fungi on the seedling growths include poor germination and less vigorous seedling.

Seed health plays an important role for successful cultivation and yield exploitation of a crop species. Among various factors that affect seed health, the

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most important are the seed borne fungi that not only lower seed germination but also reduce seed vigor resulting in low yield. Healthy seed plays an important role not only for successful cultivation but also for increasing yield of crop [10]. Seed-borne pathogens of wheat are responsible to cause variation in plant morphology and also reducing yield up to 15-90% if untreated seeds are grown in the field [11]. Seed borne infections of fungal pathogens are important not only for its association with the seeds which cause germination failure and/or causing disease to the newly emerged seedling or growing plants but also contaminate the soil by establishing its inocula permanently [12].

The aflatoxins are a group of mycotoxins produced by certain *Aspergillus* species, in particular *A. parasiticus*, *A. flavus*, *A. nomius* and *A. pseudotamarii* [13]. *Aspergillus flavus* is an important *Aspergillus* species with AF (aflatoxin) producing capability. AFs (Aflatoxins) B1, B2, G1 and G2 (AFB1, AFB2, AFG1 and AFG2) are a group of toxic, mutagenic, carcinogenic and teratogenic polypeptide secondary metabolites with health hazards to humans and animals and can adversely affect agricultural productivity [14, 15]. AFB1 was evaluated as a class 1 human carcinogen [13]. The incidence of AF in food and feed is relatively high in tropical and subtropical regions where the warm and humid climate provides optimal conditions for the growth of moulds [16]. Because moulds are present in soil and plant debris and are spread by wind currents, insects and rain, they are frequently found in/on foods together with their associated mycotoxins [13]. On the other hand, the culture of various species of shrimp has an economical importance in many parts of the world as well as in Iran. One of the problems facing the culture of shrimp farming is widespread disease which leads to heavy losses to industry. Additionally, disease caused by other factors, such as culture environments and feed, also influences the success of shrimp culture [17]. One such factor with a global food safety concern is AF, a contaminant produced by *Aspergillus* during the processing and storage of feed. The green tiger shrimp (*Penaeus semisulcatus*) is the endemic species of Persian Gulf and is cultured in Helleh shrimp farm complex, located in the humid tropical environment of Bushehr in south of Iran. Therefore, aflatoxin producing potency of the *A. flavus* isolates from above mentioned shrimps and water of their culture ponds were examined.

Among the toxigenic moulds, *Aspergillus flavus* is the most frequently isolated and is known as aflatoxins producer, which are carcinogenic compounds. All isolates of *A. flavus* were screened for the ability to produce aflatoxins by the inoculation in coconut agar medium. There are a number of reports that show the toxigenic potential essay of *A. flavus* isolated in different products or foods, Lima et al. [18] reported that of the 19 isolates of *A. flavus* recovered in rice, 52.6% were aflatoxigenic, producing aflatoxins B1 and B2. Aquino et al. [19] demonstrated that the dose of 10 kGy was effective on aflatoxin degradation in maize. The highly reactive free radicals can readily attack aflatoxins at the terminal furan ring, giving products of lower biological activity. The mutagenic activity of aflatoxin B1 in an aqueous solution (5 $g \cdot \mu L^{-1}$ water) was reduced by 34%, 44%, 74% and 100% after exposure to gamma rays at 2.5, 5.0, 10.0 and 20.0 kGy, respectively.

Aflatoxins and ochratoxin A (OTA) are the most important naturally occurring mycotoxins in agricultural products. Aflatoxins are produced by several species of *Aspergillus* (*Aspergillus bombycis*, *Aspergillus flavus*, *Aspergillus nomius*, *Aspergillus ochraceoroseus*, *Aspergillus parasiticus* and *Aspergillus psudotamarii*) [20, 21]. Chemically, aflatoxins are difuranocoumarin derivatives produced via a polyketide pathway [22]. OTA was discovered as a metabolite of *Aspergillus ochraceus* in 1965. It is a nephrocarcinogenic and teratogenic mycotoxin that has been detected in several food products [23-25].

Rapid techniques for detection of aflatoxigenic and

non-aflatoxigenic *Aspergillus* using ammonia vapour [25, 26] and methyllated β-cyclodextrine [27] as indicators on *Aspergillus* colonies grown on yeast extract sucrose (YES) agar have been described. In these methods, aflatoxigenic strains produced a pink color against ammonium vapour and green-blue fluorescence under UV light on the reverse side of colonies. Late planting, sick soils, brackish sub-soil water, use of inadequate amount of fertilizers and diseases are the factors responsible for the low yield in our country [28]. Fungal infected seeds transmit most of the major diseases of wheat crop. Seed-borne pathogens include *Alternaria alternata*, *Fusarium graminearum*, *Helminthosporium sativum*, *Drechslera sorokiniana*, *D. tetramera*, *Cladosporium oxysporum* and *Curvularia lunata*, and storage fungi include species of *Aspergillus* and *Penicillium* [28-30].

Several seed-borne pathogens are known to be associated with wheat seed which are responsible for deteriorating seed quality during storage. Kamal et al. [31] and Khan et al. [32] noted the presence of several fungi, i.e., *Alternaria*, *Helminthosporium*, *Fusarium*, *Curvularia*, *Stemphylium*, *Rhizopus*, *Cladosporium*, *Aspergillus* and *Penecillium* species in wheat seeds. Gill et al. [33] recorded 30%-40% incidence of Alternaria *tenuis* on high yielding wheat varieties in some districts of Punjab. Khan et al. [34] and Bhutta et al. [29] isolated *Drecra* sorokiniana and *Fusarium* moniliforme as major pathogens from 1267 and 24*hsle*6 wheat seed lots during 1985-90 and 1993-94 to 1996-1997 respectively. Grzelak et al. [35] isolated *Alternaria tenuis*, *Botrytis cinerea*, *Fusarium aynaceum* and *F. culmorum* predominant from triticale seeds. Singh [36] recorded *Aspergillus* spp., followed by *Drechslera*, *Penicillium* and *Fusarium* spp., associated with wheat seeds. Martin et al. [37] and Oppitz et al. [38] isolated *Alternaria*, *Curularia*, *Fusarium*, *Aspergillus* and *Penicilium* spp., as major storage fungi from wheat grains. Ghosh et al. [39] reported that several of *Aspergillus* and *Penicilium* jenseni are responsible for deteriorating wheat grains during storage. Kunwar [40] isolated *Aspergillus* spp., *Penicilium* spp., followed by *Alternaria alternata* from 50% samples of the stored wheat seeds. Dharmvir et al. [41] observed that fungi colonized during storage were responsible for reducing plant population by 42% in the field. The main objective of this study is isolation, identification and rapid detection of aflatoxin from wheat seed borne fungi.

2. Materials and Methods

2.1 Seed Health Testing

SHT (Seed health testing) for seed borne fungi was carried out following the rules of international seed testing association [42] which were used for the detection and isolation of seed borne fungi of barley by using agar plate method [43]. In agar plate method, PDA (potato dextrose agar) was used which was prepared by dissolving 39 mg of PDA powder in 1 L distil water and autoclaved at 121 °C, 1.5 bar pressure for 15 min, and adding chloramphenicol antibiotic for prevention of bacterial growth. Ten seeds were distributed on 9 cm Petri dish totaling one hundred per each cultivar. The seeds were surface sterilized with 1% sodium hypochlorite for 2-3 min and thoroughly washed in sterilized distil water before plating them on PDA [44]. The plates (10 seed/plate) were then incubated at 25 °C ± 2 and after 7 days were examined [43].

2.2 Isolation of Aflatoxin Producing A. flavus from Seed Samples

Using agar plate method [45], *A. flavus* was isolated from all the seed samples. Four hundred seeds from each sample were plated on one-half strength potato dextrose agar (PDA) medium containing chloramphenicol [46, 47]. The plates were incubated at room temperature and the presence of *A. flavus* was observed after 6 days. The *A. flavus* isolated from samples were further purified individually by sub culturing PDA slants. They were then identified according to Ref. [48].

2.3 Identification of Fungi

After incubation the growth characters as well as percentage of infection were recorded. In order to isolate these fungi into pure culture, Potato Dextrose Agar (PDA) was prepared and the fungi were inoculated onto sterile PDA and incubated for 7 days at the end of which the fungi were identified based in there colour, spore morphology and mycelia growth using the light microscope [49-51]. The fungi were identified by Thom and Barnett et al. [52].

2.4 Detection Tests

2.4.1 Coconut Based Medium Test

Coconut media agar (CMA) was prepared with local coconut milk (protein 1 g, carbohydrate 3 g, fat 4 g/50 mL based on company report). pH of both media was adjusted to 6.9, agar (10 g/500 mL) was added, the mixture heated to boiling and autoclaved for 15 min at 1.5 bar, and poured into sterile plates. The plate centre was inoculated with 5 μL of spore suspension and incubated in the dark at 28 °C. The presence or absence of a fluorescence ring in the agar surrounding the colonies under UV light after 7 days incubation was noted and the results were scored as positive or negative.

2.4.2 Ammonia Vapour Test

Aspergillus isolates were grown on CMA as single colonies in the centre of plate and incubated in the dark at 28 °C. After 10 min, the undersides of aflatoxin producing isolates turned into pink to red color. But no color change occurred in the non-toxic isolates.

Ultraviolet light could also be used as a rapid method for the identification of aflatoxin-producing isolates. The aflatoxin-producing isolates appeared as florescence colonies in the UV light, whereas nonproducing isolates appeared as white colonies. For this method a single colony is grown in the center of a Petri dish containing medium such as coconut milk agar. The dish was inverted and 1 or 2 drops of concentrated ammonium hydroxide solution are placed on the inside of the lid. The undersides of aflatoxin-producing colonies quickly turn plum-red after the bottom of the Petri dish has been inverted over the lid containing the ammonium hydroxide. Essentially no color change occurs on the undersides of colonies that are not producing aflatoxins [26].

3. Results and Discussion

Among various factor that effect seed health, the most important are the seed borne fungi that not only lower seed germination but also reduce seed vigor resulting in low yield eight genera of pathogen fungi isolated from wheat cultivor (site mol 14), as showed in Table 1, the predominant fungi was *Alternaria* sp. The mean & S.D. was (5.5 ± 1.69) and the lower frequency were *dreschslera* sp. & *pecillium* sp. The mean & S.D. was obtained by Refs. [29, 32].

The results from Table 2 show isolation save genera of pathogen fungi from wheat cultivor (aksad), most frequent seed borne fungi was *Alternar* sp. & *Cladosporium* sp. which record the maximum mean & S.D. (0.7 ± 0.55) while the *Aureobasidium* sp. & *Mucor* sp. record minimum, mean & S.D. (0.2 ± 0.23) This result was agreed with those results obtained by Refs. [29, 31, 32].

Table 1 Seed borne fungi isolated cultivars of wheat (*Triticum aestivum*) (Site Mol 14).

No.	Isolate fungi	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Total	Mean	S.D.
1	*Alternaria* sp.	6	7	6	6	6	6	3	8	2	5	55	5.5	1.69
2	*Aspergillus* sp.	_	_	_	_	1	1	_	_	_	_	2	0.2	0.46
3	*Aureobasidium* sp.	_	_	1	_	_	_	2	_	_	1	4	0.4	0.66
4	*Cladosporium* sp.	_	_	_	1	_	1	3	_	2	1	8	0.8	0.86
5	*Dreschslera* sp.	_	_	_	_	1	_	_	_	_	_	1	0.1	0.64
6	*Penicillium* sp.	_	_	_	_	_	_	_	1	_	_	1	0.1	0.64
7	*Rhizoctonia* sp.	_	_	1	_	_	_	_	_	3	1	5	0.5	1.11
8	*Stemphylium* sp.	2	1	_	2	_	2	_	_	1	_	8	0.8	0.59

Table 2 Seed borne fungi isolated cultivars of wheat (*Triticum durum*) (Aksad).

No.	Isolate fungi	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Total	Mean	S.D.
1	*Alternaria* sp.	_	1	1	_	_	_	2	_	1	2	7	0.7	0.55
2	*Aspergillus* sp.	1	1	_	2	1	_	1	_	_	_	6	0.6	0.45
3	*Aureobasidium* sp.	_	1	_	_	_	1	_	_	_	_	2	0.2	0.23
4	*Cladosporium* sp.	3	_	1	1	_	_	_	1	1	_	7	0.7	0.94
5	*Mucor* sp.	_	_	_	_	2	_	_	_	_	_	2	0.2	0.92
6	*Penicillium* sp.	_	1	_	_	1	_	_	1	_	_	3	0.3	0.40
7	*Rhizoctonia* sp.	1	_	_	_	_	2	1	1	1	_	6	0.6	0.54

Table 3 Seed borne fungi isolated cultivars of wheat (*Triticum aestivum*) (R Zgui).

No.	Isolate fungi	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Total	Mean	S.D.
1	*Alternaria* sp.	9	9	1	9	6	4	7	8	7	5	65	6.5	2.46
2	*Aureobasidium* sp.	_	_	2	_	5	5	_	1	1	4	18	1.8	1.79
3	*Cladosporium* sp.	_	_	_	_	_	_	1	_	1	_	2	0.2	0.46
4	*Dreschslera* sp.	_	1	_	_	_	_	_	_	_	_	1	0.1	0.64
5	*Rhizoctonia* sp.	_	_	1	_	_	1	1	_	_	_	3	0.3	0.35
6	*Rhizopus* sp.	_	_	1	_	_	_	_	_	_	_	1	0.1	0.64
7	*Stemphylium* sp.	1	1	_	_	_	_	_	1	_	1	4	0.4	0.27

Table 4 Seed borne fungi isolated cultivars of wheat (*Triticum durum*) (Smito).

No.	Isolate fungi	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Total	Mean	S.D.
1	*Alternaria* sp.	2	_	1	1	2	3	2	_	2	1	14	1.4	0.67
2	*Aureobasidium* sp.	3	1	_	1	1	_	_	1	_	_	7	0.7	0.85
3	*Cladosporium* sp.	3	2	2	2	1	1	2	2	3	2	20	2	0.63
4	*Rhizoctonia* sp.	_	1	_	1	_	_	_	_	_	1	3	0.3	0.35
5	*Stemphylium* sp.	_	_	_	1	_	_	_	_	_	_	1	0.1	0.64

Table 5 Detection of aflatoxin fom *Aspergillus* sp. by UV light and ammonium hydroxide method isolated from Aksad wheat cultivar.

Fungi	Control	UV light	Ammonium hydroxide
Aspergillus sp.	P1CO4	P1CO4	P1CO4
Aspergillus sp.	P2CO2	P2CO2	P2CO2
Aspergillus sp.	P4CO1	P4CO1	P4CO1
Aspergillus sp.	P4CO3	P4CO3	P4CO3
Aspergillus sp.	P5CO4	P5CO4(+)	P5CO4(+)
Aspergillus sp.	P7CO4	P7CO4(+)	P7CO4

Seven seed borne fungi isolated from wheat cultivor seed (site Rzgui), the most frequent seed born fungi was *Alternaria* sp. which obtain the maximum mean & S.D. (6.5 ± 2.46) but *Dreschslera* sp. *&* *Rhizopus* sp. (0.1 ± 0.64), as explain in Table 3, this result was confirmed with those recorded by Refs. [29, 31, 32].

Table 4 indicates five seed born fungi isolated from wheat cultivor (site smito) & the predominant fungi was *Ladosporium* sp., which record highest frequent the mean & S.D. (2 ± 0.63) while *Stemphylium* sp. record minimum mean & S.D. (0.1 ± 0.64), this result is similar to those results obtained by Refs. [29, 31, 32].

The rapid techniques detect aflatoxigenic & non-aflatoxigenic *Aspergilluus* sp. by using amonoia vapor. In the method, aflatoxigenic strains produced a pink color against ammonium vapor & green-blue florescence under UV light on the reverse side of colonies^{+}. The result in Table 5 reveal to positive results recorded from plates-5 colony-4 which mean the presence of aflatoxin as compaired to the control

which shows the negative results, this result is similar with those obtained by Saito et al. [26].

4. Conclusion

From the present study it was concluded that rapid method for detection of aflatoxin which used especially in development country, it was easy and not expesive than other methods.

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Background Activity of the Cerebral Cortex and Superficial Muscles of the Fingers in Men with Different Levels of Individual EEG α-Frequency

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Abstract: A test group consisting of 104 healthy men from the ages of 19 to 21 was divided into two groups according to the magnitude of their IAF (individual EEG α-frequency) median—groups with high (n = 53, IA ≥ 10.04 Hz) and low (n = 51, IAF ≤ 10.03 Hz) levels of IAF. Differences between the groups in terms of the power and coherence of EEG oscillations and the EMG signal average amplitude at rest were estimated. It was found that the dominant role of the thalamocortical interactions (thalamus and cortex) in the group of testees having a high IAF causes the greater differentiation and rationality of the downstream effects on muscles and provides more economic baselines and contractile capabilities of the leading hand flexor muscles. Increased non-specific influences of the limbic system and reticular formation in providing the testees' cortical activity under conditions of a low frequency are accompanied by a smaller lateral and reciprocal specificity in the downstream impacts, and by a lower performance of supraspinal innervations.

Key words: Functional status of rest, individual alpha-frequency, EEG power and coherency, muscle tone.

1. Introduction

The modern world is characterized by the growth of occupations with critical individual functional potentials of the sensory and motor systems and the brain activity of the person involved. A direct reflection of the individual characteristics of the brain is its background electrical activity [1, 2]. The α-rhythm maximum peak frequency has the most information value among its other parameters [3-5]. It is believed that various α-sub-bands are differed by the specific brain generators, functional significance, and varying degrees related to the major systems of the brain activation [6, 7]. A low or high range of the α-rhythm superiority in the background encephalogram of the person can cause his/her psychomotor and cognitive abilities [8, 9].

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Simultaneously, it has been found that there is a lack of information concerning features of the regulatory processes in the brains of persons with different individual α-frequencies and their impact on the processes of descending innervations of the distal arm muscles. The data obtained are insufficient to create an integrated system of ideas dealing with this subject matter. Nevertheless, this issue is urgent and has not only theoretical but also obvious practical importance. The purpose of the study is to identify the characteristics of the background electrical activity of the cerebral cortex and superficial muscles of fingers as indicators of the brain regulatory mechanisms and the downstream regulation of the neuromuscular periphery in testees who have different individual α-frequency EEG rhythms.

2. Methodology

The participants in this study were 104 male

volunteers from the ages of 19 to 21. Biomedical ethics rules in accordance with the Helsinki Declaration of the World Medical Association on the Ethical Principles of Scientific and Medical Research involving Human Subjects were adhered to during the experiment. All the testees were healthy with regard to the judgment and advisory conclusions of their medical professionals, and had normal hearing in their own estimation, as well as a suitable profile of manual and auditory asymmetries. The latter was determined by nature of their responses during the interview process, and their execution of motor and psychoacoustic tests [10].

The testees were in a quiescent state with their eyes closed and in a reclining position with their limbs relaxed and not crossed during the EEG and EMG testing. The experiment was carried out in a room which was sound-proof and light-proof.

Active electrodes were placed in accordance with the international system 10/20 in 19 points on the scalp of the head during the electroencephalogram (EEG "Neurocom", and the Certificate of State registration # 6038/2007 from January 26, 2007) recording. The performance of the EEG recording was monopolar, with the use of ear electrodes as a reference. The duration of the test was 40 s. The Fourier analysis era was 4 s with a 50% overlap. ICA-procedure analysis was used for the rejection of EEG anomalies.

Both the power (μV^2) and the coherence of the brain electrical activity in the θ-, α-, β- and γ-frequency intervals were also evaluated. Taking into consideration the functional heterogeneity of different sub-bands of the EEG α- and β-rhythms, the changes in the power and coherence of each of them were considered, and coefficients of coherence above 0.5 were analyzed as well.

The individual alpha-frequency of the EEG was determined in each testee by the method of W. Klimesch et al. [7] (IAF, Hz). Groups with a high or a low IAF were formed on the basis of the magnitude of the median averaged in all parts of the cerebral cortex individual α-frequency:

Groups with a high IAF, (n = 53, IAF ≥ 10.04 Hz);

Groups with a low IAF (n = 51, IAF < 10.04 HZ).

The frequency interval limits were determined individually, relying on the value of the testee's IAF. The following algorithm [7] was used and the truth of which was that the upper limit of α3-subband was set to the right side of the IAF in increments of 2 Hz. It corresponded to the lower limit of the β1-band. The upper limit of the β1-sub-band was defined according to the standard concepts as 25 Hz. The lower limit of the α2- band was determined in steps of 2 Hz to the left of the peak, and the α1- band in 4-Hz steps, as well as θ-frequencies in 6 Hz. Limits of β2- and γ-bands were recognized as standard, properly, 26-35 Hz and 36-45 Hz.

The resulting individual values of the power and coherence of EEG oscillations within the selected groups of men were averaged for each lead.

The electromyogram (EMG, "Neurosoft", EC-Declaration of Conformity # RQ093102-V, issued by the EUROCAT Institute for Certification and Testing and valid until 07 November 2014) was registered for the superficial flexor muscles (musculus flexor digitorum superfacialis) and the extensor muscles (musculus extensor digitorum) of the right and left hand fingers. A bipolar lead with surface electrodes was used in the EMG recording. The duration of the EMG test was 40 s. EMG signals in the off-line regime were processed by means of filtering and amplitude-frequency analysis. High-frequency filters were set at a level of 5 kHz, and low-cut filters at a level of 2 Hz. The band-rejection filter was 50-60 Hz. The epoch of the analysis was 3 s with an ADC frequency request of 10 kHz. The average amplitude (mV) of fluctuations in EMG was also detected.

Statistically, significant differences between groups of subjects were considered at $P \leq 0.05$ and $P \leq 0.001$.

3. Results and Discussion

Testees having a high IAF at rest are characterized by a lower power of θ-, α1-, α2- and β1-EEG

oscillations and higher in α3-, β2- and γ-bands than individuals having a low frequency (Fig. 1). According to the scientific resources, the θ-power loss and a low α-activity are associated with the state of readiness, support and mental alertness whilst the expression of α3-, β- and γ-vibrations with the organization of specific forms of attention required for a higher cognitive functions [7, 11].

On this basis, the differences in the power of the EEG frequency ranges defined among groups of men may indicate the status of a relatively high activation of the cortex tone and its readiness for the activity and monitoring of information and mental processes in individuals having a high IAF. These features of cortex background functioning became the criteria of the encephalon activity modeling [12]. Neuropsychological studies [13] have showed that the interaction between the thalamus and neocortex provides the process of the attention, the level of its activity and orientation as the best background for the occurrence of a mental process and points to the crucial role of the thalamocortical links (thalamus and cortex) in patients having a high α-frequency while providing quiescence.

Relatively lower background cortex activation tones are accompanied by higher results in the EEG coherent oscillations, especially in the frontal, central and temporal lobes in the group of testees with low rates. Thus, conditions for compensatory facilitation in the spread of excitation among particles of the cortex under these conditions are created. The functional meaning of this type of spatial synchronization is associated with higher levels of cortical stress [14]. The study carried out by I.N. Knipst and other contributing authors [15] showed that such an increase in the spatial synchronization of cortical potentials are modulated by the tonic effects of nonspecific brain systems—the limbic system in the low-frequency range and the reticular formation in the EEG high frequency band.

A passive tone of the right hand muscles in all testees is provided by a significant predominance of the EMG mean amplitude of extensor muscles ($P \leq 0.001$), as shown in Fig. 2.

All of this points to the activation of a relatively large quantity of motor units in the extensor muscle and their higher passive tone [16]. These common factors have a higher significance in men who have a high IAF. An increased extensor tone is accompanied by a reciprocal relaxation of flexors.

A passive tone of the left hand muscles in all men is accompanied by an increase in tension in the flexors ($P \leq 0.05$) but it is complemented by a relative decrease in the extensor tone ($P \leq 0.05$) in comparison with the right hand, in testees who have a high IAF. Distinguishing features in the measurements of the left hand antagonistic muscles has not been detected.

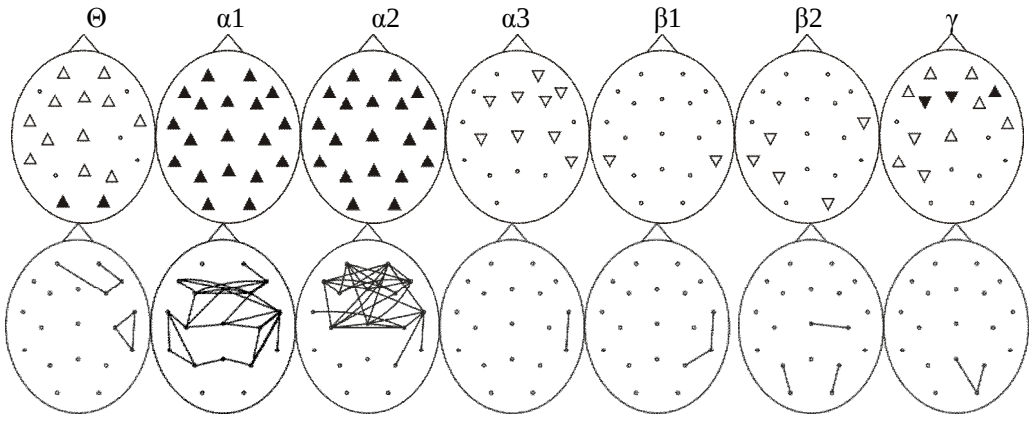

Fig. 1 Intergroup differences in EEG power and coherence.

△▲— higher rates in patients with a low IAF, $P \leq 0.05$, $P \leq 0.001$; ▽▼— lower rates in patients with a low IAF, $P \leq 0.05$, $P \leq 0.001$; —— — higher rates in patients having a low IAF, $P \leq 0.05$, $P \leq 0.001$.

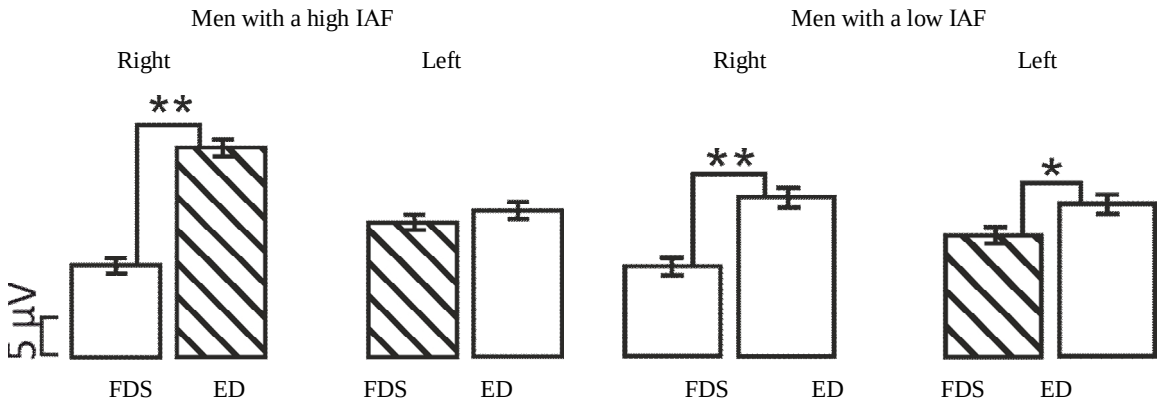

Fig. 2 Measures of the mean EMG amplitude (mV) of the surface flexor muscles (musculus flexor digitorum superfacialis, FDS) and extensor muscles (musculus extensor digitorum, ED) of the right and left hand fingers in the groups of testees.
*, **—significant differences between parameters of the antagonists, respectively $P \leq 0.05$ and $P \leq 0.001$;
▨—significant differences between parameters of the right hand muscles and the left hand muscles, respectively $P \leq 0.05$.

Table 1 Group differences in the mean EMG amplitude (M ± m, μV) of superficial muscles of fingers in the surveyed groups.

Subgroups of muscles	Men with a high level of IAF (n = 53)		Men with low level of IAF (n = 51)	
	Right hand muscles	Left hand muscles	Right hand muscles	Left hand muscles
Flexors	10.37 ± 0.74	15.26 ± 1.03*	10.14 ± 0.92	12.75 ± 0.95
Extensors	23.7 ± 2.00*	16.54 ± 1.25	16.65 ± 1.59	15.95 ± 1.82

Note: * – inter-group differences in EMG parameters, respectively $P \leq 0.05$.

Individuals with a low IAF have an extensor tone prevalence ($P \leq 0.05$) resulting in lower values by comparison with the right hand. These features indicate less differentiated mechanisms regulating the activity of both left and right hand antagonistic muscles in the low-frequency surveys. Lateral differences detected in the passive tone of flexor and extensor muscles in each testee may indicate a more economical baseline of innervations in the muscles of the right (leading) hand and reflect their greater fitness and strength.

The analysis of intergroup differences suggests that men having a high IAF are characterized by a higher tension of the extensor muscles of the right hand ($P \leq 0.05$) than testees with a low frequency, as shown in Table 1. This provides proof that a greater relaxation of flexors exists and it can display their best contractile capabilities.

4. Conclusions

Both the superiority of thalamocortical interactions and their significance in the group of testees with a high IAF lead to a greater differentiation and rationality of downstream effects on muscle. This provides a more economic baseline and contractile capabilities of the leading hand flexor muscles.

Increased non-specific influences on the part of the limbic system as well as a reticular formation in the cortical activity of testees in the low-frequency surveys are accompanied by less lateral and reciprocal specificity of the downstream impacts as well as a reduced efficiency and rationality of descending innervations.

5. Recommendations

In accordance with the results which were obtained, the aim of the future scientific research is to establish the characteristics of the initial regulatory brain processes and their influence on the process of descending innervations of the distal hand muscles in women who have different individual EEG α-frequencies.

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Liver Damage by the Interaction of Malathion with Cimetidine in Rat

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Abstract: In this study the authors aimed to evaluate the oxidative stress enzymes indicative of liver damage in rats exposed to malathion (M), subchronic form using cimetidine (C) and cimetidine plus malathion (M + C). Malathion, widely used organophosphorus insecticide worldwide, induces oxidative liver damage type; cimetidine is an antagonist of histamine H2-receptor, it has been shown to be an inhibitor of various CYP_{450} isoforms. Male Wistar rats weighing 200-250 g were studied, exposed to malathion orally for 3 weeks (0.15 mg/kg/day, 2 mg/kg/day, 15 mg/kg/day) and cimetidine 10 mg/kg/day. Malathion plus cimetidine affect susceptibility to oxidative stress and possibly modifies the antioxidant defense capacity directly or indirectly.

Key words: Malathion, cimetidine, lipid peroxidation, malondialdehyde, ALT.

1. Introduction

In the World Health Organization (WHO) worldwide, estimates that there were a million cases of serious unintentional pesticide poisoning, of which 70% occurred by occupational exposure [1]. In Mexico, pursuant to the Single Information System for Epidemiological Surveillance (*Sistema Único de Información para la Vigilancia Epidemiológica SUIVE*) of acute pesticide poisoning, have shown an upward trend, reporting 3,849 cases for 2003, a figure which increased to 3,902 by 2005 [2]. Malathion induces lipid peroxidation by free radicals as a mechanism for subchronic toxicity [3]. Pesticides can directly or indirectly alter the antioxidant defense capacity of exposed subjects affecting their susceptibility to oxidative stress [4]. Most OPs (organophosphates) pesticides being used dependent activation of CYP_{450} in that cause acute toxicity in mammals. CYP_{450} isoforms play a major role in mediating the toxicity of Malathion and related insecticides. Cimetidine is an H-2 antagonist which has been shown to inhibit several isoforms CYP_{450} cimetidine administration resulted in significant drug interactions with a wide variety of drugs [5]. Insecticides (OPs) cause liver damage and lipid peroxidation is one of the mechanisms involved in the toxicity induced by OPs [6]. Other pesticides as chlorpyrifos on the other hand, can cause nicotine effects: In patients with mild to moderate poisoning, the nicotinic effects may include tachycardia, hypertension, mydriasis and muscle cramps [7].

2. Materials and Methods

It was studied male Wistar rats weighing 200-250 g, exposure to malathion orally for 3 weeks (0.15 mg/kg/day, 2 mg/kg/day and 15 mg/kg/day) and cimetidine 10 mg/kg/day; experimental groups: (1) vehicle (corn oil), (2) malathion, (3) malathion + cimetidine and (4) cimetidine. Indicators: Oxidative, enzymatic and structural. 4 groups of rats: control group, malathion group, cimetidine group and malathion + cimetidine group, to which were administered three different doses of malathion

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orally (0.15 mg/kg/day, 2 mg/kg/day and 15 mg/kg/day) and cimetidine 10 mg/kg/day for 3 weeks. Dose response curves were performed to determine the dose, taking into account values level no observed adverse effect level (NOAEL) and LD_{50}. Following significant ANOVA, and using Bonferroni test comparing every mean with every other mean, we established the level of the statistically significant differences ($P < 0.05$).

3. Results and Analysis

Technical administrative of malathion and cimetidine plus malathion increased hepatotoxicity. The malondialdehyde (MDA) in liver was highly significant ($P = 0.0006$) in treated animals compared to the control group after 21 days of exposure to malathion alone and cimetidine malathion more than 21 days of administration of these compounds; MDA concentrations remained high with a statistical significance of $P = 0.0009$.

3.1 Lipoperoxidation

Significant changes were found in the MDA levels in the malathion (M) groups and malathion + cimetidine (M + C) ones, compared with control, and observed dose-dependent increase in MDA (Fig. 1).

3.2 Enzimatic

The serum gamma-glutamyl transpeptidase activity increased in group M, compared to control, as the M+C group (Fig. 2). Not only but also, the ALT resulted incremented in both of the treated groups compared with the control one (Fig. 3); values are expressed as mean ± SE ($P < 0.05$).

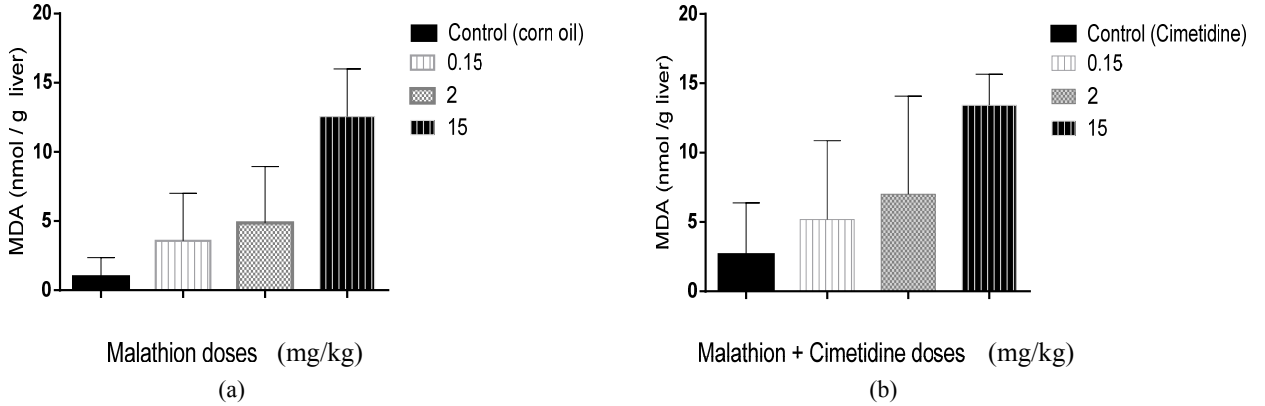


Fig. 1 (A) Significant alterations ($P = 0.0006$) were founding, the levels of MDA in the groups M and M + C compared with control, and observed dose-dependent increase in (B) MDA ($P = 0.0009$).

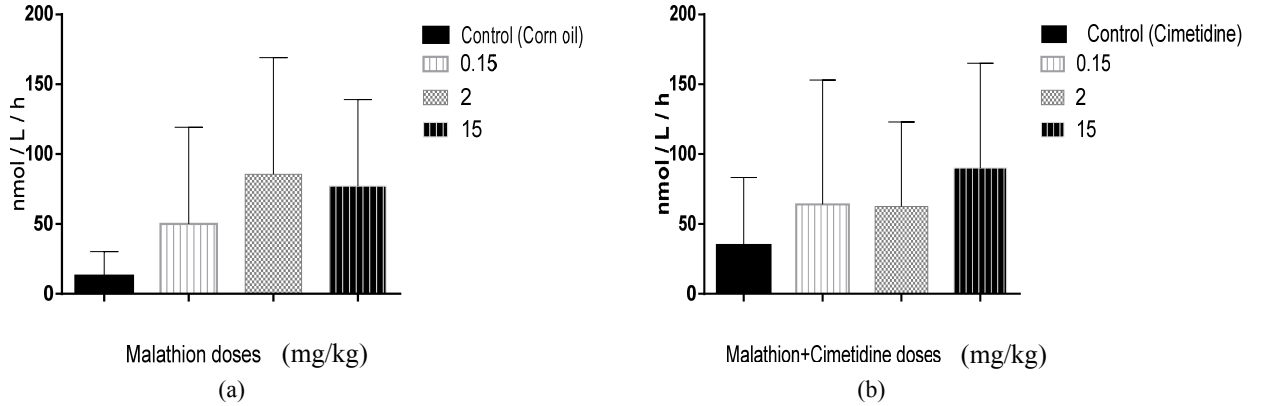


Fig. 2 (A) The serum gamma-glutamyl transpeptidase activity increased in group M, compared to control, (B) M + C group showed increased enzyme activity and dose-dependent compared with the control was more significant increase: $P < 0.001$.

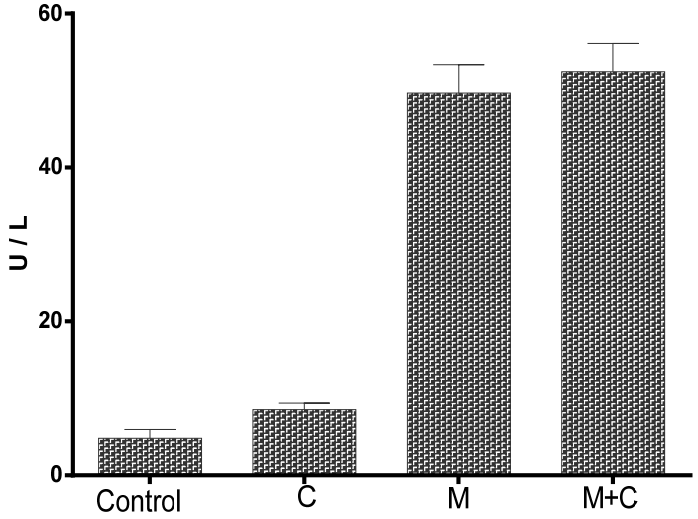
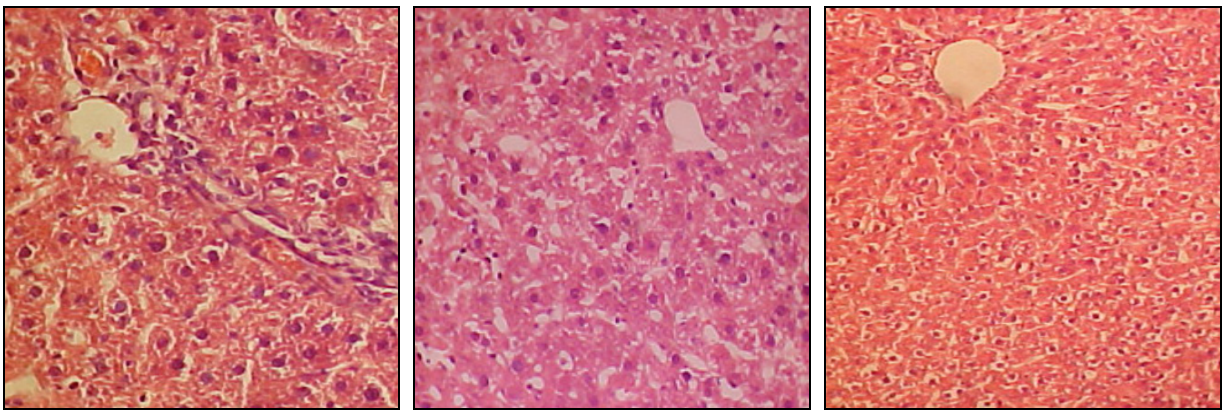


Fig. 3 The activity of alanine aminotransferase (ALT) was increased in groups M and M + C compared to control. It was very significant: $P = 0.00001$.



(a) (b) (c)

Fig. 4 Liver histopathological study presents morphological changes in liver tissue which indicate reactive hepatitis (a) and toxic hepatitis (b) suggesting organophosphate-induced liver damage; compared with the control group (c).

3.3 Structural

Liver histopathological study presented morphological changes in tissue which indicate reactive and toxic hepatitis suggesting organophosphate-induced liver damage compared with the control group (Fig. 4).

4. Discussion

There is great interest in the role played by lipid peroxidation and other free radical reactions in toxicology and human diseases [8]. Oxidative stress induced by pesticides is given by multiple steps and pathways to be a final manifestation, resulting in an imbalance between pro-oxidant defense mechanisms and antioxidant. Concomitantly pesticide poisoning occur breakdown of certain antioxidant mechanisms in different tissues, including alterations in antioxidant enzymes and glutathione redox systems [3]. The findings of this study agree with Banerjee [4]; as the authors found an increase in MDA as end product of lipid peroxidation of arachidonic acid, malathion alone caused an increase in MDA levels, this being more significant when combining with the enzyme inhibitor cimetidine. Bio-transformer activity is also determined by the interaction with other drugs or exposure to other agents that can cause an induction or

repression of the enzymes of phase I.

The radicals are generated by a variety of processes that can attack molecular targets manifesting their toxic actions. These targets include proteins especially thiol groups, DNA and RNA, and lipoprotein complexes with cholesterol [9], DNA alterations can be the result of such interactions, the effect cimetidine combined with malathion was observed, this may be indicative of the degree regenerative liver cells to repair the damage.

Lipid peroxidation has been suggested as one of the possible mechanisms in which chemicals may cause membrane damage and cell death [10]. Koner et al. [11] suggest the biochemical mechanism of toxicity of malathion in lymphocytes and possible role in immunosuppression γGTP. The results show increases γGTP activity in serum, this being more significant malathion alone and M + C, which can indicate the effects prooxidant of the γGTP activity and its role in promoting established cell precursors GSH for resynthesis therefore followed by recovery of cellular antioxidant defense.

The susceptibility of hepatocytes to oxidative stress due to exposure to Malathion is a function of the overall balance between the degree of oxidative stress and antioxidant defense capacity. Malathion may directly or indirectly alter the antioxidant defense capacity and then affect susceptibility to oxidative stress in rats.

5. Conclusion

Malathion affects susceptibility to oxidative stress and possibly modifies the antioxidant defense capacity directly or indirectly. This study will suggest alternatives for the clinical diagnosis and treatment of liver disease induced by these pesticides and proposed sharpen subsequent studies on oxidative stress in subjects exposed to organophosphate and design intervention strategies. Histopathology very forceful throws results which shows structural damage of the liver parenchyma.

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Diversity and Antimicrobial Activity of Cultivable Halophilic Archaea from Three Algerian Sites

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Abstract: Hypersaline environments are colonized by communities of microorganisms, which developed adaptations from halotolerant to extreme halophilic lifestyles. Twenty-four halophilic archaeal strains were isolated from three Algerian hypersaline sites, including the solar salterns in Ichekaben and two natural salt lakes at Ouargla and Oran. Almost full-length 16S rRNA genes were amplified using archaeal-specific primers, sequenced and phylogenetically analyzed. Three isolates from the salt lake of Ouargla were affiliated to *Halorubrum* sp., while six isolates from Ichekaben were *Natrinema* sp.. The remaining strains belonged to the genus *Haloarcula*, which was identified in the three Algerian sites. A phylogenetic tree of *Haloarcula* sp. including the two copies of the 16S rRNA gene, showed that two strains group with *Haloarcula hispanica*, while the other 13 cluster with OHF-1/OHF-2 isolated from French commercial salt. Both *Natrinema* sp. and *Haloarcula* sp. were persistent at Ichekaben. For the six *Natrinema* sp. isolates, this observation correlates with their capacity to produce antimicrobial compounds, and a DNA fragment closely related to the halocin C8 gene was amplified for five strains.

Key words: *Haloarcula*, *Natrinema*, 16S rRNA gene divergence, halocin C8, hypersaline.

1. Introduction

Hypersaline environments such as salt lakes or brinesand those of anthropic origin including salterns [1] are colonized by communities of microorganisms from the three domains of life, which have developed adaptations ranging from halotolerant to extreme halophilic lifestyles [2]. The most abundant inhabitants of hypersaline environments are archaeal members of the order *Halobacteriales*, extreme halophiles that grow optimally above 2.5 M NaCl [2-4]. *Halobacteriales* [5] currently consists of 36 genera [6]. Both culture-dependent studies and molecular data confirm the dominance of these halophilic archaea in hypersaline environments [7-9].

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In hypersaline environments, halophilic archaea are challenged with numerous competitors. One means to become the dominant colonizer is the production of halocins, which can be defined as protein antimicrobial agents produced by halophilic microorganisms [10]. However, their ecological role is controversial and one field study suggests that the contribution of halocins to microbial competitions would be negligible [11]. To date halocins have been isolated exclusively from the domain *Archaea* [10, 12], the production of halocins being considered as a nearly universal feature of halophilic archaea [13, 14]. Among the eleven halocins described so far, seven halocins (A4, C8, H1, H4, H6/H7, R1 and S8) have been characterized more or less thoroughly [12] and their mechanisms of action clearly deciphered only for one: halocin H6/H7 [15]. Since adaptation to life in extreme conditions may give

rise to an increasing diversity of structures and modes of action, the study of halocins is of particular interest both in terms of fundamental knowledge and biotechnological potential.

Microbial diversity in natural environments can be assessed either by molecular methods based on sequencing of PCR-amplified 16S rRNA genes or isolation and identification of the cultivable microflora. Both approaches involve bias and are often complementary as they do not generate identical results. The present study aimed to examine the cultivable haloarchaeal communities in salty terrestrial Algerian ecosystems, with a focus on halocin production. While many of the halophilic strains maintained in culture collections come from widely studied sites, such as the Dead Sea or the Great Salt Lake, only two species have been isolated from Algeria, both originating from the sebkha of Ezzemoul, a natural salt lake in the North-East of the country [16, 17]. In the present study, the samples were collected from three sites in Algeria, a saltern at Ichekaben and sebkhas (natural salt lakes) at Ouargla and Oran. The authors report the isolation and identification of cultivable halophilic archaea from these Algerian environments and discuss the phylogeny of 13 *Haloarcula* strains. The production of halocins by strains of the genus *Natrinema* and their persistence over a three-year period also are described.

2. Materials and Methods

2.1 Sampling of Hypersaline Environments

The salterns in the village of Ichekaben (36°33′N, 4°50′E) are located in the Wilaya of Bejaia, approximately 300 km South-East from Algiers. The saline source springing out of the mountain have been exploited traditionally since the antiquity by the inhabitants of Ichekaben to extract salt, using natural evaporation in saltern ponds. The sebkha of Ouargla is located in the Sahara 800 km South of Algiers (31°57′N, 5°20′E) and is surrounded by several oases harbouring palm groves irrigated by artesian wells. Approximately 450 km West of Algiers, the sebkha of Oran is a depression (35°42′N, 0°38′W), which starts at 110 m altitude and ends 80 m above the sea level. It is surrounded by mountains from which the water flows into the sebkha to form a shallow lake, which may be completely evaporated during summer, depending on the intensity of rainfalls.

Samples (salted water, soil, salt crystals) were collected in spring during the period 2007-2009 (Ichekaben), in 1997 and 2000 (Ouargla), and in 2009 (Oran). The samples were analysed for determination of pH and NaCl contents immediately after collection. The methods used were flame photometry for Na^+ and titration of Cl^- with $AgNO_3$. Analyses were performed by the laboratory Labo-Idres (Béjaia, Algeria).

2.2 Isolation and Growth of Halophilic Strains

Following collection, strains were isolated in culture media favouring the growth of halophilic microorganisms. A basal medium (M) containing 1% (w/v) peptone (Merck), 0.1% (w/v) meat extract (Merck), 0.2% (w/v) yeast extract (Merck), was supplemented with increasing amounts of NaCl (15% w/v or 2.6 M, 20% w/v or 3.4 M, 25% w/v or 4.3 M). The pH was adjusted to 7.2. Because some halophilic microorganisms may require the presence of $MgSO_4$ and alkaline conditions for growth [1], additional sterile medium containing 15% or 20% (w/v) NaCl, 0.1% to 5% (w/v) $MgSO_4$ adjusted to pH 7.2 or 9.0 were also prepared from medium M.

For growth of the microorganisms present in salted water samples, 0.1 mL of crude sample was plated directly on a Petri dish containing the appropriate medium and 20 g/L agar (Fluka). For growth of the microorganisms present in solid samples (soil, salt crystals), 10 g of crude sample were grinded, and resuspended in 90 mL sterile 2.6 M NaCl. The resulting suspensions were diluted 10 to 10^5 fold prior to inoculation of 0.1 mL on solid medium. The Petri dishes were incubated for two weeks at 30 °C, 37 °C or 43 °C, in order to select halophilic archaea. Pure

cultures were obtained by subsequent transfer of representative pigmented colonies to agar plates of the same medium composition.

Sensitivity of the archaeal isolates to antibiotics was determined using a double-layer diffusion assay as described below, except that 2 µL of each antibiotic were spotted onto the microbial lawn. Concentrations of the antibiotics were as follows: 10 mg/mL for ampicillin, 5 mg/mL for chloramphenicol and 1 mg/mL for novobiocin.

Isolates were named by capital letters according to both the type of sample and the sampling site: SWI (salted water), SSI (salted soil) and SI (salt crystals) from Ichekaben, SWO (salted water) and SSO (salted soil) from Ouargla, and salted water (SWW) from Oran (Wahran in Arabic). Liquid cultures of archaeal isolates and strains *Halobacterium salinarum* DSM 3754 and *Haloferax mediterranei* DSM 1411 were grown in DSMZ Halobacteria medium 372 (http://www.dsmz.de/) at 37 °C for 7-14 days.

2.3 Production of Halocins

Screening of haloarchaeal strains for the production of compounds displaying antimicrobial activity was carried out by using the agar double-layer diffusion assay. 5 mL of melted soft agar (8 g/L agar in medium 372 maintained at 50 °C) were inoculated with 1-10 µL of exponential phase culture of the target strain (OD_{600} = 0.4-0.8) to a final OD_{600} = 0.0001 or 0.00005, and poured into a Petri dish over medium 372 agar (20 g/L). Upon solidification, 5 µL of exponential or stationary phase culture of the potentially producing strain were spotted onto the plate. Incubation was carried out until observing a homogenous microbial lawn. Inhibition halos were measured in mm.

In order to determine whether the antimicrobial activity was of protein nature, sensitivity to several proteases (papain, pepsin, proteinase K and trypsin) was assayed. Following centrifugation of cultures of the producing strains, the supernatant and the pellet were recovered. The pellet was resuspended in the same volume of medium 372 as the initial culture. All protease assays were performed in a final volume of 10 µL, using 1-2 µL of resuspended pellet or supernatant, thus allowing a 5-10 fold dilution of the sample, to ensure that the concentration of NaCl was compatible with the activity of the proteases. The proteases were from Sigma, and the digestions were performed in the recommended buffers with final protease concentrations of 0.5-4 mg/mL. All protease assays were incubated for 1 h at 37 °C, except for papain for which incubation was performed at 25 °C. Controls were performed using untreated resuspended pellet and supernatant, as well as medium 372 treated likewise. Following protease treatment, 5 µL of each sample was spotted onto plates inoculated with the target strains, as described above.

2.4 Amplification and Sequencing of 16S rDNA

PCR were performed using VWR reagents (France). Template DNA was extracted from cell pellets resuspended in MilliQ water by boiling for 5 min, followed by centrifugation. Each 50 µL reaction mixture contained 5 µL of 10× PCR buffer, 1 µL of deoxyribonucleotide triphosphate mixture (10 µM each), 1 µL of each primer (10 µM), 0.25 µL (1.25 U) Taq polymerase and 1 µL of template DNA. The following PCR program was used: 95 °C for 30 s followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, followed by an extension step of 7 min at 72 °C. The oligonucleotide primers used were the specific 16S rDNA archaeal primers Arch 21F (5'-TTCCGGTTGATCCYGCCGGA-3') and Arch 958R (5'-YCCGGCGTTGAMTCCAATT-3') or the eubacterial 16S rDNA primers Eubac 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and Eubac 1492R (5'-GGTTACCTTGTTACGACTT-3') [18]. The primers used for the amplification of the almost complete archaeal 16S rRNA genes were S-D-Arch -0025-a-S-17 (5'-CTGGTTGATCCTGCCAG-3') and S-*-Univ- 1517-a-A-21 (5'-ACGGCTACCTTGTTAC GACTT-3') [19]. Positive controls were performed

using *Hbt. salinarum* DSM 3754 or *Hfx. Mediterranei* DSM 1411. The primers used to amplify a 327 bp fragment of the gene encoding halocin C8 were C8-9 (5'-CCGACGGGGTGCAGTCC-3') and C8-10 (5'-CG TCACCGCAACCGCTGT-3') [20]. Amplified DNA was submitted to electrophoresis on 1% (w/v) agarose gels in Tris-acetic acid-EDTA (TAE) buffer containing 1X GelRed® (Biotim) and visualised under UV light.

PCR products were purified using the GeneClean Turbo® kit (MPBio) and cloned into the pGEM-T Easy vector following the manufacturer's instructions (Promega). Plasmid DNA from four to six positive clones for each strain was extracted using the QuickLyse® kit (Qiagen) and sequenced by Beckman Coulter Genomics or Eurofins MWG Operon. The sequences were compared with the GenBank database using the BLASTN algorithm [21]. Nucleotide sequences of the 16S rRNA genes of the halophilic archaea from this study have been deposited in the Genbank database under the accession numbers HQ641743 to HQ641750, HQ844527, JN873316 to JN873337 and JQ281789 to JQ281796, as shown in Table 1.

2. 5 Phylogenetic Analysis of Haloarcula sp.

The phylogenetic analyses were performed on the following website: http://www.phylogeny.fr [22]. Multiple alignments were generated using ClustalW [23] and refined with Gblocks. Phylogenetic tree was reconstructed using the Neighbour-Joining method (BioNJ software) [24] with the Kimura 2-parameter, using 1,000 replicates in the bootstrap test. The tree was visualised using TreeDyn and rooted using the 16S rRNA gene sequence from strain SWO33 (this study) as the functional outgroup.

3. Results

3.1 Diversity and Persistence of the Cultivable Archaea in Three Algerian Hypersaline Sites

The physico-chemical characteristics of the three sites (pH = 7.2-8.1; NaCl concentrations 150-280 $g \cdot L^{-1}$) were consistent with growth requirements of halophilic microorganisms. The isolation conditions chosen for the selection of strains (two-weeks incubation in high salt concentration media) favoured the growth of halophilic archaea. Slow-growing pigmented colonies were selected. They were subsequently checked for their belonging to the archaeal domain by PCR amplification using archaeal and eubacterial specific primers. Morphological and isolation characteristics of the 24 archaeal isolates thus provided by this study are reported in Table 1. All isolates are resistant to ampicillin and chloramphenicol but sensitive to novobiocin. Sequencing of the almost full-length 16S rRNA genes showed that the 24 archaeal strains belonged to *Haloarcula*, *Halorubrum* or *Natrinema*, three genera of the *Halobacteriaceae* family (Table 1 and Fig. 1). *Haloarcula* sp. was found in the three Algerian environments, while *Halorubrum* sp. and *Natrinema* sp. were isolated specifically from Ouargla and Ichekaben, respectively.

BLASTN analysis of the almost full-length 16S rRNA genes assigned (> 99.5% identity with full-length 16S rRNA genes in the GenBank database) strains SWO33, SWO40 and SWO41 to the species *Halorubrum tebenquichense*, *Hrr. chaoviator* and *Hrr. ezzemoulense*, respectively (Table 1). As for *Natrinema* isolates, analysis of the 16S rRNA gene was not sufficient to clearly assign the Algerian strains to a species, mostly because many sequences in the database are identified only to the genus level. The presence of *Natrinema* appears as a characteristic feature of the salterns of Ichekaben, as it was isolated from both 2007 and 2009 samplings (Table 1).The majority of the strains (15 out of the 24 isolates) belonged to the genus *Haloarcula*, which was found in the three sites (Table 1 and Fig. 1). In Ichekaben salterns, *Haloarcula* isolates were retrieved from the 2007, 2008 and 2009 samplings (Table 1). For most strains it was not possible to refine the determination to the species level, due to the presence of two copies

Table 1 Features of the 24 halophilic isolates from this study.

Strain	Sampling site	Sampling year	Conditions of isolation[c]			Cell morphology[d]	Pigmentation	Identification (16S rRNA gene accesion n°)
			Temperature (°C)	NaCl (M)	$MgSO_4$ (mg/L)			
SI2	Ichekaben	2007[a]	30	4.3	20	Pleomorphic	Red	*Haloarcula* sp. (JN873319/JN873320)
SI4	Ichekaben	2007[a]	30	4.3	20	Coccoid to short rods	Pink	*Natrinema* sp. (JN873337)
SI11	Ichekaben	2008[b]	43	4.3	20	Coccoid	Red	*Haloarcula* sp. (JN873325/JN873326)
SI14	Ichekaben	2007[a]	30	4.3	20	Coccoid to short rods	Pink	*Natrinema* sp. (JN873317)
SSI1	Ichekaben	2009[a]	43	4.3	20	Pleomorphic	Red	*Haloarcula* sp. (JQ281789/JN873318)
SSI3	Ichekaben	2007[a]	30	4.3	20	Coccoid to short rods	Pink	*Natrinema* sp. (JN873336)
SSI7	Ichekaben	2009[a]	43	4.3	20	Coccoid	Pink	*Haloarcula hispanica* (JN873322/JN873323)
SSI9	Ichekaben	2009[a]	43	2.6	20	Coccoid to short rods	Pink	*Natrinema* sp. (JN873324)
SWI5	Ichekaben	2009[a]	43	2.6	0	Pleomorphic	Red	*Haloarcula* sp. (HQ641744/HQ641743)
SWI6	Ichekaben	2009[a]	43	2.6	0	Coccoid to short rods	Pink	*Natrinema* sp. (JN873316)
SWI8	Ichekaben	2009[a]	43	3.4	20	Pleomorphic	Red	*Haloarcula* sp. (HQ641746/HQ641745)
SWI15	Ichekaben	2007[a]	30	4.3	20	Coccoid to short rods	Pink	*Natrinema* sp. (JN873335)
SWI51	Ichekaben	2007[a]	30	4.3	20	Pleomorphic	Pink	*Haloarcula hispanica* (JN873321/JQ281794)
SSO54	Ouargla	1997[a]	43	4.3	20	Coccoid	Red	*Haloarcula* sp. (JN873329/JN873330)
SWO25	Ouargla	1997[a]	37	4.3	0	Pleomorphic	Red	*Haloarcula* sp. (HQ844527/HQ641747)
SWO32	Ouargla	1997[a]	43	4.3	0	Pleomorphic	Red	*Haloarcula* sp. (HQ641749/HQ641748)
SWO33	Ouargla	1997[a]	43	4.3	0	Pleomorphic	Orange/Red	*Halorubrum tebenquichense* (HQ641750)
SWO40	Ouargla	2000[a]	43	4.3	20	Pleomorphic	Red	*Halorubrum chaoviator* (JN873333)
SWO41	Ouargla	2000[a]	43	4.3	20	Pleomorphic	Red	*Halorubrum ezzemoulense* (JN873334)
SWW35	Oran (Wahran)	2009[a]	43	4.3	20	Pleomorphic	Red	*Haloarcula* sp. (JQ281790/JQ281791)
SWW43	Oran (Wahran)	2009[a]	43	4.3	20	Pleomorphic	Red	*Haloarcula* sp. (JQ281795/JQ281796)
SWW45	Oran (Wahran)	2009[a]	43	4.3	20	Pleomorphic	Red	*Haloarcula* sp. (JN873327/JN873328)
SWW46	Oran (Wahran)	2009[a]	43	4.3	20	Pleomorphic	Red	*Haloarcula* sp. (JQ281792/JQ281793)
SWW55	Oran (Wahran)	2009[a]	43	4.3	20	Pleomorphic	Red	*Haloarcula* sp. (JN873331/JN873332)

[a] Sampling in spring, [b] Sampling in summer, [c] pH of the media is 7.2, [d] Cell morphology as observed on medium 372 (DSMZ).

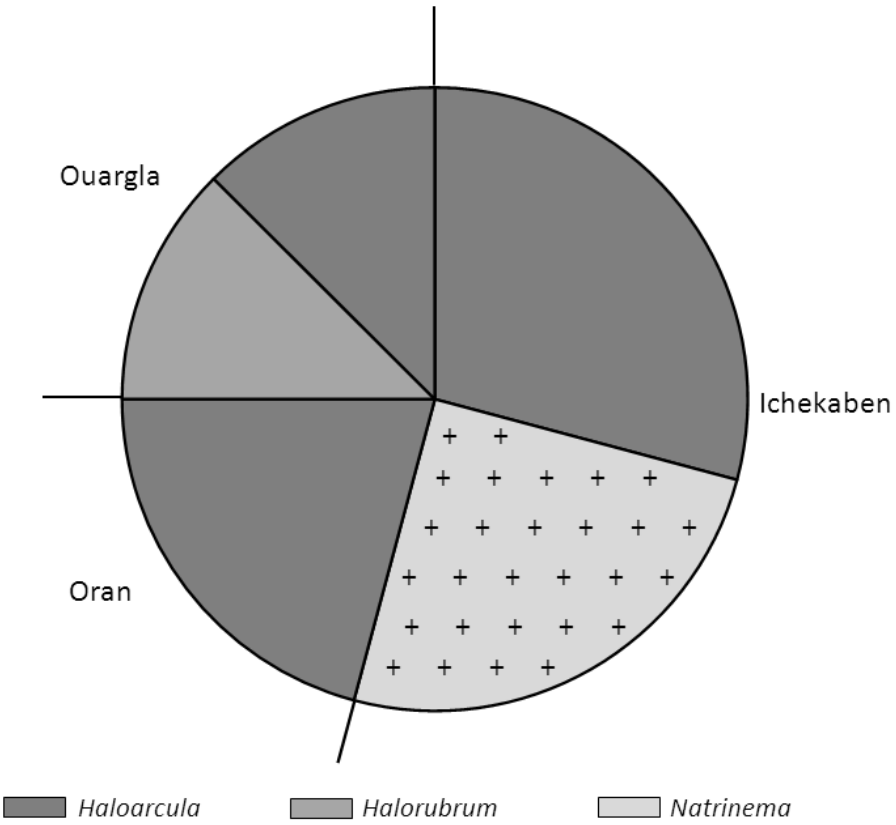

Fig. 1 Genus distribution of the cultivable haloarchaeal strains isolated along this study, according to their origin. Strains displaying antimicrobial activity are indicated by +.

of the 16S rRNA gene detected by the sequencing of four to six clones for each strain. A divergence of 5.0-6.0% was observed between the two copies of 16S rRNA gene sequence. Intragenomic 16S rRNA gene heterogeneity, with approximately 5% nucleotide substitutions between the two copies of the gene, was first detected in *Haloarcula marismortui* [25] and was then further shown as characteristic of *Haloarcula* strains [26].

3.2 Phylogenetic Analysis of Haloarcula Strains

The authors performed the phylogenetic analysis of the *Haloarcula* strains, including sequences of the fifteen strains isolated along this study and sequences of the type strain(s) of each defined *Haloarcula* species (Fig. 2). The authors also included relevant sequences such as those from species that remain taxonomically unvalidated and those from *Haloarcula* sp. OHF-1 and OHF-2 [27], which showed as the nearest relatives to most of the *Haloarcula* strains isolated in this study from BLASTN analysis. The phylogenetic tree of *Haloarcula* sp. based on 16S rRNA gene divides into two main branches, containing type I (*rrnA*) and type II (*rrnB*) sequences (Fig. 2) as defined by Cui et al. [26]. Two strains from the Ichekaben salterns (SWI51 and SSI7) are clearly affiliated with *Haloarcula hispanica*. These two strains were also morphologically distinct from the other *Haloarcula* isolates as they displayed a lighter pigmentation (pink rather than red, Table 1). Moreover, strains SWI51 and SSI7 were isolated from samples collected in 2007 and 2009, respectively, thus suggesting the regular presence of *Har. hispanica* in this saltern.

The phylogenetic analysis reveals that, as concerns one of the copies of the 16S rRNA gene, the other thirteen strains form one stable clade with a 94% bootstrap value and cluster with sequences arising

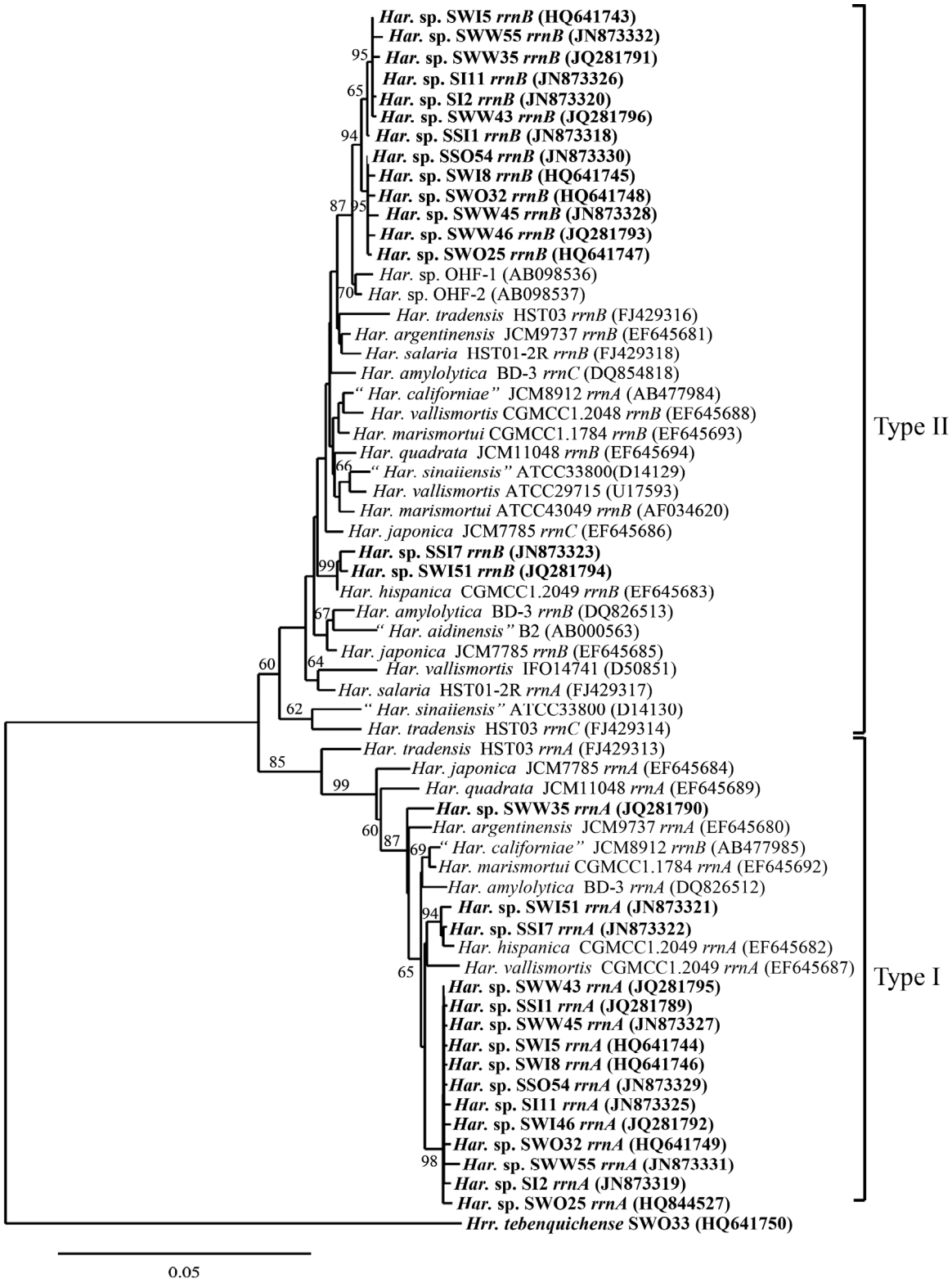

Fig. 2 Neighbour-joining phylogenetic tree of *Haloarcula*, based on 16S rRNA gene sequences. Accession numbers of the sequences are indicated in parenthesis. Taxonomically unvalidated species names are noted in quotation marks. Bold names correspond to the sequences generated in this study. Bootstrap values are based on 1,000 replicates and are noted on main branches with over 60% bootstrap support. Bar represents 0.05 expected changes per site.

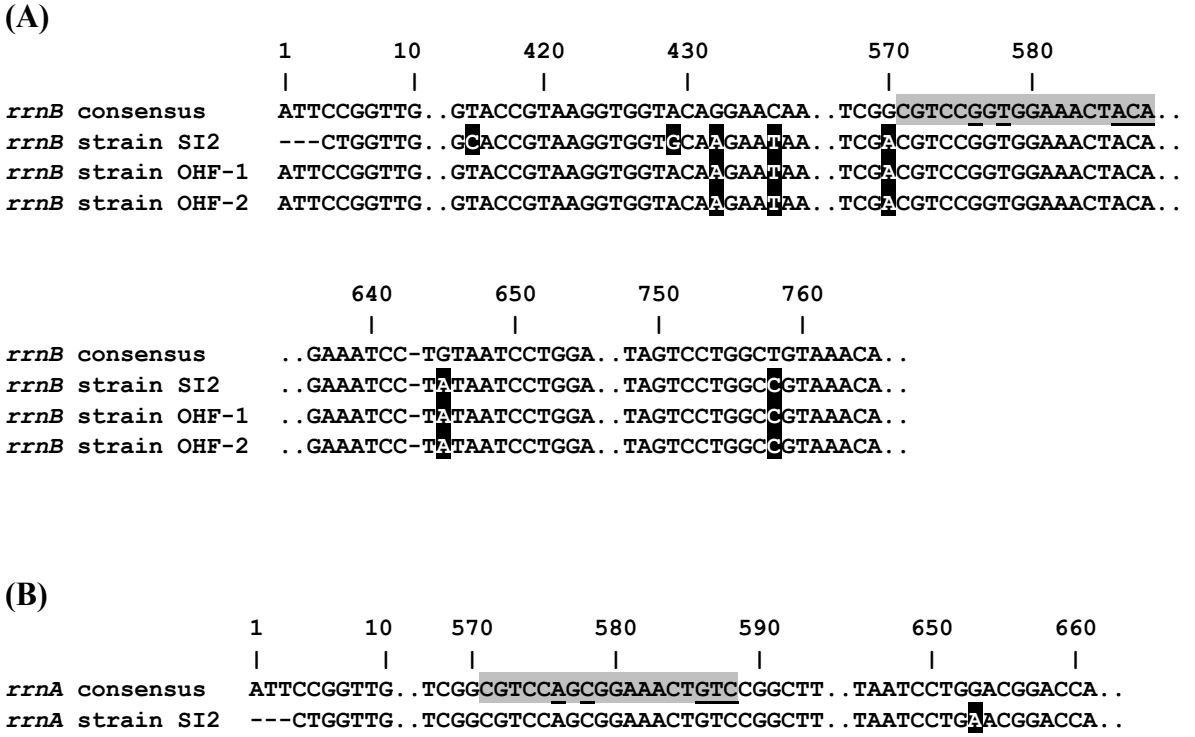

Fig. 3 Alignment of type II (A, *rrnB*) and type I (B, *rrnA*) 16S rRNA gene sequences from *Haloarcula* sp. Consensus *rrn* sequences (*rrnA* consensus: 9 sequences and *rrnB* consensus: 20 sequences) are common to all *Haloarcula* 16S rRNA genes available in GenBank except OHF-1, OHF-2 and the thirteen *Haloarcula* sp. from Algerian environments isolated in this study. For the positions shown in this alignment, the *rrnA* and *rrnB* sequences from strain SI2 are identical to those from strains SI11, SSI1, SWI5, SWI8, SSO54, SWO25, SWO32, SWW35, SWW43, SWW45, SWW46 and SWW55 (this study). Multiple alignments of sixty-one 16S rRNA genes were carried out by Clustal W [23]. Grey background indicates the localization of primers H1R and H2R used by Cui et al. [26] for detection of type I and type II 16S rRNA genes with the specific positions to each type underlined. Black background highlights nucleotides of thirteen *Haloarcula* sp. from Algerian environments, OHF-1 and OHF2 sequences, differing from the consensus sequences common to all *Haloarcula* 16S rRNA genes.

from strains OHF-1 and OHF-2 (Fig. 2). Since these sequences are included in the type II sequences branch, they were named *rrnB*. Our alignment of the *HaloarcularrnB* gene sequences unveils five positions which are very specific to the sequences of this *Haloarcula* cluster (Fig. 3A). Furthermore, two specific positions are observed in the *rrnB* gene sequences from the thirteen *Haloarcula* sp. from this study (Fig. 3A). The second copy of the 16S rRNA genes available in the 13 strains (minus one, SWW35, the *rrnA* sequence of which is divergent) cluster with a 98% bootstrap value and are included in the type I branch; therefore they were likewise named *rrnA*. In the *rrnA* gene sequences, one position is very specific to the thirteen *Haloarcula* sp. from this study (Fig. 3B) but cannot be compared to OHF-1 and OHF-2 since the sequences are not available. Thus, thirteen out of the *Haloarcula* strains isolated from the three Algerian hypersaline environments of this study form a phylogenetically coherent cluster.

3.3 Production of Halocins

In order to identify halocin-producing archaea, the authors performed antimicrobial assays of the archaeal strains against other isolates from this study and strains *Hbt. salinarum* DSM 3754 and *Hfx. mediterranei* DSM 1411. Only the six *Natrinema* strains displayed the capacity to inhibit significantly the growth of several of the halophilic archaea isolated in this study, including *Haloarcula* sp. SWI5 and the

Table 2 Features of halophilic strains displaying antimicrobial activity.

Producing strain[x]	Halocin C8 gene[a]	Target sensitive strains[b]
Natrinema sp. SI4, SI14, SSI3, SWI6, SWI15	+	*Haloarcula* sp. SWI5, SWI8, SWO32 *Natrinema* sp. SSI9 *Halobacterium salinarum* DSM 3754 *Haloferax mediterranei* DSM 1411
Natrinema sp. SSI9	-	*Haloarcula* sp. SWI5 *Natrinema* sp. SI4, SI14, SSI3, SWI6, SWI15 *Halobacterium salinarum* DSM 3754 *Haloferax mediterranei* DSM 1411

[a] Presence of the halocin C8 gene was assayed by PCR amplification using primers C8-9 and C8-10, followed by sequencing of the 327 bp amplicon, [b] Strains for which inhibition halos were > 5 mm.

culture collection strains *Hbt. salinarum* DSM 3754 and *Hfx. mediterranei* DSM 1411 (Inhibition halos 15-30 mm, data not shown, Table 2 and Fig. 1). This inhibition was comparable to that observed in similar experimental conditions using a producer strain *Hfx. mediterranei* DSM 1411 which is known to produce halocin H4 [28]. Furthermore, sensitivity of the antimicrobial compounds produced by *Natrinema* sp. SWI6 and SSI9 to papain, pepsin, proteinase K, and trypsin was assayed. While both cell pellet and culture supernatant from strains SWI6 and SSI9 produced inhibition halos over a confluent lawn of *Hbt. salinarum* DSM 3754 cells, the antimicrobial activity was annihilated after treatment with papain. By contrast, no sensitivity of the antimicrobial compound(s) produced by strains SWI6 and SSI9 to pepsin, proteinase K, or trypsin could be detected.

The *Natrinema* strains could be separated in two groups based on their antimicrobial spectra, in particular concerning *Natrinema* cross-inhibition. Indeed, strain SSI9 inhibited the growth of strains SI4, SI14, SSI3, SWI6 and SWI15. The five latter strains could not inhibit the growth of each other but all presented inhibition halos when grown on lawns of strain SSI9. One of the sequences presenting high identities (99.2-99.6%) with the 16S rRNA gene of the six *Natrinema* sp. strains from this study was that of strain AS7092, identified in the Genbank database as *Halobacterium*. However, this Chinese strain from which halocin C8 has been purified [29] and the corresponding gene identified [30], belongs in fact to the genus *Natrinema* [31]. Therefore, the *Natrinema* strains were investigated for the presence of the gene encoding halocin C8 by PCR amplification with specific primers [20]. An amplicon of the expected size (327 bp) was detected for strains SI4, SI14, SSI3, SWI6 and SWI15 but not for strain SSI9 (Table 2). Sequencing of this amplicon confirmed that the five strains possess an identical DNA fragment with a sequence harbouring 99% identity with that of the halocin C8 gene (data not shown).

4. Discussion

The cultivable halophilic archaea isolated from the three Algerian environments belong to three genera of the *Halobacteriaceae*. While *Haloarcula* was isolated from all sites, *Halorubrum* and *Natrinema* were found only at Ouargla and Ichekaben, respectively. Although cultivation bias cannot be overruled, this is not the usual pattern observed in cultivation-based studies of halophilic archaea, which generally demonstrate the predominance of *Haloferax* and *Halorubrum* isolates [7, 32-34]. Microbial diversity of Algerian salt lakes has seldom been studied. One molecular study of two Algerian saharian sebkhas has revealed the presence of *Haloferax*, *Halorubrum*, *Halalkalicoccus*, *Haladaptatus*, *Halobacterium* and *Halosarcina* but not of *Haloarcula* and *Natrinema* [35]. Reports comparing cultivation and molecular approaches have shown that the presence of *Halorubrum* sp. and *Haloarcula* sp. is normally identified by both methods [32, 36]. The results emphasize the statement that molecular and cultivation based methods are complementary to estimate microbial diversity in

natural environments. To our knowledge, *Natrinema* strains have been isolated from China, Thailand, India, and a deep salt lake in the Mediterranean Sea [37-42] but this study is the first report of the presence of *Natrinema* sp. in North Africa.

Hrr. ezzemoulense, which was isolated from Ouargla in the sample collected in 2000, was originally described from the sebkha of Ezzemoul in the North of Algeria [16]. It thus appears that several species of *Halorubrum* are present in Algerian salt lakes with at least *Hrr. ezzemoulense* being present both in lakes at the North of the country and in Saharan areas. As for *Haloarcula*, two phylogenetically-consistent groups of strains were isolated. The *Har. hispanica* SWI51 and SSI7 from the 2007 and 2009 samples of Ichekaben demonstrate the persistence of this species in this region of Algeria. *Har. hispanica* was originally isolated from a Spanish solar saltern [43]. It is thus possible that this species has developed particular adaptations that allow its survival in salterns, an environment that has been created by mankind. The 13 remaining *Haloarcula* strains isolated from the three hypersaline environments form a stable monophyletic cluster. This conclusion remains true for the two copies of the 16S rRNA gene except for SWW35 whose *rrnA* sequence is divergent. This suggests that the *Haloarcula* population in Algerian hypersaline environment is largely dominated by this phylogenetic cluster, which would be particularly persistent in Ichekaben salterns as it was retrieved from three successive sampling campaigns.

Since *Haloarcula* sp. and *Natrinema* sp. were regular inhabitants of the salterns of Ichekaben during the three-year period of the study, the authors investigated whether this could be linked to the production of antimicrobial compounds. Only the *Natrinema* isolates displayed inhibitory activities. In the case of strain SWI6, the sensitivity of the antimicrobial activity to papain implies a proteinaceous nature, thus pointing to the presence of a halocin. Five strains (SI4, SI14, SSI3, SWI6 and SWI15) would produce a compound related to halocin C8, previously isolated from the Chaidan salt lake in China. In *Natrinema* AS7092, the gene cluster includes a gene encoding a precursor protein which is further cleaved into an immunity protein (which protects the strain against its own toxic production) and the active halocin, along with genes involved in transport and regulation [20, 30]. It thus appears that geographically distant sites (Algeria/China) would be inhabited by phylogenetically-related archaea producing a common antimicrobial peptide. This statement contrasts with previous observations in the other prokaryotic domain *Bacteria*. The existence of families of antimicrobial peptides is well documented in the bacterial domain and the corresponding genes are generally incongruently distributed among phylogenetic groups. Genes involved in biosynthesis of bacterial antimicrobial peptides are often carried by plasmids and/or localized close to mobile elements.

Although the *Natrinema* sp. SSI9 displayed strong antimicrobial activity against *Natrinema* sp. SI4, SI14, SSI3, SWI6 and SWI15, the authors were not able to amplify the halocin C8 gene. Following the definition of bacteriocins [44] which are generally active on phylogenetically related strains, this constitutes a first indication that the activity of SSI9 would be due to a halocin. This statement is strengthened by the sensitivity of the antimicrobial compound to papain. Strain SSI9 might either produce another halocin or a variant of halocin C8 with sequence divergence and/or a different immunity system. One other strain of *Natrinema* isolated from Indian salt pans has been reported to produce a poorly characterized halocin [45]. It thus appears that the *Natrinema* genus may prove to be prolific in terms of production of antimicrobial compounds and that it might explain their persistence in the site of Ichekaben compared to other hypersaline sites for which *Natrinema* sp. isolates have been seldom described.

The ecological role of halocins remains unclear.

While it has been stated that production of halocins is a universal feature of halophilic archaea [14], one study reports that halocin activity in hypersaline ponds is negligible [11]. However, the latter work checked for the presence of halocins in cell-free water samples. The authors have observed, and it has also been reported in other works [20], that the antimicrobial activity remained partly associated to the membrane of the producing cells. In the case of halocin C8, it has been suggested that the immunity protein anchored to the membrane could be sequestering the antimicrobial peptide [31]. In the field, it is possible that halocins are released upon environmental signals that remain to be identified.

5. Conclusion

Cultivable halophilic archaea were investigated from two natural salt lakes (sebkhas) and a traditional solar saltern in Algeria. Algerian hypersaline environments are colonized by a persistent new phylogenetic cluster of *Haloarcula*, among which no isolate displayed the capacity to inhibit the growth of other halophilic strains. This work also provides the first evidence concerning the existence of conserved halocin gene clusters among haloarchaeal strains isolated from distant sites and may be correlated to the particular persistence of the *Natrinema* genus in the Ichekaben salterns. In the future, further investigations will certainly unveil new aspects of the emerging field of archaeal antimicrobial compounds and their ecological impact.

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Physiological Races of *Fusarium oxysporum* f. sp. *Ciceris* in Iraq

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Abstract: Twenty isolates of *Fusarium oxysporum* f. sp. *ciceris* were isolated from wilted chickpea plants obtained from different districts of north part of Iraq to assess variability in pathogenicity of the populations. Each isolate was tested on 12 differential chickpea varieties. Isolates showed highly significant variation in wilt severity on the differential varieties. Based on the reaction types that induced on differential varieties, isolates were grouped into four groups, First group included isolates FocS1, FocQ7, FocQ10, FocF13, FocH17 and FocH18; the second group included isolates FocS2, FocS3, FocS4, FocQ5, FocQ8, FocQ9, FocF11, FocF12, FocF14 and FocH19; the third group included isolates FocF15, FocH16, FocH20; where the isolate FocQ6 was placed in the fourth group. Results showed that the percentage of genetic similarity was ranged 42% to 100% and was 42% between the first group and other groups and 72% between the three groups the rest and thus this indicate the presence of four races of the fungus which are 0, 4, 5 and 1B/C, this represent the first record of these races in Iraq.

Key words: Chickpea wilt, *Cicer arietinum*, *Fusarium oxysporum* f. sp. *ciceri*s, pathogenic variability.

1. Introduction

Chickpea (*Cicer arietinum* L.) is an important food legume in Iraq and is grown mainly in the north part of the country for domestic consumption purposes. In the 2007 cropping season, chickpea was cultivated on 10500 ha of land with a productivity of about 700 kg/ha as compared to 3 t/ha in the world [1]. This low productivity was due to a variety of a biotic and biotic stresses. Chickpea is attacked by a number of soil-borne and foliar diseases as well as field and storage insect pests. Among the soil-borne diseases affecting chickpea, Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceris* is the major yield-limiting factor, wherever the crop is grown in north Iraq. The wilt can be observed in susceptible genotype within 25 days after sowing in the field. The pathogens attack the roots of plants and cause wilting as a result the whole plant shows drooping of leaves and paler color than healthy plants. The plant finally collapses and dies. Such plants do not show external rotting and look healthy, when cut vertically downward from the collar region, show brown streak of the internal tissues [2, 3].

Throughout the world, annual chickpea yield losses due to Fusarium wilt vary from 10% to 15% [4] but can reach even 100% under certain conditions [5]. In Ethiopia, about 30% yield loss of chickpea due to wilt/root rot has been reported, where *F. oxysporum* f. sp. *ciceris* was isolated from more than 50% of the root samples. Fusarium wilt of chickpea can be managed using resistant cultivars, adjusting sowing dates, and fungicidal seed treatment [6].

The occurrence of pathogenic races in *F. oxysporum* f. sp. *ciceris* is known from other parts of the world [7-11], however, until now the presence of pathogenic variability in populations of *F. oxysporum* f. sp. *ciceris* in Iraq has not been studied. Therefore, in

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order to strengthen the breeding efforts that aim at boosting chickpea productivity and production through the development of wilt resistant chickpea varieties, this study was undertaken with the aims of assessing the pathogenic variability in isolates of *F. oxysporum* f. sp. *ciceris*, causing chickpea wilt.

2. Materials and Methods

2.1 Collection of Wilted Chickpea Plant Samples

Wilted chickpea plant samples were collected from four major chickpea-growing districts, which are in the production domain of north Iraq (Ninevah and Erbil provinces). In each district, ten chickpea fields were observed and wilted plants were systematically collected by traversing each field diagonally.

2.2 Isolation, Purification and Identification of Fusarium Isolates

Five pieces of plant tissue (1-2 mm) were taken from the collar region of each wilt-affected plant and the remaining part was split lengthwise and checked for browning of vascular tissue to confirm fusarial wilting [12] pieces of plant tissue, taken only from plants that showed clear vascular discoloration, were surface disinfected with 1% sodium hypochlorite solution for 2-3 min, rinsed twice in distilled sterilized water, dried on filter paper and were plated on 2% water agar in 9 cm Petri dishes [7].

The plates were incubated at 25 °C for 5 days. After incubation, a loopful of conidial mass was transferred from actively growing *Fusarium* colony to 10 mL of distilled and sterilized water to prepare conidial suspension. The conidial suspension was diluted 10 times to obtain well-separated conidia, and from the final dilution one loopful was taken and streaked on solidified 2% water agar medium. After 24 h of incubation at 25 °C, a single colony was cut from well-separated colonies and transferred to a Petri dish containing solidified potato dextrose agar (PDA). A total of 20 isolates of Foc were isolated. FocS1-FocS4 from Shakhan, FocQ5-FosQ10 from Al-Qush, FosF11-FosF15 from Fyda and FosH16-FosH20 from Harer regions. *Fusarium* isolates were identified microscopically by their morphological characteristics such as abundance of micro-and fewer macroconidia, short and unbranched monophialides, white to creamy-white color on PDA medium and production of chlamy dospores.

2.3 Inoculum Preparation and Infestation of Soil

Each isolate of Foc was multiplied on Petri dishes (9 cm diameter) containing 20 mL of PDA by transferring a small amount of infested sand from the test tube. From 7-day-old culture, two discs (1 cm diameter each) were removed with sterile cork borer and transferred to 100 g of sand: maize meal (9:1, autoclaved twice at 1,218 °C for 20 min) in marmalade glass jars and incubated for 14 days at 25 °C [13]. The inoculum raised in each marmalade jar was mixed thoroughly with 2 kg sterilized soil in 15 cm plastic pots. The pots were disinfested with 2.5% sodium hypochlorite solution for 5 min, rinsed in distilled water and air-dried before use. The isolates were allowed to become established in the infested soil/sand mixture for 1 week before planting of chickpea seeds.

Seeds of different chickpea differential varieties were surface-sterilized by immersion in 2.5% sodium hypochlorite solution for 2-3 min, rinsed twice in distilled sterilized water, air-dried in the laboratory and planted in each pot containing previously sterilized and then artificially infested with pathogen soil-sand mixture.

Pots were arranged in a randomized complete block design with three replications for each isolate-differential line or isolate-variety combination and maintained on greenhouse benches for 60 days.

Data on the responses of all differential varieties to deferent isolates of the pathogen was recorded at weekly intervals. Disease development on differential varieties was scored on the disease reactions were graded as resistant (0%-20% wilt), moderately

susceptible (> 20% to 50% wilt) and susceptible (> 50% wilt). Vascular discoloration was checked by splitting the stem and tap roots of two to three plants chosen randomly to confirm that the disease is caused by Foc [12]. Based on similarity/dissimilarity of reactions of differential varieties, isolates were designated into deferent groups. These values (0 and 1) were entered in the program (NTSYS). A result of the analysis a matrix included transactions genetic similarity between isolates, including the work of the genetic family tree Dendrogram of all isolates that collected in groups based on the extent of similarity or genetic variation among them, and using UPGMA (Unweighted Pair Group Method with Arithmetic).

3. Results and Discussion

3.1 Pathogenic Variability in F. oxysporum f. sp. Ciceris

All the 20 isolates of *F. oxysporum* f. sp. *ciceris* (Foc) collected from farmer's fields' infected and produced deferent levels of disease severity on the deferential varieties of chickpea. Reaction types calculated also deferred among isolates. The isolates showed highly significant variation.

In a study of genetic variability for 20 isolates of the fungus Foc collected from different areas of chickpea planted in Nineveh and Irbil provinces. The results presented in Tables 1 and 2 showed that there was difference in the pathogenicity of these isolates in effecting chickpea differential varieties. The isolates were classified in four groups depending on the reaction of differential varieties. The first group, which included isolates FocS1, FocQ7, FocQ10, FocF13, FocH17 and FocH18, from the results obtained it was concluded that these isolates belong to the 0 race which is known internationally [8]. The race 0 was non-pathogenic to the JG-62 variety, while this variety was susceptible to other races of the pathogen [7, 14-17].

The isolates caused severe and progressive

Table 1 Reaction of 12 chickpea differential varieties to the 20 isolates of *Fusarium oxysporum* f. sp. *ciceris*.

Isolate No.	Deferential varieties											
	1	2	3	4	5	6	7	8	9	10	11	12
FocS1	S	R	S	R	S	S	R	S	MS	S	S	R
FocS2	S	S	S	R	MS	S	MS	S	S	MS	MS	R
FocS3	S	S	S	R	MS	S	MS	S	S	MS	MS	R
FocS4	S	S	S	R	MS	S	MS	S	S	MS	MS	R
FocQ5	S	S	S	R	MS	S	MS	S	S	MS	MS	R
FocQ6	S	S	S	MS	S	S	MS	S	R	MS	R	R
FocQ7	S	R	S	R	S	S	R	S	MS	S	S	R
FocQ8	S	S	S	R	MS	S	MS	S	S	MS	MS	R
FocQ9	S	S	S	R	MS	S	MS	S	S	MS	MS	R
FocQ10	S	R	S	R	S	S	R	S	MS	S	S	R
FocF11	S	S	S	R	MS	S	MS	S	S	MS	MS	R
FocF12	S	S	S	R	MS	S	MS	S	S	MS	MS	R
FocF13	S	R	S	R	S	S	R	S	MS	S	S	R
FocF14	S	S	S	R	MS	S	MS	S	S	MS	MS	R
FocF15	S	S	S	MS	MS	S	S	S	R	MS	R	R
FocH16	S	S	S	MS	MS	S	S	S	R	MS	R	R
FocH17	S	R	S	R	S	S	R	S	MS	S	S	R
FocH18	S	R	S	R	S	S	R	S	MS	S	S	R
FocH19	S	S	S	R	MS	S	MS	S	S	MS	MS	R
FocH20	S	S	S	MS	MS	S	S	S	R	MS	R	R

1: Annigeri; 2: JG-62; 3: ICC4475; 4: K850; 5: UC27; 6: CHAPP2; 7: PCH-15; 8: C-104; 9: L550; 10: CPS-1; 11: BJ-212 and 12: WR-315.

Table 2 Reaction of 12 chickpea differential varieties to four races of *Fusarium oxysporum* f. sp. *ciceris*.

Deferential varieties	Races			
	0	4	5	1B/C
Annigeri	S	S	S	S
JG-62	R	S	S	S
ICC4475	S	S	S	S
K850	R	R	MS	MS
UC27	S	MS	MS	S
CHAPP2	S	S	S	S
PCH-15	R	MS	S	MS
C-104	S	S	S	S
L550	MS	S	R	R
CPS-1	S	MS	MS	MS
BJ212	S	MS	R	R
WR-315	R	R	R	R

yellowing without wilting on L-550 variety, this is fully accomplished with the finding of Ref. [11] that the 0 race cause yellowing symptoms without wilting. The isolates FocS2, FocS3, FocS4, FocQ5, FocQ8, FocQ9, FocF11, FocF12, FocF14 and FocH19 which was conceive pathogenicity placed in the second group, which gave results comparable to race four [9, 16-18], these isolates which were able to affected all varieties except WR-315 and K850, which were resistant to these isolates. These isolates caused severe infection on six varieties (Annigeri, JG-62, ICC4475, CHAPP2, L550 and C-104). The third group, consists FocF15, FocH16 and FocH20 which it gave different reactions in the infection of chickpea differential varieties and depending on resistance reaction of WR-315 and BJ212 varieties and moderate susceptibility of the varieties K850, UC27 and CPS-1, It can be considered that these isolates are very closed to race 5 [14, 16, 19]. These isolates caused severe infection on the chickpea differential varieties (Annigeri, JG-62, ICC4475, CHAPP2, PCH-15 and C-104). Where the isolate FocQ6 has placed in the fourth group, which belong to 1B/C race depending on the reaction of the UC27 verities which was susceptible to the isolate FocQ6 where the two varieties K850 and PCH-15 were moderate susceptible [14, 19].

Through the results it is clear that 30% of the isolates belonged to the first group which is known as race 0 and 50% of the isolates belonging to the second group which is known as race 4 and 15% of the isolates belonging to the third group which is known as race 5 and 5% of the isolates belonged to the fourth group which is known as race 1B/C and these results are in agreement to those results mentioned by Haware and Nene [7], which they revealed that there were four races of the fungus in India. Dolar [20] reported the existence of races 0, 2 and 3 in Turkey, while [21] by screening 24 isolates of *F. oxysporum* f. sp. *ciceris* were isolated from wilted chickpea plants obtained from deferent districts and "wilt sick plots" of central Ethiopia to assess variability in pathogenicity of the populations, isolates were grouped into four corresponding races of the fungus and there are four races of reasoned patients in Ethiopia.

Depending on the pathogenicity of these isolates a dendrogram was constructed to show the genetic variation of isolates based on the extent of the genetic variation among them, the results of genetic analysis have given four groups (Fig. 1). The first group included isolates FocS1, FocQ7, FocQ10, FocF13, FocH17 and FocH18, the second group included isolates FocS2, FocS3, FocS4, FocQ5, FocQ8, FocQ9, FocF11, FocF12, FocF14 and FocH19, the third group satisfaction to isolates FocF15, FocH16 and FocH20,

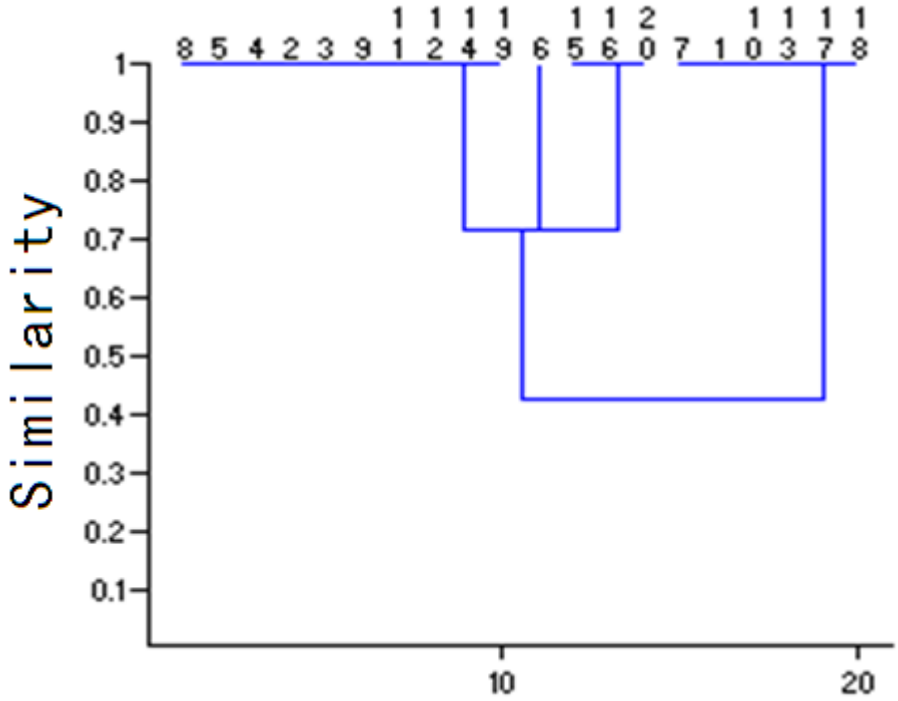

Fig. 1 The genetic tree of 20 isolates of the fungus *Fusarium oxysporum* f. sp. *ciceris* from Ninevah and Erbil provinces using UPEGMA programs showing similarity between groups.

and the fourth group included one isolate (FocQ6). It is clear from this dendrogram that the proportion of genetic similarity among these isolates was from 42% to 100%.

It was 42% between the first group and the other groups and 72% between the rest three groups. Results in Table 1 indicate the presence of four races of the fungus, which are given in Table 2 namely (0), (4), (5) and (1B/C). These results represent the first record of these races in Iraq. It is clear that there are differences in the pathogenicity of the isolates of different regions, which is due to the difference in the phenomenon of sexual reproduction in fungi accounting for the accumulation of variables and the occurrence of genetic differences and as a result globally there are eight races which are distributed in the world [7, 10, 19].

4. Conclusion

The determination of race distribution of *F. oxysporum* f. sp. *ciceris* is fundamental to guide the development of appropriate strategies for disease management according to different regions. However, there are no reports about the determination of *F. oxysporum* f. sp. *ciceris* races in different chickpea growing areas in Iraq. In this study, variability of *F. oxysporum* f. sp. *ciceris* was examined in the important chickpea growing areas of North Iraq for the first time. Four different races were determined among the 20 isolates. These results will be useful in developing of integrated strategies for diseases management and breeding programs to Fusarium wilt.

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varieties used in this study.

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Impacts of Anthropogenic Stresses on Biodiversity: A Case Study of Lumbini, Nepal

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Abstract: Biodiversity conservation denotes the protection, management and utilization of resources available on or below the surface of the earth. The sustainability of biodiversity resources rely on the ecological balance. A sound planning based on ecological background and human needs provide successful management of natural resources. The study area, Lumbini harbors rich biodiversity and provide significant habitats for several species of birds, mammals, herpetofauna and fish. There are four major habitat types in Lumbini area-farmland, forest, grassland and wetland. The resources outside the Lumbini Development Trust and surrounding villages have already depleted due to excessive human interference in natural resources. Lumbini and its vicinity are rich in natural resources but poor in terms of infrastructure and delivery of services. The areas are losing natural wealth to pay for development. Rural areas around Lumbini are confronted with worsening poverty, ill health, illiteracy and continuing deterioration of natural ecosystems on which the local residents depend for their well-being. The only way to assure a safer and more prosperous future in Lumbini area is to deal with environment and development issues in a balanced manner. This research work attested that the local stakeholders feel ownership on people centered conservation.

Key words: Biodiversity, Lumbini, Laxmipur, plot, vegetation, density.

1. Introduction

Nepal is a small country which has total geographical area of 147,181 km^2 and has about 30 million populations [1]. The population growth rate is 2.24 percent per annum that figures double rate of 31 years [2]. Nepal is a least developed country which has been ranked as one of the world's poorest countries with per capita income US $220. It is a landlocked country, roughly rectangular in shape; the land extends approximately 885 km east-west and 145 km at its narrowest to 241 km at its broadest north-south. The country is bordered by Tibet, the Autonomous Region of China in the north and by India in the east, south and west.

The country is divided into three ecological regions: the snowcapped high Himalayas, the mountainous region and the flat sub-tropical Terai region. The high Himalayan region extends in the north from west to east at an altitude of 4,000 to 8,848 m. The mountainous region consists of mountain varying in height from 1,525 to 4,877 m with long terraced slopes leading to fertile valleys. Below these ranges lies the Churia range from 610 to 1,524 m. fertile valleys of various widths and altitudes lie between these mountains and hill ranges.

Since decades back, the loss of biodiversity in Nepal has drawn the attention of the government of Nepal to prepare national biodiversity strategy considering the resources degradation. The fragile ecosystem of hilly area has been under intense pressure for decades. It is due to excessive number of livestock, rapidly increasing population (2.6%), unsustainable exploitation of natural resources, inadequate management practices and development of unsound infrastructures are damaging the precariously

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balanced ecosystems of hilly region of Nepal [3]. The increasing poverty (32%) of the people forces them to encroach vulnerable marginal lands for cultivation, deforestation and over grazing that aggravate the wholesome environment. As a result, the country is losing fertile soils, irrigation systems, dams, hydro-powers, roads and bridges, severe loss on socio-economic development and tragic loss of life and property increased year after year.

It is therefore imperative to find immediate and medium term solution to avoid further loss of biodiversity around the country. Several areas have already degraded or are being degraded due to lack of participatory conservation plan. The current study focuses on the particular area of the country, the Lumbini area.

The reason to choose Lumbini and Laxmipur as study area is to preserve this area and eventually reverse the trend of loss of biodiversity in the adjacent Laxmipur area and surrounding villages. This study will find out the impacts of human pressure on biodiversity and the ways to mainstream the local residents in conservation and development programs. It is a cultural heritage site and has profuse biodiversity. This research will focus the biodiversity issues only and not the archeological aspect. The establishment of Lumbini Development Trust in 1985 is an important endeavor to conserve the flora, fauna and archeological remains of Lumbini.

Laxmipur settlement is one of the rural settlements with average family size is 7.09 [4] situated nearby Lumbini Development Trust. The village can be reached by gravel and earthen road. The natural resources such as forests and grasslands are rarely found. As a result, most of the households have used cow dung cake for cooking and heating since more than two decades in Lumbini area [5]. It is reported that women have to work 14.15 h daily-the heavy workload ever recorded in Terai region [6]. The reason of the workload is due to large involvement in preparing cow dung cake, drying, harvesting and so forth. The agricultural productivity is decreasing and use of chemical fertilizers to grow more food is increasing [7].

1.1 Modified Whittaker Plots for Vegetation Enumeration

The Modified-Whittaker plot design is a vegetation sampling design frequently used to assess plant communities at various scales [8]. The plots to be established measure 1000 m^2. (50 m × 20 m) which has sub plots of three sizes. The one plot measures 100 m^2 (20 m × 5 m) in the center and two other plots measure 10 m^2 (5 m × 2 m) are established in corners opposite. There are ten smaller subplots of 1 m^2 (2 m × 0.5 m) arranged in a way that six subplots fall inside the main plot and adjacent to the 100 m^2 subplots perimeter. Plots are placed parallel to the major environmental gradient of the vegetation type being sampled to encompass the most heterogeneity. Rectangular plots placed parallel to the major environmental gradient of a vegetation type encompass more heterogeneity and recover greater species richness than round or square quadrats. Because this holds true at all scales, this shape is kept consistent for the plot and its nested subplots [8].

1.2 Description of the Study Area (Lumbini Area)

Lumbini area lies in Terai physiographic region of Nepal. The climate is hot in summer and cold in winter with high humidity (99%). The temperature could go up to 44 °C and drops to 1 °C. The topography of the study area is plain. The soil type varies from sandy loam to clay and clay loam. The clay type soil has marsh lands and loam soil has extensive agricultural farms. The location (latitude, longitude and altitude) of the study area is shown in Table 1.

2. Statement of the Problem

The recent and past human activities have made conservation efforts a serious threat. Many other sites have been degraded and others are prone to

Table 1 Coordinates of Lumbini development trust.

Direction	Latitude (North)	Longitude (East)	Altitude (Feet)
SE	27°27′45.23″	83°17′02″	316
SW	17°27′45.22″	83°16′03.51″	320
NE	27°30′20.12″	83°17′02.88″	350
NW	27°30′21.46″	83°16′05.46″	323

Coordinates of Laxmipur village.

Latitude (North)	Longitude (East)	Altitude (Feet)
27°27′23.19″	83°17′59.19″	
To	To	318-332
27°27′30.62″	83°17′57.96″	

Source: google earth software, downloaded on March 31, 2008 [9].

degradation. The factors contributing to the loss of biodiversity are excessive population growth in the areas (6.01 per households), excessive livestock grazing in the vicinity, and unsustainable use of forest resources and potential development of the eco-tourism sites [10]. These are causing environmental degradation. To address the above burning issues, rigorous and concrete conservation plans are needed.

The alarming loss of biodiversity of other sites has drawn serious attention to Lumbini. The continued depletion of biological resources and its consequences are being suffered by the residents of these places. Realizing the deleterious results, the Lumbini Development Trust [11] and the local people are trying to conserve the invaluable resources. To help with this endeavor, the current study developed a user friendly biodiversity conservation plan that will be in effect after July 2009.

Biodiversity conservation is strongly tied with people's livelihoods because it is a source of food, medicine, revenue, employment and other values [12]. Despite its potentials to contribute to poverty reduction in Nepal, it is under threat from direct and indirect causes. These causes are typically multiple and synergistic. The levels of causality include proximate causes (land clearing for agriculture, overgrazing, slash and burn and poaching directly induces loss of biodiversity), intermediate causes (inappropriate economic policies and land tenure and lack of understanding on the total economic values of biodiversity) and ultimate causes (population growth, poverty, low standards of living, lack of social development and empowerment which increase pressure on natural resources thus resulting in their over consumption) [13].

The main objective of the proposed research is to help the local population and the government of Nepal to develop a biodiversity conservation plan that would help preserves or improve the existing ecological diversity of plant and animal species in Lumbini region. Specifically, this study aims to conduct a biodiversity assessment of Lumbini and adjacent area.

3. Materials and Methods

The major objective of this research was to assess the biodiversity of the two sites (one is protected and the other is degraded) and help the local population and the government to develop a conservation plan that would help preserve the existing diversity in plant and animal species in Lumbini region.

The Lumbini development area is protected by the Lumbini Development Trust Act 2042 of His Majesty's Government of Nepal [11]. The other area is the human settlement with extensive agricultural lands and has already been degraded. The vegetation species of each designated plots are inventoried and analyzed. The loss of biodiversity of Laxmipur village and surroundings is due to accelerated human interference during the last four decades [14]. The malaria eradication in Terai region during 1960s is the

obvious reason which attracts thousands of permanent settlers in the area. The settlers migrated from hilly region which was safe from the communicable diseases at that time. The settlers deforested the area as much as they could to expand for agricultural production. The excessive number of livestock and intensive cultivation reduced the fertility of soil and now these lands are deteriorating at unsustainable rate [15].

The inventory of plants is done using a method common in USA. The Modified-Whittaker plot method is rarely used in Nepal to inventory plant communities in research works. This research takes Modified-Whittaker plot method as a tool to compare vegetation in two sites of the same locality, as shown in Fig. 1.

The size of the surveyed plots is shown in Table 2.

The floral and faunal database of the study sites has been obtained through previous literatures and on-site observation (see annexure). The enumeration of plants was completed using the total enumeration counting in the Modified-Whittaker Plot (detailed enumeration is available on request from the author).

The Modified Whittaker Plot is 0.1 ha (50 m × 20 m) and within this area, there are subplots in the center and corners. In the center, the subplot is 0.01 ha (20 m × 5 m) and two subplots are 0.001 ha (5 m × 2 m) in opposite corners. The smallest subplots are 0.0001 ha (2 m × 0.5 m) in two opposite corners and along the perimeter of the largest plot.

4. Results and Discussion

As vegetation information obtained, the study shows differential status in species richness, diversity and similarity. Using the Modified Whittaker Plot method, vegetation has been sampled in both sites. Since the sites are located in the same plain region, the locality offers less species diversity. The diversity and similarity indices are explained by statistical analyses.

The soil characteristics of the study sites are sandy loam and loamy soil that support vegetation and agricultural plants. Soil physical characteristics have not been studied yet and previous researches do not have soil description of Lumbini area. The soil is neither too acidic nor too basic as reported by district agriculture office. Because there are small rivers in and nearby Lumbini, sandy loam soil is found along the bank of river. The areas are rich in biodiversity and form a micro climatic site.

The area of study considering only Modified Whittaker Plots is 3.0 hectares taking two sites. Sampling is done in 210 Modified Whittaker Plots (140 in LD area and 70 in Laxmipur due to existing site conditions that do not allow lying plots).

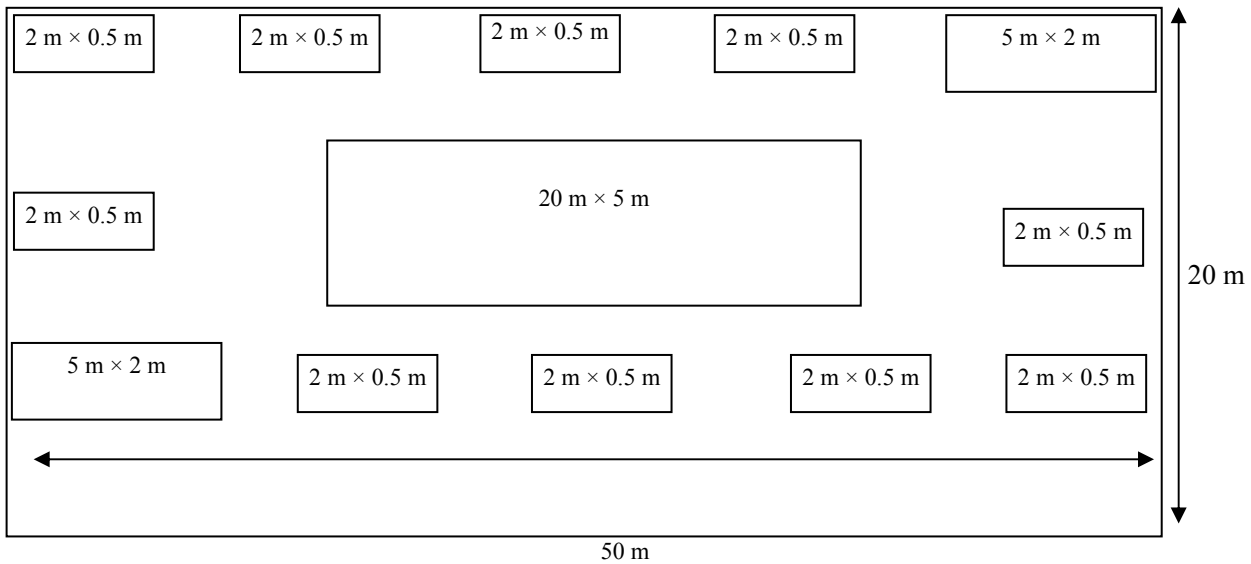

Fig. 1 Modified Whittaker Plots. (Adapted from Stohlgren 1995).

Table 2 The size of the surveyed plots.

Size of plot	Area (ha)	Total number of plots
50 m × 20 m	0.1	1
20 m × 5 m	0.01	1
5 m × 2 m	0.001	2
2 m × 0.5 m	0.0001	10

4.1 Biodiversity Index

The plant diversity is estimated to compare the distribution pattern in different plots or transects, either heterogeneous or homogenous, Simpson's index and Shannon Weaver index are widely used. This research has used both these indices to determine diversity species wise in each plot. The species diversity is estimated using diversity index. In practice, two important diversity indices has been used i.e. Simpson's diversity index and Shannon Weaver function. Both these indices are calculated in MWP in this research.

4.2 Simpson's Diversity Index (SDI)

The Simpson's Diversity Index gives the probability when individuals selected at random from communities that belong to different species. It considers number of species and total number of individuals of all species. The Simpson's Diversity Index is calculated applying the formula, $SDI = \Sigma\, n\,(n - 1)/N\,(N - 1)$ where N is the total number of individuals of all species, SDI is diversity index, and n is the number of individuals belonging to a species.

Simpson's Diversity Index shows inverse relationship with heterogeneity, i.e., index values decrease (or increase) as diversity increase (or decrease), i.e., higher (or lower) index probability values correspond to higher (or lower) diversity values. To account for this it has been proposed that Simpson' Index should be subtracted from its maximum possible value of 1 (Pielou 1977 cited in Schemnitz, S.D. 1980). Therefore, when a sample is being considered as a random sample from an infinitely large population, Simpson's diversity index is $D = 1 - \text{SDI}$.

4.3 Shannon Weiner Index

In most cases, it is not possible to identify and count every individual in the community. Therefore, it is necessary to take a random sample of individuals from a population of all species, the Shannon Weiner (1949) Index theory is a good estimate of diversity. It is simple and extensively used to determine diversity. The formula for the Shannon Weiner Index function is $H' = -n/N \times \ln (n/N)$ where ln is the natural log, n is the number of individuals of a species, and N is the total number of individuals of all species in the sample, as shown in Table 3.

4.4 Species Richness

The number of species on an area is called species richness of the area (Kreb, 1978). Richness is an indicator of the relative wealth of species in a community. From the available plot data, it is confirmed that Lumbini Development area has greater richness (87) than Laxmipur area (67).

4.5 Estimation of Similarity

The similarity index is used to compare two plant communities and determine how much they are similar. The measure of similarity can be used to examine temporal (time base), spatial (space base) in vegetation studies. The similarity index between two sites is 13.96%, as shown in Table 4.

The species diversity, similarity and richness in two sites (Lumbini Development Trust area and Laxmipur village) show a distinct variation in biodiversity. The LD area has higher species diversity evidenced by statistical results than Laxmipur. The species similarity between two sites is indexed at 13.96% and the Lumbini Development area has a greater richness

Table 3 Summary results of species diversity.

Site	Plot density	Plot size (m^2)	Shannon Weiner Index	Simpsons Index
Lumbini Development area	Dense	50 × 20	2.737	0.0983
		20 × 5	2.002	0.190
		5 × 2	1.388	0.345
		2 × 0.5	1.787	0.282
	Medium dense	50 × 20	1.812	0.183
		20 × 5	1.563	0.206
		5 × 2	1.517	0.167
		2 × 0.5	1.432	0.209
Laxmipur	Dense	50 × 20	2.235	3.110
		20 × 5	1.712	0.300
		5 × 2	1.727	0.228
		2 × 0.5	1.243	0.363
	Medium dense	50 × 20	1.428	0.254
		20 × 5	0.956	0.333
		5 × 2	1.054	0.200
		2 × 0.5	1.560	0.067

The SWI species diversity results indicate that LDT area has rich diversity than the species diversity in Laxmipur, however 5×2 dense plot in Laxmipur has rich diversity than in LDT. It may be due to the selection of vegetation in dense plot (5×2) has more diverse species in Laxmipur. Similarly, the SI diversity in LDT has lesser value indicating that vegetation in enumerated plots are more similar than out of sample plots. The results are consistent with the vegetation found in preserved area in LDT. The vegetation in LDT are basically an artificially regenerated with species in plots. While the vegetation in Laxmipur are planted with mixed species and therefore has higher SI diversity.

Table 4 Statistical results of the two sites based on four parameters.

Plot	Size of plot	Number of species found in both sites		SD	Skewness	*t*-test	Correlation
		LD area	Laxmipur				
Dense	50 m × 20 m	29	20	6.363961	1.287189	0.18843	0.873793
	20 m × 5 m	14	14	0			
	5 m ×2 m	7	10	2.12132			
	2 m × 0.5 m	12	7	3.535534			
		62	51	7.778175			
Medium dense	50 m × 20 m	9	5	2.828427	1.127599	0.039	0.457496
	20 m × 5 m	6	3	2.12132			
	5 m × 2 m	5	3	1.414214			
	2 m × 0.5 m	5	5	0			
		25	16	6.363961			

(87) of species than Laxmipur area (67).

The determination of various parameters of diversity, species richness, similarity has shown that the sites are not in similar. The MWP is used for both sites to assess from the sampled plots were analyzed using statistical methods.

The statistical results show that there is no significant variation in species diversity between sites, however, there is greater species richness in LD area than Laxmipur. The statistical results only interpret the species diversity and species similarity indices. The wildlife studies have not been done as this should be another broader research. The wild animals' movement is concentrated in Lumbini Development area because this area offers many refuges (wetland, forest and grassland) while Laxmipur does not provide as such.

5. Conclusion

The research concluded the impacts of human activities on biodiversity in Lumbini as a pressing environmental problem and it must be stopped by implementing participatory conservation and development plan. The socio-economic condition of local residents of Laxmipur settlement needs considerable development in order to mainstream them in biodiversity conservation.

The biodiversity of Lumbini Development Trust is relatively okey and needs more attention to increase the species diversity by replacing monoculture species such as *Dalbergia sissoo*. The trust should request the government of Nepal to translocate one third of the blue bull population (not discussed here) either to Bardia National Park or Koshi Tappu Wildlife Reserve. Illegal poaching of blue bull, python snake and Sarus crane must be stopped by establishing anti-poaching units. A joint committee consisting local leaders and government authorities should be set up to monitor and govern the biodiversity conservation programs in Lumbini Development Trust and vicinity areas.

Habitat management based on some targeted birds, mammals, reptiles and fish species is required to increase the numbers of sarus crane, eurasian eagle owl, gray hornbill, white-rumped and slender billed vultures, lesser spotted eagle, lesser adjunct, open-billed storks. Similarly, blue bull antelope, black buck and spotted deer will be an attraction for visitors, if kept in the open closure.

Conservation of biodiversity through participatory management invites common goal of conservation and development simultaneously. Major recommendations include but not limit to the following points:

- The biodiversity conservation plan should prioritize protection of flora and fauna in the area;
- The funding matrix, mechanisms of implementation and profile of resources should be generated and documented;
- Scientific management such as carrying capacity of blue bull, translocation of blue bull and multiple species vegetation management should be started immediately;
- Sarus Crane Sanctuary located in Lumbini Development area should be prohibited to visitors except scientific studies;
- Grass cutting should be limited and be guided through comprehensive study. Palatable grasses such as napier, clover, lucerne and maize crops should be introduced and continued in order to stop blue bull going out to nearby agricultural land for foraging.

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Titration Method Validation of Live Vaccines against Infectious Bursal Disease

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Abstract: Validating a method of analysis goes through different steps, which aims at testing the normality of measurements distribution, estimating the uncertainty of the components of a measurement (i.e., accuracy and correctness), and finally, define the control tests of non degradation of the method performances. This paper outlines the steps for validating a biological method of analysis. It involves the construction of an experimental design, a statistical model, and the preparation of an interne laboratory reference material (pilot vaccine). The latter is used to study the impact of deviation and variation factors, in order to, optimize the analytical method, to evaluate the bias (random error), and to calculate the uncertainty of measurement, and make the control charts. This method is applied in the titration of live viral vaccines of Gumboro disease on chicken's embryos fibroblasts. The experimental results show that potential influence factors related to the titration method had no significant influence on the obtained results. Taking into account these results, an operating mode has been elaborated. The finalized method proved to be faithful to standard deviation of repeatability and reproducibility of 0.21 and 0.22, respectively, with a confidence level of 95%. The calculated uncertainty of measurement is equal to 0.2, which represents the average error level of a titer. A homogeneous stock of interne laboratory reference vaccine (MRIL), with an average titer of 5.9 log DIT 50, was produced and the control chart set in away to provide the laboratory with an important tool of control and monitoring of the viral titers evolution in time, as well as, the mastery of the validated titration method performances.

Key words: Live vaccine of infectious bursal disease, titration method characterization, experimental design, method validation, accuracy, uncertainty, statistics, control chart.

1. Introduction

All bioassays require procedures of dosing methods, titration, calibration and results validation. It is recognized around the world that a laboratory must take the appropriate dispositions to make sure that it is capable of providing the data of the required quality. Such dispositions include the use of qualified interne control procedures and validated analytical methods [1-3]. When a laboratory uses non-standard methods or outside the limit of the standard application, a method validation and verification of the analytical performance must be performed [4]. This obviously shows that there is a very close relationship between the analysis quality and the validation of the method making it [5].

The choice of the method is the real starting point of validation. It is the fruit of a compromise between the instrumental possibilities of the laboratory, the cost of measurement and the required performance [6, 7].

In bioanalysis, validation aims at demonstrating the performance of the analytical method and proving that the obtained results are reliable, and this within well-defined limits. Validation consits, therefore, of a series of experimental studies permitting to prove that the method is in accord with its application domain. This field of application is a description made by the analyst about different analytes, acceptable levels of

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uncertainty (correctness and precision), and sometimes, certain conditions of use that one is entitled to expect from the method [8].

Hence, the control of quality of a method of analysis involves a statistical mastery of its performance in time [9]. Yet, there is a normal variability in experimental measurements [8]. Any analyst knows that making several measurements on the same sample with the same method gets different values [5]. The principal of a statistical control of a method consists of verifying whether this variability will remain within the acceptable limits of uncertainty during the validation. The control charts are a very classic example of tool, used to check at a given point, if the performance will remain within the pre-fixed limits [8]. All these details are grouped in a folder that contains the objectives of the undertaken studies and the acquired results [10]. It is, therefore, useful to provide an organization of the validation [6], which corresponds to a balance between costs, risks, technical possibilities and time [11]. However, any routinely internal control method must be validated against a reference method in order to get reliable, interpretable, valid results, and finally declare its conformity or non conformity [12, 13]. In this context, the present work sheds light to the experimental protocols and statistical methods used to validate an analytical method. Certain recommendations for specific aspects in method validation were presented in this study. The method was evaluated for the purpose of supporting the production of vaccine against Gumboro disease; the vaccine that prevents an immunosuppressive disease in young chickens that has been responsible for major economic losses in the poultry industry worldwide. The virus titer determination is the most critical of all vaccine quality control methods, so the validation of the method is essential. Validation of this method was the process by which the reliability and relevance of method was proved.

The approach is applied here to the titration of live vaccines used against Gumboro disease or infectious bursal disease virus (IBDV). It can be extended easily to other methods to check, on the one hand, the reliability of results and the quality of vaccines used in the field, and on the other hand, to minimize the risk of reanalysis by lack of confidence in the validity of results. More precisely, the objectives of this paper are to statistically assess the validity and reliability of the titration method in its domain of application, describe the procedures for bias adjustment, calculate the uncertainty of measurements by using interne laboratory reference material, and finally, establish the control charts of the reference vaccine in order to demonstrate its efficiency and reliability vis a vis regulatory and standards requirements.

To achieve this objective, the validation procedure will be set in two stages. The first will be based on the identification, evaluation and optimization of the method variation factors. The second will involve the quality criteria, namely: repeatability, reproducibility and the uncertainty of measurement of the titration method.

2. Materials and Methods

The method validation consists of defining the objectives of analytic performances, characterizing the system performances and discussing the results, by combining the experimental or bibliographic research and, especially, by justifying the choices.

2.1 Research Strategy and Study Design

2.1.1 Research Strategy

The strategy adopted for this study has been devoted, in the first instance, to observing the implementation of the method in order to identify, evaluate and optimize the factors having an impact on the result or the uncertainty of measurement to provide a defined and reliable operating mode; secondly, to evaluating the quality criteria of the method prefixed by the laboratory.

2.1.2 Study Design

(1) Study of the method implementation to identify

the variation factors influencing the assay results;

(2) preparation of the pilot vaccine which represents the interne laboratory reference material;

(3) evaluation, using the pilot vaccine, of the identified variation factors to make an optimal adjustment of the factors influencing the analytical method;

(4) comparison protocol by comparative titrations;

(5) statistical analysis of the results;

(6) completion of the operating mode;

(7) evaluation, using the pilot vaccine and within a normative framework, of the method quality criteria, namely: repeatability, reproducibility and uncertainty of measurement;

(8) establishment of the control chart of the interne laboratory reference material.

2.2 Biological Material

2.2.1 Eggs and Chickens

Embryonated eggs from specific pathogen-free (SPF) chickens (VALO1, Lohmann LSL-LITE) were purchased from Lohmann Tierzucht (Cuxhaven, Germany). Chickens were hatched and reared in filtered air positive pressure isolation units (National Laboratory of Veterinary Drug Control, Rabat, Maroc) for the duration of the study. The birds were given food and water ad libitum.

2.2.2 Chicken Fibroblast Culture

Primary culture of chicken embryo fibroblasts was obtained from 5 to 12 days-old chicken embryos. The secondary culture of chicken fibroblast was used in the method. Secondary culture was prepared by trypsinization and filtration of primary culture. Suspension was dispensed in 24-well microplates and incubated for 24 h before titration.

2.2.3 Viral Vaccine Strain

An intermediate commercial IBDV vaccine containing the 2512 strain was used in this study (Bursa Blen MTM, Merial Laboratories and Gainesville, GA).

2.2.4 Interne Laboratory Reference Vaccine (Pilot Vaccine = MRIL)

The interne reference vaccine will be produced by multiplying the vaccinal virus (vaccine strain 2512) in the Chicken's Embryos Fibroblasts (See details in paragraph: 2.3.3).

2.3. Experimental Methods

2.3.1 Titration Method Description of IBDV Live Vaccines

Dilutions of virus titer were inoculated in secondary chicken's embryo fibroblasts. The inoculated cells were incubated at 37 °C and with 5% of CO_2 for 5-7 days. The criterion of infectivity of the IBDV is identified with the presence of cytopathogenic effect in the inoculated chicken embryo fibroblasts. The infectious titer is expressed in infectious dose 50% of the cultivation of tissue (TCID 50).

2.3.2 Evaluation of the Titration Method

The treatment of the analytical sample is, usually, the key step of the assay method. It contains most of the analytical error and represents a limiting factor in terms of speed and automation [14].

The variation factors of the method were identified by studying the existing procedure in laboratory and by observing the performance of this procedure by several operators.

The approach seeks to emphasize anything that might be a source of variation in the results. Among the factors which could influence the reliability of the results are: factors linked to accidental deviation in the application method, and factors of real variation [5].

2.3.3 Preparation of the Interne Laboratory Reference Material (MRIL)

The interne reference vaccine will be produced by multiplying the vaccinal virus in the Chicken's Embryos Fibroblasts. The primary seed lot represents the cultivation of IBDV, whose uniformity and stability are assured. The liquid is kept under a temperature of at least -70 °C.

The seed lot of work is the cultivation of the same virus derived from the primary seed lot, and intended

to be used in the production of internal reference vaccine. The seed lot of work will be later distributed in containers and stored at -80 °C. Series of freezing, thawing and centrifugation lead to collect a supernatant, containing the viral particles. To avoid any risk of contamination, the supernatant was filtered with 0.45 μm and 2% of antibiotic (penicillin and streptomycin 1,000 IU/mg) and antifungal (Fungizone 1,000 IU/mg) was added.

At each passage, a comparative assay of the supernatant was carried out to check if there is an increase, decrease or stabilization of the viral titer, so as to use either the first, second or third passage as reference vaccine. To validate the assay method of live IBDV vaccine, a homogeneous stock is prepared from the selected passage and 2% of penicillin and streptomycin (1,000 IU/mg) and Fungizone (1,000 IU/mg) was added and aliquoted in sterile recipients of 600 μL/recipient, at a temperature of -80 °C.

2.3.4 Pre-validation Steps (Optimization of Procedure Phase)

2.3.4.1 Identification of Variation Factors

Having not defined the variation factors which are components of the procedure might be a source of error or would amplify the uncertainty of measurement [8]. These influencing factors were identified during the execution of the preliminary procedure, based on bibliographic data, namely: the manufacturers' records, the European Pharmacopoeia, the U.S. federal code, and the laboratory method Ploufragan (France). The approach consists of finding out anything that might be a source of variation and deviation in the assay result while making the titration of IBDV vaccine.

2.3.4.2 Evaluation of the Identified Variations Factors

Factors will be subject to a number of essays. Through the results of these assays and via a statistical analysis, the authors can determine whether these variation factors are actually influencing or not.

(1) Evaluation of the Age Factor of Chicken's Embryonated Eggs

As prescribed in the American reference monograph [15], titration of live virus against Gumboro disease can be performed using chicken's embryos aging from 9 to 11 days. However, the essay will be performed on fibroblasts prepared from chicken embryos of 5 days, 9 days, 11 days and 12 days.

(2) Evaluation of Cell Concentration Factor

The cell concentrations tested are respectively: 3×10^4 cells/mL, 6×10^4 cells/mL, 10^5 cells/mL and 10^6 cells/mL.

(3) Evaluation of Incubation Period Factor

The incubation interval described in reference monographs [15-17], varies from 5 to 7 days in the case of titration of live virus vaccines of IBD. To gain time, the approach was to make a series of titration during an incubation interval of 5 days and another set of titration during 7 days. After inoculation, if the difference between the two intervals is significant, the incubation interval of 6 days will be evaluated.

(4) Evaluation of pH Factor

In general, the pH of the milieu, where the cells were cultivated, represents a limiting factor for cells growth. The pH tested in this study is 6.4, 6.8, 7.2 and 7.8, respectively.

2.3.5 Finalization of the Procedure

Once all these factors set, the finalized procedure could be completed while avoiding the variation factors attributed to the method. Thus, the titration method will be operational.

2.3.6 Validation Steps

2.3.6.1 Performance and Criteria for Selecting a Method of Analysis

Validating an analytical method is to define the titration features of a substance in a substrate. The main parameters to verify are: fidelity with the condition of repeatability and the intermediate precision.

According to the ISO 3534-1 [18], fidelity is the closeness of agreement between independent test

results obtained under stipulated conditions. Fidelity refers only to random errors and it is not linked to the true or specified value. The fidelity measurement is expressed in terms of infidelity and is calculated using the standard deviation of the assay results. More this standard deviation is important, the weaker fidelity will be. The fidelity measures rely closely on stipulated conditions where repeatability and reproducibility conditions represent the extremes. It can be studied by successively passing the samples for repeatability, and during a long period for the intermediate fidelity (intra-laboratory reproducibility). It is interpreted by the coefficient of variation *CV* (in %). Note that the analysis of variance (ANOVA) can optimize the time of the study.

(1) Evaluation of Repeatability

This test consists of the titration of the viral particles contained in the interne vaccine of reference under identical conditions: same operator, same lot of reagents, same equipment, same calibration and within a short time [19]. The test will be repeated 20 times, knowing that the recommended number of trials is between 10 to 30 times for optimal statistical interpretation. The exploitation of the results involves the calculation of the mean (X), standard deviation (σ) and the coefficient of variation (CV) of the experimental values of each category, as shown in Eq. (1).

$$CV_R = (\sigma/X) \times 100 \quad (1)$$

(2) Evaluation of the Reproducibility

The test consists of dosing the viral particles contained in the interne vaccine of reference, as well as, varying the operators, days, equipments and the aliquots of the pilot vaccine [19]. The titrations will be performed by three operators as described previously (12 titrations per operator). Repeating the test several times leads to an adequate statistical analysis. The results will be interpreted according to the standard deviation of reproducibility, and especially via the CV (coefficient of variation) of reproducibility.

The calculated *CV*, expressed in %, allows an evaluation of the repeatability and reproducibility of the method. It is used to check the acceptability of the test results of repeatability and reproducibility. The calculated *CV* is compared to the pre-selected accepted *CV* limit: $CV_R < 5\%$ [20]. It can be concluded that if the *CV* test result of repeatability and reproducibility is less than 5%, the method is repeatable and reproducible, with a risk of 5%. The ANOVA test 1 is, also, used to confirm our results.

(3) Uncertainty of Measurement

Uncertainty of measurement is a parameter associated with the measuring result. It characterizes the dispersion of values that could reasonably be attributed to the measured [21-23].

Thus, the uncertainty of measurement is issued from the uncertainties confirmed by experiments and/or from estimated uncertainties. It must cover the entire test method [24-26]. If the result refers to a homogenized sample, the uncertainty of measurement deals only with the analytical part.

Hence, all methods of analysis convey results with a degree of uncertainty, which must be taken into account when selecting the method to use for a particular purpose.

This uncertainty may have important implications when the concentration of a given substance constitutes a level of intervention. The client and the analyst should consent on how the data must be used to produce results of "quality" (i.e., proper to use). The situation deserves further investigation and must take into consideration the main factors leading to variability in experimental measurements and influencing, also, the test result [5].

The interpretation of results in terms of compliance/non-compliance of a vaccine titer depends on the measurement span. In fact, if the span is great, the interpretation of the obtained results, in terms of compliance, is virtually impossible.

To solve the problem of variability in titration measures and make the interpretation less delicate, the uncertainty of measurements of the method must be determined.

The calculation of the uncertainty relies on the calculation of the general standard deviation, which is defined as the average square root of the standard deviation in all measurements of repeatability and reproducibility [27].

In the same conditions, the standard deviation will be applied to all the assay results of the IBD live vaccine, and will indicate the level of accuracy of the result.

2.3.7 Control Graphs

The control chart is a graphical tool, commonly used in the statistical mastering process. It is used to monitor the fluctuations in a process by distinguishing predictable and random variability causes.

The natural variations of this process have to be characterized assignable and due to identifiable factors, in order to be possible to intervene. Once operational, the control chart allows monitoring the stability of this process previously defined by its center (mean) and its dispersion (standard deviation). It seeks prevention, to immediately visualize abnormal fluctuations and deviations [6].

The interne laboratory reference sample is the subject of several titrations (at least six). On this basis, the mean X and the standard deviation σ are calculated. The mean average is used at the midpoint of the diagram and the alert levels are set between +2 and -2 of standard deviation. Discharge limits are set at +3 and -3 of standard deviations. Based on normal distribution and as described by shwane, 95.5% of the subsequent average titration series must be within the range of +2 and -2 of standard deviation, and 99.7% must be in the range of +3 and -3 of standard deviation.

The interne laboratory reference sample is regularly analyzed, and the measurements are reported on the graph in chronological order of their collection. If the chart shows variations in the expected value, the authors can, either, assume that the reference vaccine titer is unstable, the essay is not valid, or the method of analysis is not well managed. Noticing 7 consecutive points, all above the average or all below it, can be explained by: bad preparation of the reference sample, defective preparation of reagents, poor calibration of instruments, an error of the analyst, or due to test contamination [5].

2.3.8 Used Statistical Tests

Any research, whose results are translated into digital data derived from repeated experiments, requires the use of the statistical method. This requirement is more imperative in biological research, knowing that, the vital phenomena are essentially characterized by their fluctuations and variability [28].

The selection of a statistical method that collects comparable data, with the minimum error, is the very beginning of any study. The comparison of the averages and the variances will contribute to the analysis and interpretation of the obtained results during this study. The calculated average from a control material is an estimation of the central value of the results distribution. Any changes in the accuracy will result in a change in the control average. The standard deviation, related to the results distribution around the mean, reflects the random error and confirms, also, the accuracy of the technique [29].

Through ANOVA test, two sources of variation were compared: the variance due to the error (random variance of the sample mean) and systematic or factorial variance (variance due to an independent variable).

In practice, the estimation of the systematic variance can not be done, independently, of the variance due to the error (t) [31]. In fact, it is estimated:

- An intra-group variance which is due to the error alone.
- An inter-group variance is due to both the error and the systematic variance.

If the systematic inter-group variance is greater than the intra-group variance, there is, obviously, something other than the variance due to the error that influences the difference between the means [30, 31].

Statistically, the inter-group variance significance is tested to the intra-group variance, as shown in Eq. (2)

$$F_{obs} = \text{inter-group variance } (CM_a)/\text{intra-group variance } (CM_e) \quad (2)$$

follows a law of distribution of Fischer:

• If $F_{obs} < F_{th}$: no possible rejection of H_0 for risk α (coefficient/significant model).

• If $F_{obs} > F_{th}$: Rejection of H_0 for risk α (coefficient/insignificant model).

3. Results

3.1 Pre-Validation Steps (Method Optimization)

3.1.1 Evaluation of the Age Factor of the Embryonated Eggs

The titrations of the CV were performed on fibroblasts, prepared from chicken's embryonated eggs, ranging from 4% to 4.34%. The average coefficient of variation is of 3.75%. The results were reported in Fig. 1 and Table 1.

The statistical test ANOVA 1 showed that: CM_e = 0.06; CM_a = 0.01; F_{obs} = 0.16 and critical F = 3.01, to admit the hypothesis H_0 ($\alpha_i = 0 \forall_i$), is to admit that the age factor of chicken's embryonated eggs does not, significantly, affect the result, as shown in Eq. (3)

$$F_{obs} = CM_a/CM_e = 0.16 \quad (3)$$

Is to be compared to the limit $F_{th} = F_{0.95} = 3.01$, and according to Fisher Snedecore law: F_{obs} (= 0.16) < F_{th} (= 3.01), H_0 is accepted; and, consequently, this parameter has no significant impact on the titration result.

It is noted that there is no significant difference between the results of assays performed on secondary culture of chicken fibroblast of different ages.

3.1.2 Evaluation of the Cell Concentration Factor

The coefficients of variation of the viral particles titers, contained in the reference sample, were obtained by different titrations, with four different cells concentrations. They range from 6.01% in high concentrations to 3.15% in low concentrations, with an average CV of 4.26%. The results were reported in Table 2.

During the titration tests, it is noticed that when the concentration of fibroblasts is high, the titers are low. However, when the titers are high, the concentration of fibroblasts is low.

The statistical test ANOVA1 indicates that: CM_e = 0.10; CM_a = 2.63; F_{obs} = 25.35 and critical F =3.88.

According to the hypothesis H_0 ($\alpha_i = 0 \forall_i$), the cell concentration factor has no significant influence on the result. $F_{(0.05)}$ = 25.35 > = 3.88F_{th} × F_{obs} = CM_a/CM_e = 2.63/0.10 = 25.35.

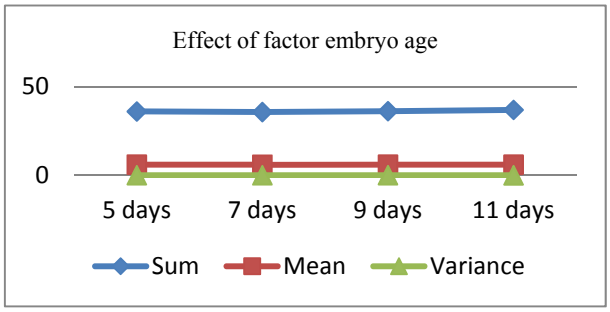

Fig. 1 Statistical analysis of data via ANOVA.

Table 1 Effect of factor age embryonated eggs.

Embryo age in days	5	7	9	11
	5.8	5.9	6.3	5.7
	6.2	6.1	6.1	5.9
Titer in log $DICT_{50}$/mL	5.7	6.3	5.9	6.3
	5.9	5.9	6.2	6.1
	6.3	5.7	5.8	6.2
*Mean log $DICT_{50}$/mL	6.0	5.96	6.03	5.98
*Standard deviation(s)	0.24	0.21	0.19	0.26
*CV%	4	3.52	3.15	4.34

*Parametric statistics: mean, standard deviation, coefficients of variation (CV%).

Table 2 Impact of cell concentration.

Cell concentration	3×10^4	6×10^4	10^5	10^6
	6.1	6.3	5.8	5.1
	5.7	5.9	5.2	4.9
Titer in log $TCID_{50}$/mL	6.2	5.8	5.7	5.2
	5.8	6.2	5.1	4.7
	6.3	5.7	5.9	6.42
*Mean log $TCID_{50}$/mL	6.02	5.98	5.54	5.26
*Standard deviation (s)	0.19	0.21	0.26	0.29
*CV%	3.15	3.51	4 .37	6.01

*Parametric statistics: mean, standard deviation, coefficients of variation in % (CV%).

Table 3 Multiple comparisons of averages according to Duncan test.

Elementary statistics	Duncan test; variable: VAR1 (Titers); significant differences marked of $P < 0.05$			
VAR2 (concentration)	(1) $M = 6.02$	(2) $M = 5.98$	(3) $M = 5.54$	(4) $M = 5.26$
G1:1 (3×10^4)	0.000503*		0.000205*	
G2:1 (6×10^4)	0.000205*		0.000609*	
G3:1 (10^5)		0.000604*	0.000503*	
G4:1 (10^6)	0.000101*	0.000609*		

*Significant difference.

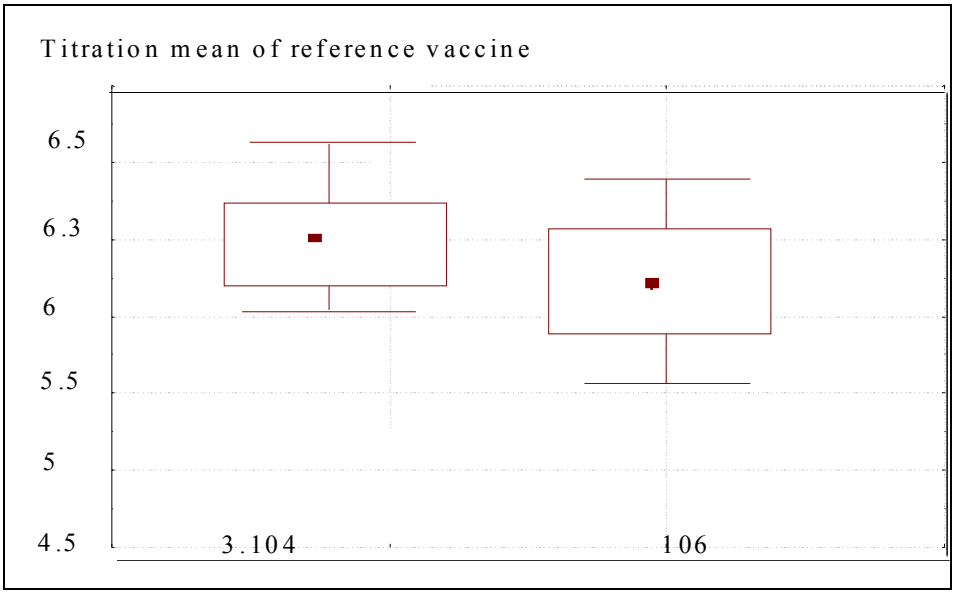

Fig. 2 Significant difference in mean viral titers of cell concentrations tested ($P < 0.05$).

Compared to the limit $F_{th} = F_{0.95} = 3.88$, and according to Fisher Snedecore law: $F_{obs} > F_{th.}$

In reference to both conditions laid down in the statistical test to evaluate the different influencing parameters, the H_0 is rejected, i.e., the cell concentration factor has a significant impact on the titration, with a risk of 5%.

The analysis of variance allows parallel comparison of means, taking into account possible interaction effect. The negative aspect of it is that the test is global. If we conclude that there is a significant difference, it would be necessary to conduct conventional tests, comparing averages pairwise, to identify those which really differ. Duncan test was applied to the results to make multiple comparisons of averages. The results are summarized in Table 3 and illustrated in Fig. 2.

The analysis of variance identifies the existence of a

significant difference between the four cell concentrations. The pair wise comparison of averages shows a gradual and proportional heterogeneity in the cell concentration used by the pilot vaccine.

3.1.3 Evaluation of the Incubation Period Factor

The coefficients of variation ranged from 3.98% to 2.82%, with an average *CV* of 3.4%. The evolution of viral particles titers in the control sample over time is shown in Table 4.

Statistical analysis of the data showed that F_{obs} = 0.01 < F_{th} = 4.6. So, this factor has no significant influence on the expected result. For the procedure, the shortest incubation period (5th day) was selected to save both time and give the result as soon as possible to customers.

3.1.4 Evaluation of the pH Factor in the Cell Culture Milieu

The coefficients of variation of the pilot vaccine titers range from 8.55 in acidic pH to 9.25 in basic pH, with an average coefficient of 6.17%. The results are reported in Table 5 and illustrated in Fig. 3.

The titers are different in varied pH values. In the histogram, the titers means are almost the same when the pH of the milieu is neutral (7 ± 0.2), whereas, those made in acidic pH (6.4) or basic pH (7.9) are very low (Fig. 3).

The statistical test ANOVA1 showed that: F_{obs} = 32.86; critical F = 3.24; F_{obs} = 32.86 > F_{th} = 3.24.

Considering the hypothesis H_0 ($\alpha_i = 0 \forall_i$) is to admit that the pH effect in the cultivation milieu has no significant impact on the result. $F_{obs} = CM_a/CM_e$ = 32.86.

Compared to the limit $F_{th} = F_{0.95}$ = 3.24, and according to Fisher Snedecore law: $F_{obs} > F_{th}$.

Regarding the two conditions set in the statistical test to evaluate the different influencing parameters,

Table 4 Impact of the incubation period.

Incubation period in days	5 days	7 days
Titers in log $TCID_{50}$/mL	5.9	5.8
	6.3	5.9
	5.8	6.2
	6.3	6.3
	6.1	5.9
	5.8	6.1
	6.3	5.9
	5.8	6.1
*Mean log $TCID_{50}$/mL	6.03	6.02
*Standard deviation (s)	0.24	0.17
*CV%	3.98	2.82

*Parametric statistics: mean, standard deviation, coefficients of variation in % (CV%).

Table 5 Impact of pH on the cell culture medium.

PH in the cell culture medium	3×10^4	6×10^4	10^5	10^6
Titer in log $TCID_{50}$/mL	4.2	6.2	5.8	4.9
	4.9	6.3	6.1	4.3
	5.1	5.9	5.7	5
	4.3	6.1	5.8	4.1
	4.7	5.8	6.2	4.2
	4.2	6.1	6.3	4.1
*Mean log $TCID_{50}$/mL	4.56	6.1	5.98	4.43
*Standard deviation(s)	0.39	0.18	0.25	0.41
*CV%	8.55	2.95	3.96	9.25

*Parametric statistics: mean, standard deviation, coefficients of variation (CV%).

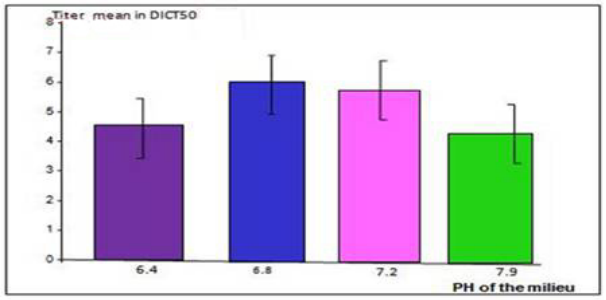

Fig. 3 Impact of the pH on the cell culture medium.

H_0 is rejected. In other words, the pH factor of the cultivation milieu has a significant impact on the final viral titer outcome, with a risk of 5%. So, the use of a cultivation milieu of neutral pH (7 ± 0.2) is always recommended.

3.2 Establishment of an Optimized Procedure

The observations and the results indicate that the deviations are common in the application of procedures. Besides, these deviations have an impact on the quality of results. So, the test method should be formalized correctly to prevent them.

This first part leads to the establishment of an optimized procedure. In Table 6, the evolutionary elements are described and defined precisely, taking into account: the results, the optimization of axes of different variation factors, and the remarks perceived during the practice of the method. This optimized procedure allows for a more reliable and effective results.

3.3 Validation Steps

3.3.1 Evaluation of Repeatability

The calculated coefficient of variation in repeatability conditions is equal to 1.52% (Table 7), compared to the acceptable level of variation equal to 5%. The *CV* obtained is largely lower than 5%. So, the titration method is repeatable.

3.3.2 Evaluation of Reproducibility

The titration test results from reproducibility, performed indifferent days and by three different operators and are summarized in Table 8, and illustrated in Fig. 4 and Fig. 5.

The calculated coefficient of variation in reproducibility conditions is equal to 3.8%, compared to the acceptable level of variation equal to 5%. The *CV* of reproducibility is largely lower than 5%. So, the titration method is reproducible.

Analyzing the titers results performed by the three operators in Fig. 4, it is noticed that the three curves overlap. But, the difference cannot be declared significant between the three operators unless the statistical test ANOVA1 is used.

The statistical test ANOVA 1 demonstrates that: $CM_e = 0.029$, $CM_a = 0.003$, $F_{obs} = 0.12$, and critical $F = 4.30$.

The hypothesis H_0 ($\alpha_i = 0 \forall_i$) means that the operator effect has no significant impact on the result:

$$F_{obs} = 0.12 < F_{th} = 4.30.$$

Table 6 Parameters of the procedure modified by the implementation of the titration method.

Cell type	Chicken's embryos fibroblasts of 24 h
Age of embryonated egg	Egg from 5 to 11 days might be used
Cell concentration	3×10^4 cell/mL
Serum concentration in the cell culture medium	10% Foetal Bovine Serum (FBS)
Serum concentration in virus inoculation medium	2% Foetal Bovine Serum (FBS)
Inoculated volume	Inoculate 0.1 mL of each dilution prepared from the first dose
pH of MEM medium	7 ± 0.2 (check pH in case of doubt)
Incubation period	Incubation is performed during 5 days
Vaccine reconstitution	The lyophilized live vaccine is reconstituted in sterile distilled water or in the vaccine diluent
Effect of temperature	Manipulate in air conditioned isolated room and use an ice bath for the test tubes containing the vaccine dilutions.
Metrology	Preparation of a dilution range using micropipettes: 100-1,000 µL checked by the responsible of metrology in the National Laboratory of Veterinary Drug Control.

Table 7 Repeatability of the control vaccine titration.

Viral titer of control vaccine (log $TCID_{50}$/mL)		
Serial number	Serial number	
5.8	6.1	
5.9	5.8	
6.1	6.1	
5.7	5.9	
5.6	6.2	
6.2	6.3	
5.8	5.6	
5.6	5.9	
5.9	5.8	
6.1	5.7	
Mean (X)*		5.90
Standard deviation (σ)*		0.21
CV%* of repeatability		1.52%

*Parametric statistics: mean, Standard deviation coefficients of variation of repeatability.

Table 8 Reproducibility test of the control vaccine titration.

Viral titer of control vaccine (log $TCID_{50}$/mL)			
Operator 1	Operator 2	Operator 3	
5.6	6.0	6.0	
5.9	6.2	5.8	
6.0	6.1	5.9	
6.1	5.8	6.0	
5.9	5.9	6.1	
5.7	6.1	5.6	
5.9	5.6	6.1	
5.7	5.8	5.8	
6.1	5.8	6.1	
6.2	6.1	5.9	
5.9	5.9	5.9	
*General mean (X)			5.89
*General standard deviation (σ)			0.22
*CV of reproducibility in %			3.8%

*Parametric statistics: mean, standard deviation, coefficients of variation.

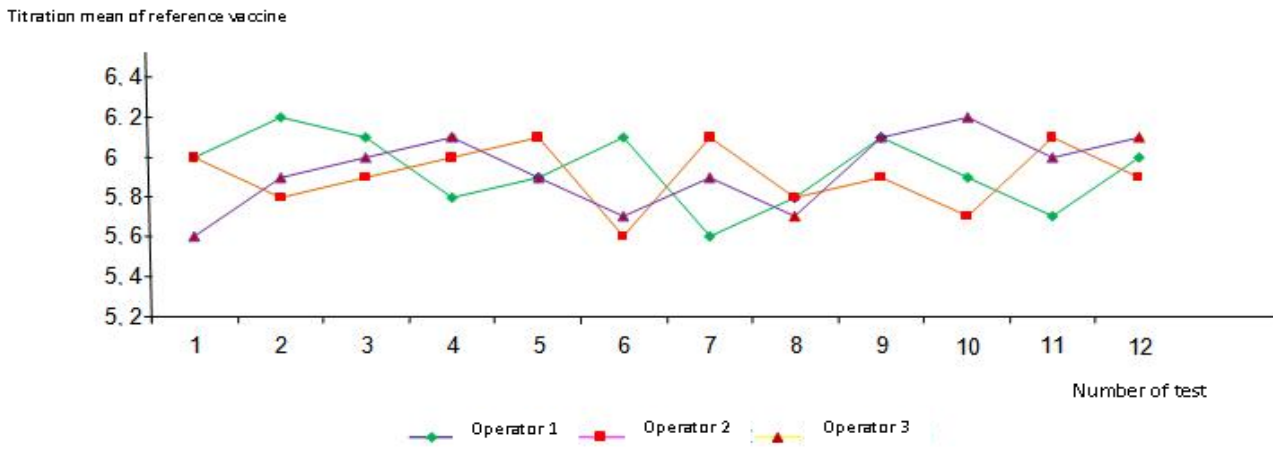

Fig. 4 Reproducibility of the reference vaccine titration tests.

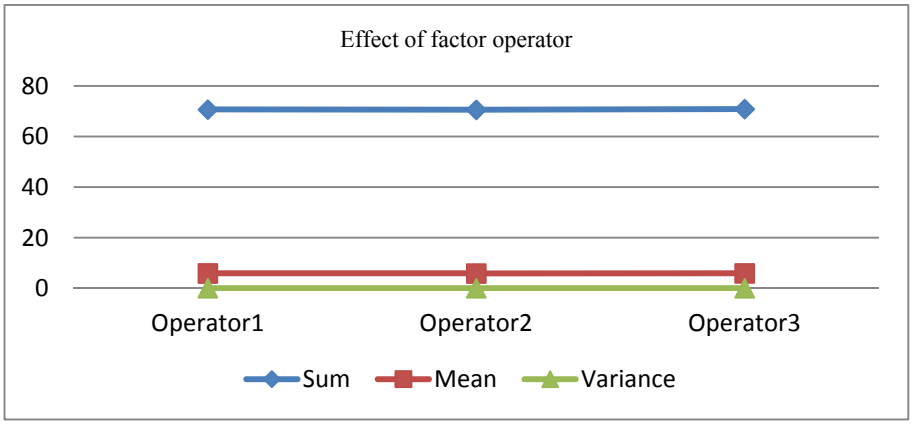

Fig. 5 Statistical analysis of data via ANOVA.

Table 9 Uncertainty of measurement of the titration tests.

Viral titer of control vaccine (log $TCID_{50}$/mL)		
Serial number	Serial number	Serial number
5.8	5.9	6.3
5.9	6.1	5.7
6.1	5.6	5.8
5.7	5.8	6.3
5.6	5.7	6.1
6.2	6.3	6.1
5.8	6.1	6.3
5.6	6.2	6.8
5.9	5.9	6.9
6.1	5.9	6.7
6.3	6.3	5.9
5.9	5.8	6.2
5.7	5.6	6.1
6.3	5.8	-
5.6	6.3	-
General mean (X)		5.9
General standard deviation (σ)		0.2

*Parametric statistics: mean, standard deviation coefficients of variation of repeatability.

Compared to the limit F_{th} = 4.30, and according to Fisher Snedecore law: $F_{obs} < F_{th}$, the hypothesis H_0 is rejected. So, the operator factor has no significant impact on the titration method (Fig. 5).

The general standard deviation is equal to 0.22. It allows us to outline the expected result (RA) and state that the true value of the desired titer has a probability of 95% to be in the interval $X - \sigma < RA < X + \sigma$ where X represents the general mean and σ is the average standard deviation between different operators.

For an average titer of 5.89 performed in reproducibility conditions, the expected results may fluctuate between $X - \sigma < RA < X + \sigma$. It can be concluded that the titration method of live virus vaccines against Gumboro disease is reliable, because it is repeatable and reproducible.

3.3.3 Evaluation of the Global Uncertainty of Measurement

According to the Standards Council of Canada [28], the uncertainty of measurement is defined as the general standard deviation of all performed tests of repeatability and reproducibility. Table 9 shows the

Table 10 Titration test results of repeatability and reproducibility.

Viral titer of control vaccine (log $TCID_{50}$/mL)			
Serial number 1	Serial number 2	Serial number 3	
5.9	5.9	5.8	
6.0	6.1	6.3	
6.1	5.9	5.9	
5.8	5.7	5.8	
6.0	6.0	5.6	
5.9	5.9	6.3	
5.8	6.0	6.0	
5.6	5.7	5.8	
6.1	6.0	6.0	
5.9	5.9	6.2	
6.2	5.7	-	
5.8	5.6	-	
*Mean (X)			5.9
*Standard deviation (σ)			0.23
LAL ($X - 1.96\sigma$)			5.44
UAL ($X + 1.96\sigma$)			6.36
LCL ($X - 3\sigma$)			5.2
UCL ($X + 3\sigma$)			6.69

*Parametric statistics: mean, standard deviation.

overall results obtained during the validation of the titration method, in order to assess the global uncertainty of measurement.

After optimizing the titration method and the mastery of all the factors of variation, the uncertainty of measurement was determined by studying the span of the test results in repeatability and reproducibility. The calculated measurement span (equal to 0.2) was narrowed. This means that the method performances are improved, and that the adopted approach leads to a logical range of measures that can be applied to other measures performed in routine.

3.4 Establishment of the Control Chart of the Pilot Vaccine

The control chart has been established to ensure the stability of the pilot vaccine titer, and allow permanent monitoring of the method performance over time Graphically (Fig. 6), it represents the quality of both the internal reference material, and the method of analysis. In fact, if the pilot vaccine titer is included in the confidence interval of the control chart, the test result is valid and the method is stable. The results are summarized in Table 10 and illustrated in Fig. 6.

4. Discussion

Validation of an analytical method is the responsibility of the laboratory, which must define its experimental protocol, depending on the objective and the needs of its customers. In fact, NF ISO 17025 imposes no experimental protocol in the laboratory to validate a new method of analysis. However, it emphasizes the objective evidences in the accuracy of results. Validation is always a balance between costs, risks and technical possibilities.

Inspired of other scientific researches, this new validation protocol is performed to ensure quality in the future results, with a pre-fixed risk, and in function to the finality of the method of analysis.

To the knowledge, the objective of a method of analysis is to accurately quantify every unknown quantity that the laboratory will have to determine.

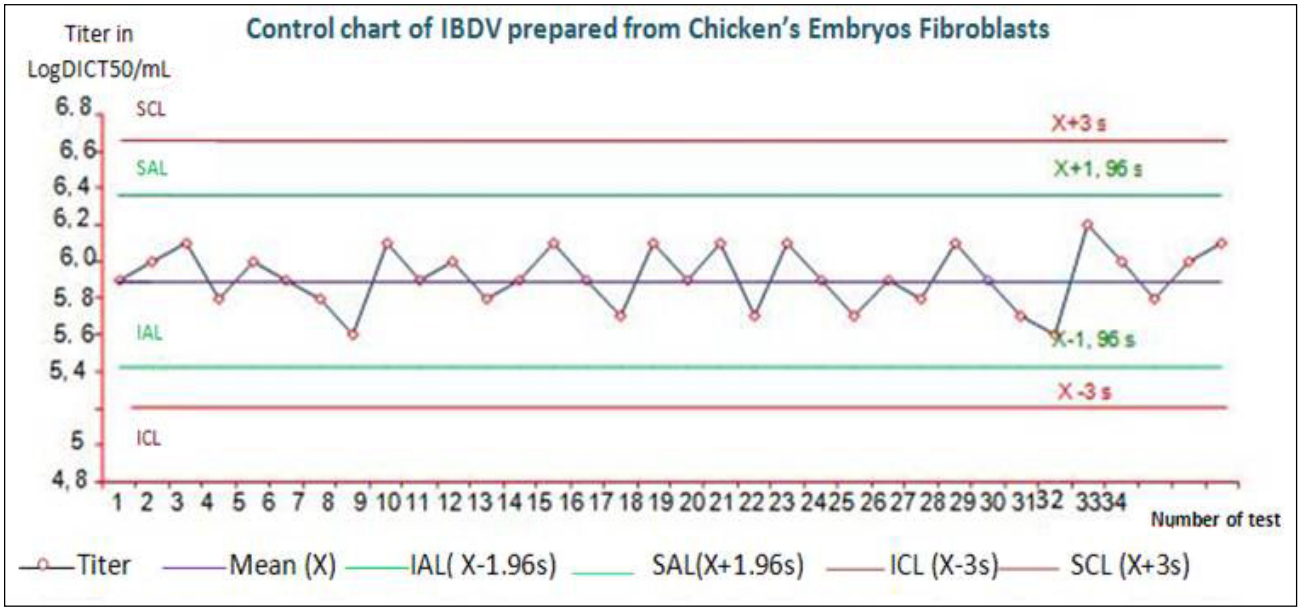

Fig. 6 Control chart of reference vaccine.

The difference between the analysis result and the unknown true value must be inferior to the limit of acceptance, which may be variable in regards to the requirements of the analyst (and/or client), and the finality of the method of analysis.

In the first part of this research, the authors tried, initially, to determine the most important factors of variation and deviation affecting the accuracy of the method of analysis in the titration of live vaccines of IBDV, by using observation and implementation of the method. Secondly, the reagents and protocols to get an operational and accurate procedure were optimized.

The evaluation of the different variation parameters has limited those affecting the result, such as: the cell concentration factor and the pH of the milieu where the cells were cultivated.

This was demonstrated by analyzing the variance of one factor (ANOVA1), which showed the existence of a highly significant difference ($F_{0.05} > F_{th}$). Moreover, it was confirmed via the test (Duncan) by comparing the titers means pairwise, which showed a gradual and proportional heterogeneity at different cell concentrations and at different pH.

As for the cell concentration, the significant decrease in viral titers observed in this study seems very logical, because the cytopathogenic effect detected in infected cells is inversely proportional to the cell concentration. Thus, the cell concentration is an important parameter for achieving and the success of the titration without difficulties. By routinely practicing the method, an adequate cell density (4×10^5 cells/mL) with cells of quality, easy for reading on slides has been established.

The cells age is a very important parameter for the titration method. In fact, the chicken's embryo fibroblasts are primary vulnerable cultures that cannot be stored for long periods even at 4 °C. It is, therefore, important to use the cells immediately after preparation.

Also, during the implementation of the method, an appropriate concentration (5%) of fetal calf serum (FCS) and/or adult calf serum was determined, containing the growth factors necessary to primary cells or cell lines, in the milieu of cells culture. This result is similar to international standards [15-17], which all recommend minimizing the use of serum in the milieu of cultivation.

In contrast, when the pH in the milieu of cell culture is neutral, the great increase of the titers

averages is quite normal. The pH represents a limiting factor for cell growth. An acidic or basic pH in the milieu of cell culture can influence the cell growth and, consequently, influence the final result of the assay. So, these two parameters mentioned above are considered limiting factors in the application of the procedure.

In addition, the evaluation of other variations factors revealed statistically no significant difference. Analyzing the age factor of the embryonated eggs, used in the preparation of fibroblasts, gave identical results on the different ages of the studied embryonated eggs. This was demonstrated by analyzing the variance of one factor (ANOVA1), which showed the existence of no significant difference ($F_{th} > F_{0.05}$).

Similarly, the length in the incubations durations may influence the quality of the cells, and affect, therefore, the final result of the assay. However, the experiments demonstrated that this factor did not influence the test from 5 to 7 days. But, in the 7th day of incubation, the authors noticed that the quality of cells deteriorated, and the cells were detached from the wall of the cupules, making reading on slides difficult. Five-day incubation duration was selected to give the result as soon as possible to clients. These results are consistent with the instructions described in international standards titration [15-17]. They evoke all the use of fibroblasts prepared from embryonated eggs ranged from 9 to 11 age day, and incubation intervals from 5 to 7 days. There was not specified, neither the absolute age of the embryonated eggs, nor an absolute incubation interval for the titration.

All parameters described above are interrelated. They influence the overall analytical performance of the titration method, and ultimately, the level of confidence in the results obtained. So, it is important to take into account the optimization phase in the method validation. Optimizing the protocols and the operating conditions is one of the main steps of validation of physico-chemical and biological methods; as it improves their performances [11].

The evaluation of this optimization step can be reached by comparing the validation features before and after optimization. However, the absence or lack of access to similar works, restrain us from comparing the results with those of other researchers.

This first part leads to the establishment of an optimized operating mode, precisely defined, and allows for more reliable and effective results.

The second part of the work aimed at validating the method of analysis by assessing the quality criteria and ensuring the continued validity of the result, namely: repeatability, reproducibility and uncertainty in measurement, compared to the recommended levels of the standards.

The approach consists of demonstrating that the coefficients of variation, obtained under conditions of intermediate fidelity, are inferior than a tolerance value; for example: 5% is selected by the laboratory according to standards requirements. The difference between the obtained result and the unknown true value of the sample should be smaller, or at least inferior than a limit of acceptance in order to make this method be valid. For that, the estimations of bias and variance, obtained through experiments with known samples (MRIL), will inform us if the titration method is able to quantify the viral particles, contained in the reference vaccine, with sufficient accuracy.

In the experimental study, the statistical analysis of the results showed that the CV calculated in terms of repeatability (1.52%), and the CV calculated in terms of reproducibility (3.8%) are very inferior to the acceptable variation threshold (5%) made in the laboratory, according to the methods validation Guide in medical biology Cofrac [20]. The results of the research are, statistically, less variable. The variability of responses can be explained by the natural variability of biological characteristics. But, there is a normal variability in experimental measurements [5]. The variability of responses happens even in the most

identical conditions, i.e., the same vaccine, performed in the same day, by the same operator, with the same technique, and exactly through the same conditions can provide quite different results.

In general, this variability increases as the number of factors increases. These results are similar to those obtained by other researchers [11]. Under these conditions, our method of analysis is considered repeatable and reproducible.

Any analytical method is characterized by a true bias (systematic error) and a true fidelity (random error measured by standard deviation). These two parameters are unknown, similarly, to the true value of the test sample. In fact, experiments, conducted during the validation, will estimate the true bias. Also, fidelity will be more reliable, if experiments with known samples (reference materials) will be adapted, and the number of tests appropriate. These estimations of bias and fidelity are not targets in themselves, they are an obligatory intermediate steps to evaluate whether the analytical method can reach its objective of being able to quantify or not, with sufficient accuracy, any sample of analysis.

To evaluate the uncertainty of measurement by statistical analysis of experimental data, the authors can employ intra laboratory test validation data. Nevertheless, when evaluating the uncertainty of measurement, it should be ensured that conditions are not fundamentally different from those existing at the time of validation. In fact, any experimental value is linked to an uncertainty of measurement that limits the method applicability. Validation describes and studies the performances and limitations of any test method. It attests if the test method is suitable and convenient to perform tasks, taking into account the uncertainties.

Determining the uncertainty of measurement depends on the analytical problem. The extent of measurement is of 0.2. It did not exceed the interne standard set by our laboratory. It allows interpreting the results of compliance/non-compliance of a vaccine as defined by the manufacturer. No similar studies are available, allowing us to compare or confirm our results. In addition, the evaluation of uncertainty of measurement by the confidence interval, recommended by other researchers, is not always accurate. Some authors have shown that the confidence interval decreases when the number of measurement increases [11]. In this case, despite the increased number of measures (44 measures), our confidence interval remains acceptable.

The application phase in routine of a validated method is naturally followed up by the establishment of control charts in order to maintain, through time, the performance of the method. In fact, the creation and use of control charts is considered, nowadays, as an essential means of mastering a process [18, 19]. The use of internal reference vaccine the authors can get s important information about the fidelity of the method, as it is used in the laboratory, and identifies any modifications. When the method appears to work correctly, performing a large number of assays of the pilot vaccine allows calculating the mean and the standard deviation of the control vaccine titers. The regularly successive results of the internal reference vaccine, made in the course of normal series of analysis, allow assessing whether the method is well controlled, i.e., if the titration of the pilot vaccine gives results corresponding to the initial estimation of the mean and standard deviation.

The authors can confirm that the interne laboratory reference vaccine, used during the validation of the method, remains stable, only if all assay results are distributed close to the target value (average), no ascendant or descendant tendency of alarm limits and control limits are observed, and no random behavior is observed in the graph. According to Max Feinberg in 1999, if one of these cases is found, all the measuring system must be reconsidered.

Overall, the validation methodology described here has worked exceptionally well in practice. The most useful feature was that it has provided a generic template for a wide range of test methods. It was

discussed in the context of cell-based methods; however, the principles outlined here can be applicable to virus titer testing on embryonated eggs, as well. This paper has shown that it was not necessary to change the method due to increasing regulatory requirements; it was sufficient to prove that the method with a long history could also be validated to serve the purpose. The benefit of having an established and valid method is evident as the preparation and the system were already well characterized and consequently the variability of the data is very low compared to those that could be expected in the similar studies. This method was validated for quality control for vaccine batch release, but if the method would be used for research purposes or for viral clearance, it would be necessary to increase the range. Maybe using living cells staining methods [32-34] or other titration method [35] should be used to explore how to detect very small amounts of virus in a sample [36].

In conclusion, the validated method and the mastering of its implementation will provide reliable results to make decision of compliance.

Overall, it is recommended to verify the accuracy of the method by using a certified reference material and by being related to a reference value. This reference value can be obtained either through an international reference vaccine of known titer, or through inter-laboratory tests to calculate a conventional true value. Unfortunately, the international reference vaccine is not currently available. So, the study of accuracy should be achieved through the establishment of an inter-laboratory test.

5. Conclusion

The titration method of live vaccine against infectious bursal disease, validated in the National Laboratory of Veterinary Drug Control in Rabat Morocco, proved accurate with a standard deviation of repeatability of 0.21, a standard deviation of reproducibility equal to 0.22, a confidence level of 95%, and with a global uncertainty of measurement equal to 0.2. This allows the laboratory to:

(1) Affirm the reliability of decisions of compliance or non-compliance in reference to the quality of vaccines used in the field;

(2) avoid or, at least, minimize the risk of additional costs due to the re-analysis in case of doubt in the reliability of results in routine;

(3) deploy appropriate technical and human resources to achieve the analyzes requested by its clients, with a recognized and reliable quality;

(4) give confidence to customers in the results that it delivers;

(5) develop tools of international recognition such as the certification [27].

In parallel, the preparation of a homogeneous interne laboratory reference vaccine and the construction of its control charts give the laboratory a pertinent reference material for monitoring and maintaining the method performances, as well as, allowing the confirmation of the test results.

Finally, it is always possible to improve the performance of a method by studying all factors having an impact on the quality of results. That is why the authors suggest completing this study by assessing the accuracy via inter-laboratory tests, and applying it to other titration methods of live virus vaccines in the National Laboratory of Veterinary Drug Control in Morocco.

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Effect of Different Concentration of Cytokinins, Carbon Source and Agar on *in vitro* Propagation of *Dahlia* sp. through One Single Node

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Abstract: An efficient *in vitro* protocol for mass production of shoot of *Dahlia* was developed by using node explant, various carbon sources such as sucrose, glucose, fructose and galactose. Agar concentrations and various growth regulators on *in vitro* shoot multiplication of *Dahlia* were studied in the present investigation. The nodal explant from the gardens grown plant were used as testing plant material to develop an efficient protocol for mass propagation of exotic *Dahlia* to enhance their production for growers and the local markets. This study determined the effect of different carbon sucrose concentrations and gelling agent on *in vitro* propagation of *Dahlia*, different carbon sources (sucrose, glucose, fructose and galactose) were investigated, each sugar was added individually to the MS culture medium at the concentrations of 15, 30 and 45 g·L^{-1}, respectively. Culture medium of each treatment was supplemented with 1.5 mg·L^{-1} BA + 1.5 mg·L^{-1} Kin + 7.0 g·L^{-1} agar. The highest number of shoots (7.00), number of leaves (11.50), number of node (6.75) and shoot length (8.24 cm) was obtained on MS medium supplemented with 30 g·L^{-1} glucose. The least number of shoots (3.38), number of leaves (5.00), number of node (3.13) and the least shoot length (2.96 cm) was obtained on 45 g·L^{-1} galactose and the least shoot length (2.29 cm) was observed on MS medium with free carbon sources. While the medium with 30 g·L^{-1} glucose and 8 g·L^{-1} agar gave the highest number of shoots (7.13), number of leaves (10.75), number of node (7.13) and shoot length (8.18 cm). However, the least number of shoots (1.50), number of leaves (1.88), number of node (1.63) and the least shoot length (1.26 cm) was obtained with 30 g·L^{-1} galactose and 12 g·L^{-1} agar. Rooting was readily achieved upon transferring the microshoots onto MS medium supplemented with 0.1 mg·L^{-1} IBA, IAA and NAA and 30 g·L^{-1} (w/v) different types of carbon sources. The percentage of rooting was less (71.88%) on MS medium containing IAA as compared with IBA or NAA. While the medium having 30 g·L^{-1} glucose with 0.1 IBA or NAA mg·L^{-1}, give the highest percentage of root (100%), and the highest number of root (3.88) and root length (3.56 cm) was obtained on MS medium containing 30 g·L^{-1} glucose with 0.1 mg·L^{-1} IBA. More than 98% of rooted plantlets were established in the greenhouse.

Key words: Carbon source, *Dahlia* sp. *in vitro*, single node.

1. Introduction

Dahlia, member of *Compositeae* family, is a beautiful gift of nature and is an important garden plant due to its diversity in colors, size, shapes, forms and profusion of flowering [1]. It is grown as both annual and perennial plant. All the *Dahlia* cultivars are derived from one immensely variable specie *D. variables* or *D. pinnata* [2]. *Dahlias* are also medicinally important as its tubers contain high amount of insulin and fructose and small quantities of medicinally active compounds such as phytin and benzoic acid. Many important diseases of *Dahlia* are caused by fungal, bacterial and viral sources leading to various types of impairment [3] and can be successfully eliminated using micro-propagated plant material [4].

Further, micropropagation of plants is a well-known strategy for efficient production and propagation of the elite plant material. It helps in the development and rapid propagation of selected plants

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with desirable characters in shortest possible time and new cultivars can also be developed by protoplast fusion and genetic modifications [5]. A good micropropagation protocol for *Dahlia*, therefore, could be useful for producing the low cost plant material in bulk. The regeneration of *Dahlia* plants has been reported either directly from explants without callus formation or indirectly through callus induction and regeneration. There are a few reports available on *in vitro* proliferation of *Dahlia* sp. However, in other flowering crops like carnation and gerbera, indirect regeneration is reported from certain explant sources like shoot meristems [6, 7], floral buds [8, 9] and cotyledon and hypocotyl explants of *Dahlia* cultivars [10] on different media and the shoot and root regeneration occurred in the calli [11]. The present study was therefore, aimed to develop an efficient protocol for mass propagation of exotic *Dahlia* to enhance their production for growers and the local markets and this study was determine the effect of different carbon sucrose concentrations and gelling agent on *in vitro* propagation of *Dahlia*.

2. Material and Methods

The present work was carried out in Plant Tissue Culture laboratory of the Horticulture Department, School of Plant Production, Faculty of Agriculture and Forestry, University of Duhok, Iraq during the period from March, 2013 to October, 2013. Single nodal segments of *Dahlia* were excised from healthy plants grown in the garden. The explants were surface disinfested by soaking them in mercuric chloride ($HgCl_2$) (0.1%) for 5 min. Finally, they were rinsed in sterile distilled water thrice for 5 min. Plantlets from the establishment stage were cultured individually by Muurashige and Skoog [12] as a basal medium supplemented with fixed concentration 2 $mg \cdot L^{-1}$ BA during the proliferation stage. The pH of the media was adjusted to 5.7 ± 0.1 autoclaved at a temperature 121 ºC at 1.05 kg/m^2 pressure for 20 min. The culture explants were incubated under 16 h of synthetic light (Fluorescent light at 1000 Lux) and 8 h of darkness at average temperature of 25 ± 2 °C. Thus, the following experiments were carried out. Each culture test tube (18 cm × 200 mm) was autoclaved and containing 15 mL of the medium.

2.1 Multiplication Stage

2.1.1 Effect of Different Concentration of Cytokinins on Shoot Multiplication

In this experiment, different cytokinins (BA and Kin) and different concentrations of 0, 1, 2, 3 and 4 $mg \cdot L^{-1}$ BA or Kin alone were added to MS medium which was containing 30 $g \cdot L^{-1}$ sucrose, 7 $g \cdot L^{-1}$ agar. After 4-6 weeks, the following data were recorded: number of shoot development, number of leaves per culture, number of node per culture and average of shoots length (cm).

2.1.2 Effect of Different Concentrations/ Combinations of BAP and Kin on Shoot Multiplication

In this experiment, different cytokinins (BA and Kin) and different concentrations of 0.5, 1.5 and 2.5 $mg \cdot L^{-1}$ BA combination with 0.5, 1.5 and 2.5 $mg \cdot L^{-1}$ Kin were added to MS medium which contained 30 $g \cdot L^{-1}$ sucrose, 7 $g \cdot L^{-1}$ agar. After 4-6 weeks, the following data were recorded: number of shoot development, number of leaves per culture, number of node per culture and average of shoots length (cm).

2.1.3 Effect of Different Concentrations/Type Carbon Sources on Shoot Multiplication

In this experiment, different carbon sources (sucrose, glucose, fructose and galactose) were investigated, each sugar was added individually to the MS culture medium at the concentrations of 15, 30, or 45 $g \cdot L^{-1}$. Culture medium of each treatment was supplemented with 1.5 $mg \cdot L^{-1}$ BA + 1.5 $mg \cdot L^{-1}$ Kin + 7.0 $g \cdot L^{-1}$ agar. After 4-6 weeks, the following data were recorded: number of shoot development, number of leaves per culture, number of node per culture and average of shoots length (cm).

2.1.4 Effect of Different Concentrations of Gelling Agents Combinations with Different Type of Carbon Sources

Agar at 4, 8 and 12 g·L^{-1} were used in MS medium supplemented with 1.5 mg·L^{-1} BA + 1.5 mg·L^{-1} Kin and containing 30 g·L^{-1} carbon sources (sucrose, glucose, fructose and galactose). After 4-6 weeks, the following data were recorded: Number of shoot development, number of leaves per culture, number of node per culture and average of shoots length (cm).

2.2 Rooting Stage

The function of the rooting stage is to prepare the plantlets for establishment outside the artificial, closed environment of culture vessel. The present work aimed to study factors affecting rooting of *Dahlia*.

2.2.1 Effect Different Type of Carbon Sources Combinations with Some Auxins on the Rooting of *Dahlia* Shoots

Dahlia shoots at 3 cm length produced from the multiplication stage were cultured on the following media. MS basal medium supplemented with different type of carbon sources (sucrose, glucose, fructose and galactose) at 30 g·L^{-1} and fixed concentrations of (IAA, IBA and NAA) at the concentration of (0.1 mg·L^{-1}) were investigated. Each treatment of above experiment was supplemented solidified with 7 mg·L^{-1} agar. In this experiment, the shoots were cultured in test tubes (25 cm × 150 mm) and incubated at 27 ± 2 °C under light provided by white fluorescent tubes giving the intensity of about 2,000 Lux for 16 h/day. After 4-6 weeks, the following data were recorded: root percentage, number of roots per explant, and average of root length (cm).

2.2.2 Effect of Some Auxins Concentrations on the Rooting of *Dahlia* Shoots

Dahlia shoots at 3 cm length produced from the multiplication stage were cultured on the following medium. MS basal medium supplemented with different with IBA (indole 3-butyric acid) or NAA (naphthalene acetic acid) at the concentration of 0.0, 0.3 and 0.5 mg·L^{-1} were investigated. Each treatment of above experiment was supplemented with 30 g·L^{-1} sucrose and solidified with 7 g·L^{-1} agar. In this experiment, the shoots were cultured in test tubes (25 cm × 150 mm) and incubated at 27 ± 2 °C under light provided by white fluorescent tubes giving the intensity of about 2,000 Lux for 16 h/day. After 4-6 weeks, the following data were recorded: root percentage, number of root per explant, and average of roots length (cm).

The experiments were designed as a factorial RCBD (randomized complete block design). The comparison between means was carried out according to Duncan's multiple range test ($P < 0.05$). Lastly, for acclimatization stage, a group of successfully rooted plantlets were removed from culture test tubes and their roots were washed with distilled water and soaked in Benlate fungicide (0.1% for 10 min) [13]. They were transferred to pots containing a wet sterilized soil mix (peatmoss + loam + styrofoam 1:1:0.5, v:v:v) under tightly controlled atmosphere of the greenhouse.

3. Results and Discussion

3.1 Effect of Different Concentration of Cytokinins on Shoot Multiplication

Results presented in Table 1 showed that increasing the concentration of BA caused an increase in the length of proliferated shoots and number of developed shoots, number of leaves and number of node. The number of shoots per explants was found in the range of 4.88 to 3.13 under different concentrations of BA and Kin when used singly and 2.0 mg·L^{-1} BA and 1.0 mg·L^{-1} Kin yielded maximum shoots. The *in vitro* experiments of the present study initially involved the establishment of nodal explants in sterilize cultures, which resulted in the induction of multiple shoot formation, elongation of shoots, development of roots for plantlet formation and finally the establishment of plantlets under *ex vitro* condition. The node explants showed different responses when they were cultured

on MS with different concentrations of cytokinins. Of the two cytokinins (BA and Kin) used, BA was found to be comparatively better for shoot proliferation (Table 1, Fig. 1). Similarly, 100% the nodal segments produced shoots on MS medium supplemented 2 $mg \cdot L^{-1}$ BA. In this medium, the highest number of shoots per culture was 4.88 and the average length of shoots per culture was 4.58 cm on MS medium with 3 $mg \cdot L^{-1}$ Kin.

The nodal explants initially produced about two to three shoots within three-four weeks after inoculation on BA supplemented medium. On the other hand, the highest of 100% cultures of nodal explants produced shoots on the medium with 3 $mg \cdot L^{-1}$ Kin. With respect to both shoot induction and increased shoot number, in BA proved better than Kin and it was found to be as the most effective cytokinin for shoot induction as well as shoot proliferation in walnuts. The findings of the present study are in conformity with those of many others where BA performed better than Kin. As a synthetic cytokinin BA has the advantage over other cytokinins in inducing *in vitro* shoot production in woody plant like *Ficus religiosa* [14].

3.2 Effect of Different Concentrations/Combinations of BA and Kin on Shoot Multiplication

It has shown in Table 2 and Fig. 2 that the high frequency regeneration of multiple shoots, a range of cytokinins either individually or in combination with other cytokinins were used. Growth becomes feasible only on the addition of one or more of these classes of hormones to the medium [15]. So, nodal segments were cultured on MS medium supplemented with various concentrations and combinations of cytokinins BA and Kin. The response of nodal segments with combinations was comparatively better than single cytokinin. Nodal explants cultured on MS medium containing BA at 1.5 $mg \cdot L^{-1}$ + Kin at 1.5 $mg \cdot L^{-1}$ produced 5.63 shoots/explants. Whereas BA 2.5 $mg \cdot L^{-1}$ + Kin 2.5 $mg \cdot L^{-1}$ produced 3.63 shoots/explant. Maximum shoot length of 6.43 cm was obtained on medium containing BA at 1.5 $mg \cdot L^{-1}$ + Kin at 1.5 $mg \cdot L^{-1}$. While, nodal explants cultured on MS medium containing BA at 1.5 $mg \cdot L^{-1}$ + Kin at 1.5 $mg \cdot L^{-1}$ produced 10.63 leaves/explants and 5.63 node/explants. The lowest number of node per explant, were obtained on medium containing (0.5 $mg \cdot L^{-1}$ BA + 0.5 $mg \cdot L^{-1}$ Kin) as (3.50) node per explant. While the lowest number of shoot per explant, leaves perexplant were obtained on medium containing (2.5 $mg \cdot L^{-1}$ BA + 1.5 $mg \cdot L^{-1}$ Kin) as (3.00 and 6.38) number of shoot per explant, leaves per explant respectively, with shoot length of (3.19 cm) were obtained on medium containing (2.5 $mg \cdot L^{-1}$ BA + 2.5 $mg \cdot L^{-1}$ Kin).

Similar response was observed with *Rosa indica* L. [16]. The least number were produced with MS medium without growth regulators (control) and MS

Table 1 Effect of different concentrations of BA and Kin on *in vitro* shoot proliferation from nodal explants of *Dahlia* sp. on MS medium after 6 weeks of culture.*

PGRs $mg \cdot L^{-1}$		No. of shoots/explant	No. of leaves/explant	No. of node/explant	Average length of shoots (cm)
Control	0	2.88bc	6.13c	2.25bc	1.61d
BA	1	3.88ab	8.75ab	3.00b	3.01b
	2	4.88a	10.38a	3.88a	2.76b
	3	4.38a	9.63a	3.00b	1.78cd
	4	4.75a	8.88ab	2.63bc	1.23d
Kin	1	3.13bc	6.88c	1.88c	2.50bc
	2	3.00bc	7.25bc	2.38bc	4.11a
	3	2.50c	6.63c	2.13bc	4.58a
	4	2.250c	6.75c	1.75cc	3.30b

* Means followed by the same letter within each character (column) do not differ significantly ($P \leq 0.05$) according to Duncan's Multiple Range Test (Duncan, 1955).

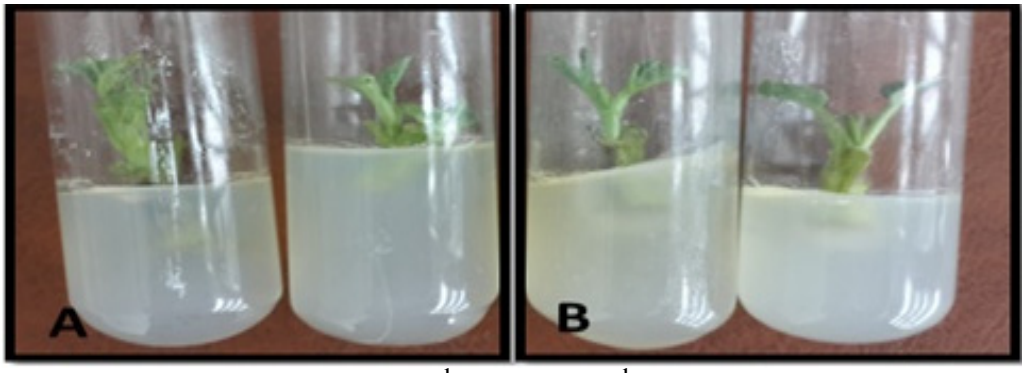

Fig. 1 Effect of different concentrations of (A) 2 mg·L^{-1} BA, (B) 1 mg·L^{-1} Kin alone on shoot multiplication of *Dahlia* sp. on MS medium after 6 weeks of culture.

Table 2 Effect of different concentrations/combinations of BA and Kin on in vitro shoot proliferation from nodal explants of *Dahlia* sp. on MS medium after 6 weeks of culture.*

PGRs mg·L^{-1}	No. of shoots/explant	No. of leaves/explant	No. of node/explant	Average length of shoots (cm)
BA + Kin				
0.5 + 0.5	4.25abc	7.63cdf	3.50d	3.41e
0.5 + 1.5	4.38abc	8.88bc	4.25bcd	4.28cde
0.5 + 2.5	4.75ab	8.25cd	4.63abc	4.70bcd
1.5 + 0.5	4.88ab	10.25ab	5.38ab	5.75ab
1.5 + 1.5	5.63a	10.63ab	5.63a	6.43a
1.5 + 2.5	4.63ab	10.88a	4.88abc	5.26abc
2.5 + 0.5	4.25abc	7.25cde	3.63cd	4.34cde
2.5 + 1.5	3.00c	6.38e	4.63abc	3.98de
2.5 + 2.5	3.63bc	6.75de	4.63abc	3.19e

* Means followed by the same letter within each character (column) do not differ significantly ($P \leq 0.05$) according to Duncan's Multiple Range Test (Duncan, 1955).

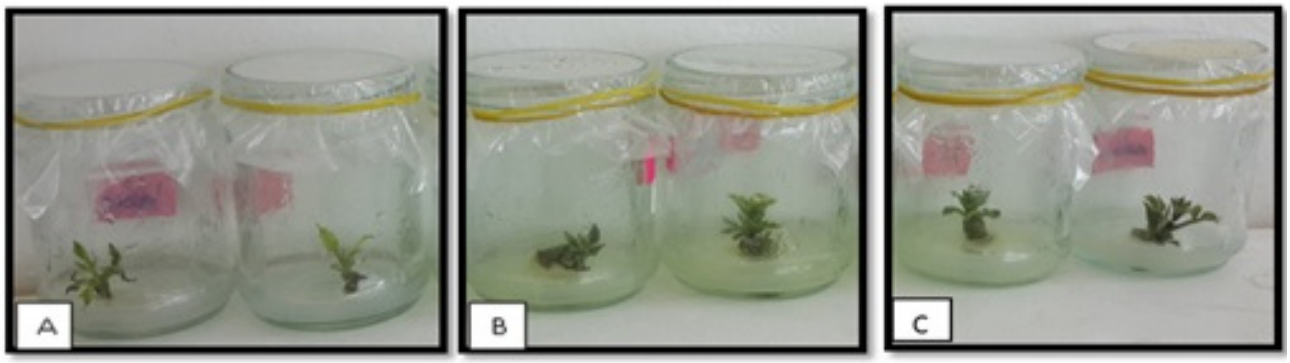

Fig. 2 Shoots multiplication of *Dahlia* sp. on MS medium supplemented with BA + Kin at different concentrations after 6 weeks of culture. (A) 0.5 mg·L^{-1} BA + 2.5 mg·L^{-1} Kin, (B) 1.5 mg·L^{-1} BA + 0.5 mg·L^{-1} Kin, (C) 1.5 mg·L^{-1} BA + 1.5 mg·L^{-1} Kin.

medium containing 3 mg·L^{-1} Kin in tissue cultures (as well as in intact plants and plant organs), cytokinins appear to be necessary for plant cell division. Cytokinins are very effective in encouraging direct or indirect shoot initiation.

To encourage the growth of auxiliary buds, and reduce apical dominance in shoot cultures, one or more cytokinins are usually incorporated into the medium at proliferation stage [17]. Shoot proliferation in this trial is comparable with those reported by Bobrowski, et al. [18]. They reported that the best medium for shoot proliferation was MS medium supplemented (1 mg·L^{-1} or 2 mg·L^{-1} BA), but Kin was not effective on multiplication rate. While Villa et al.

[19] reported that the greater numbers of shoots were produced with 1 mg·L^{-1} BA in WPM basal medium. In our experiment the best result was obtained with 2 mg·L^{-1} BA in MS basal medium. The average number of leaves per explants was recorded after 6 weeks of culture. However, the average number of leaves per explants on MS medium supplemented with BA at 2 mg·L^{-1} + Kin at 1 mg·L^{-1} was maximum as compared to some treatments.

3.3 Effect of Different Concentrations/Type Carbon Sources on Shoot Multiplication

The experiments were performed to determine the effect of different carbon sources at different concentrations with fixed concentration of BA at 1.5 mg·L^{-1} + Kin at 1.5 mg·L^{-1} on shoot multiplication from node explants of *Dahlia*. It was observed from the results that, among the different carbohydrate sources used, glucose performed well followed by sucrose, fructose and galactose in keeping shoot number constant. Highest frequency of shoot regeneration was observed both at 30 g·L^{-1} of glucose (7.00) number of shoot per explant and 30 g·L^{-1} of sucrose (6.38) number of shoot per explant (Table 3 and Fig. 3). Greater elongation 8.24 cm was obtained on MS medium supplemented with 30 g·L^{-1} of glucose and on the same medium, nodal explants produced (11.50) leaves/explants and (6.75) node/explants. The lowest number of shoot per explant was obtained on MS medium containing 15 g·L^{-1} galactose as 3.25 shoot per explant. While the lowest number of node per explant, leaves per explant was obtained on MS medium containing (2.5 mg·L^{-1} BA + 1.5 mg·L^{-1} Kin) and 45 g·L^{-1} galactose as 3.13 node per explant, 5.00 leaves per explant and 2.96 cm shoot length.

This may be due to sucrose is generally regarded as the best carbon source and is universally used as the principal energy source although in certain cases glucose and fructose may be substituted, but most other sugars are poor carbohydrate sources for the plant. These results are in coordination with the finding of Duong et al. [20] and Sujanal et al. [21] who found that as a single carbohydrate source in medium fructose exhibited a better growth when compared with sucrose. In case of the combination of two hexoses (glucose and fructose) at different concentrations the best proliferation of cell was obtained at the combination of 30 g·L^{-1} glucose and 30 g·L^{-1} fructose.

3.4 Effect of Different Concentrations of Gelling Agents Combinations with Different Type of Carbon Sources

Data in Table 4 show the effect of different concentrations of agar with fixed concentration of

Table 3 Effect of different concentrations/type carbon sources on in vitro shoot proliferation from nodal explants of *Dahlia* sp. on MS medium after 6 weeks of culture.*

Carbon source (g·L^{-1})		No. of shoots/explant	No. of leaves/explant	No. of node/explant	Average length of shoots (cm)
Control	0	4.25de	7.75de	3.13e	2.29f
Sucrose	15	5.38bcd	9.63bc	4.63cd	2.76ef
	30	6.38abc	10.63ab	6.13ab	6.30b
	45	5.88abc	8.50cd	5.88ab	4.70c
Glucose	15	6.63ab	10.38ab	6.00ab	4.68c
	30	7.00a	11.50a	6.75a	8.24a
	45	5.38bcd	10.25ab	5.00bc	4.20cd
Fructose	15	3.50e	6.88def	4.38cde	3.94cd
	30	5.38bcd	7.63de	4.25cde	3.95cd
	45	5.13cd	5.75fg	3.25e	3.74cde
Galactose	15	3.25e	5.38fg	3.63de	3.63cde
	30	3.63e	6.38efg	3.75cde	3.84cd
	45	3.38e	5.00ge	3.13e	2.96ef

* Means followed by the same letter within each character (column) do not differ significantly ($P \leq 0.05$) according to Duncan's Multiple Range Test (Duncan, 1955).

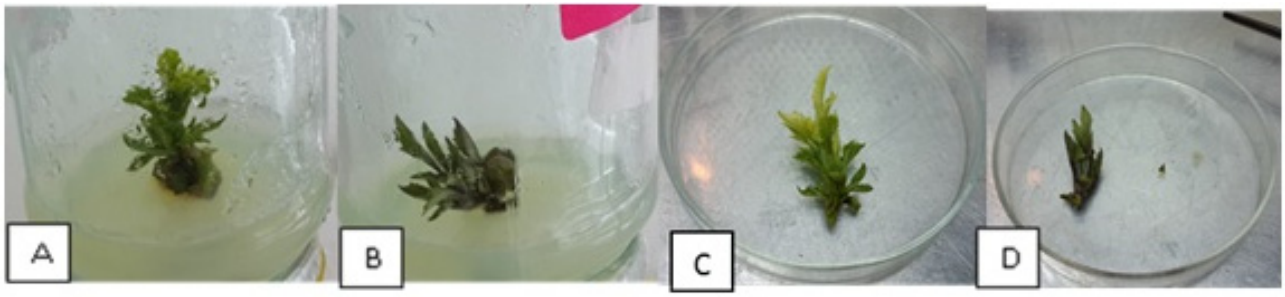

Fig. 3 Effect of carbon source concentrations on growth proliferation of *Dahlia* sp. plantlets after 6 weeks. A = 30 g·L^{-1} glucos, B = 30 g·L^{-1} sucrose, C = 30 g·L^{-1} fructose, D = 30 g·L^{-1} galactose.

carbon sources (sucrose, glucose, fructose and galactose) and the best plant growth regulators (1.5 mg·L^{-1} BA + 1.5 mg·L^{-1} Kin) on characterized of *Dahlia* shoots at multiplication. It is obvious that using agar with glucose and sucrose was significantly more superior than using agar with fructose and galactose in increasing number of shoot per explant, number of leaves per explant, number of node per explant and average of shoots length (cm).

Meanwhile, suing MS medium supplemented with 8 g·L^{-1} agar with glucose gave (7.13) shoot/explant was more effective in increasing proliferation percentage as compared with MS medium supplemented with 8 g·L^{-1} agar with sucrose (6.75) shoot/explant. The maximum shoot length of (8.18 cm) was obtained on MS medium supplemented with 8 g·L^{-1} of agar with glucose and on the same medium, nodal explants produced (10.75) leaves/explantsand (7.13) node/explants. On the other hand, statistical differences were nil between MS medium containing agar with fructose and agar with galactose when leaf number and node number parameter was considered. While the lowest number of node per explant, leaves per explant were obtained on MS medium containing 12 g·L^{-1} agar with all carbon sources as (2.50, 3.25, 1.75 and 1.50) number of shoot per explant, leaves per explant, 2.63, 2.88, 2.13 and 1.88 leaves per explant and 2.75, 2.63, 1.75 and 1.63 node per explants, respectively, with less elongation of shoot 1.41, 1.50, 1.36 and 1.26 cm were obtained on the same MS medium.

In general, the above results can recommended that agar with glucose gave the highest shoot length compared with agar with sucrose and all treatments in *Dahlia* plantlets. Meanwhile, agars with glucose as a gelling agent were better than agars with (sucrose, fructose and galactose) at proliferation stage. These results agree with the findings of Arrequi et al. [22] who found that tuberizalion was higher when phytogel (TM) was used rather than Difco Bacto agar for all potato cultivars. Also Taha [23] who found that Gelrite gave the highest average shoot number and shoot length compared with agar.

3.5 Effect Different Type of Carbon Sources Combinations with Some Auxins on the Rooting of Dahlia Shoots

Rooting only occurred when media contained glucose, sucrose, fructose and galactose. Media devoid of sugar did not produce roots indicating the importance of sugar in root formation. The results showed that type and concentration combination of carbon sources had significant effects on rooting percentage, root length, root number and survival rate. Shoots grown on medium having glucose had the highest percentage of root formation. By adding the different type auxins on carbon sources (glucose and sucrose) in the MS medium from IAA, IBA and NAA at fixed concentration 0.1 mg·L^{-1} an increase of percentage rooting, mean length of roots and mean number of roots was observed. On the contrary, by adding the different type auxins on carbon sources (fructose and galactose) in the MS medium from IAA, IBA and NAA at fixed concentration 0.1 mg·L^{-1}

Table 4 Effect different of concentrations of gelling agents combinations with different type of carbon sources on *in vitro* shoot proliferation from nodal explants of *Dahlia* sp. on MS medium after 6 weeks of culture.*

Agar concentration ($g \cdot L^{-1}$) + Carbon source ($g \cdot L^{-1}$)		No. of shoots/explant	No. of leaves/explant	No. of node /explant	Average length of shoots (cm)
4	Sucrose	3.50cd	5.00c	3.50cd	2.35de
	Glucose	4.88b	5.25c	3.88bc	3.14cd
	Fructose	2.75cde	3.50d	2.75cde	2.35de
	Galactose	2.75cde	2.88de	1.88e	1.98ef
8	Sucrose	6.75a	10.25a	6.25a	6.09b
	Glucose	7.13a	10.75a	7.13a	8.18a
	Fructose	6.00a	5.75bc	4.75b	3.83c
	Galactose	3.88bc	6.63b	3.75cd	3.48c
12	Sucrose	2.50de	2.62de	2.75cde	1.41e
	Glucose	3.25cd	2.88de	2.63de	1.50ef
	Fructose	1.75e	2.13e	1.75e	1.36e
	Galactose	1.50e	1.88e	1.63e	1.26e

* Means followed by the same letter within each character (column) do not differ significantly ($P \leq 0.05$) according to Duncan's Multiple Range Test (Duncan, 1955).

Table 5 Effect different of concentrations of gelling agents combinations with different type of carbon sources on *in vitro* shoot proliferation from nodal explants of *Dahlia* sp. on MS medium after 6 weeks of culture.*

Carbon source ($g \cdot L^{-1}$) + PGRs ($mg \cdot L^{-1}$)		% root	No. of root/explant	Average length of roots (cm)
Sucrose	IAA	81.25abcd	2.50def	2.90ab
	IBA	84.38abcd	3.00bc	3.46a
	NAA	93.75abc	2.25cde	2.39bc
Glucose	IAA	96.88ab	2.88cd	2.91ab
	IBA	100.00a	3.88a	3.56a
	NAA	100.00a	3.63ab	2.59bc
Fructose	IAA	75.00cd	1.63fg	2.28bcd
	IBA	84.38abcd	1.88efg	2.44bc
	NAA	78.13bcd	1.75fg	2.24cde
Galactose	IAA	71.88d	1.38g	1.53de
	IBA	75.00cd	1.25g	1.99cde
	NAA	75.00cd	1.13g	1.46e

* Means followed by the same letter within each character (column) do not differ significantly ($P \leq 0.05$) according to Duncan's Multiple Range Test (Duncan, 1955).

previous parameters decreased gradually. The highest root length and percentage rooting. (3.56 cm 100%) was obtained on MS medium containing carbon source (glucose) with IBA and on the same medium, plantlet produced (3.88) root/explants increase compared with all other treatments. On the other hand, statistical differences were nil between MS medium containing IAA, IBA and NAA with fructose and with galactose when root number and parameter was considered. While the lowest number of root per explant were obtained on MS medium containing fructose and galactose with both auxins, with less elongation of root and percentage rooting decreased. 1.53, 1.99 and 1.46 cm (71.88, 75.00 and 75.00) were obtained on MS medium containing galactose with both auxins respectively.

In this study, the number and length roots were also very low on media containing galactose. As described previously, galactose was the least effective sugar in terms of rooting frequency in apricot and related species [24]. On the other hand, galactose was completely ineffective in stimulating the shoot

Table 6 Effect of different concentrations of auxinson *in vitro* shoot proliferation from nodal explants of *Dahlia* sp. on MS medium after 6 weeks of culture.*

Plant growth regulator (mg·L^{-1})		% Roots/explant	No. of root/ explant	% Roots/explant
Control	0	53.13c	1.38c	1.44b
IAA	0.3	84.38ab	2.38b	2.61ab
	0.5	75.00b	2.00bc	2.13b
IBA	0.3	96.88a	4.25a	3.36ab
	0.5	100.00a	4.13a	5.53a
NAA	0.3	100.00a	2.25b	3.54ab
	0.5	96.88a	2.38b	2.83ab

* Means followed by the same letter within each character (column) do not differ significantly ($P \leq 0.05$) according to Duncan's Multiple Range Test (Duncan, 1955).

proliferation and the induction of roots in of Cork oak [25]. The negative results obtained with galactose showed that it is not efficiently metabolized by some species, or galactose in the medium is not used as carbon source at all, however, regulates osmotic potential only.

3.6 Effect of Some Auxins Concentrations on the Rooting of Dahlia Shoots

The data presented in Table 6 were recorded after 6 weeks from culturing shoot of *Dahlia*. Results indicated that, the highest percentage of root (100%) was recorded when shoots were cultured on MS salt medium supplemented with IBA at 0.5 mg·L^{-1} and NAA at 0.3 mg·L^{-1}, the highest number of roots (4.25) was recorded when shoots were cultured on MS salt medium supplemented with IBA at 0.3 mg·L^{-1}, while the lowest number of roots (1.38) was recorded when growing on MS medium supplemented with without plant growth regulators (control). The highest root length (5.53 cm) was recorded when using MS medium supplemented with 0.5 mg·L^{-1} IBA, the average root length of shootlets grown on MS medium supplemented with 0.5 mg·L^{-1} IBA was significantly higher than those grown on MS media supplemented with different concentration of IAA and NAA, whereas the least percentage of roots (53.13%) were seen in MS medium free-hormone.

Induction of rooting on the proliferated shoots on MS medium supplemented with IBA, NAA and IAA was also reported by Amin et al. [26] who found that roots induced by adding NAA to the culture media were better than using IAA in *lxorafulgens* Roxb. Sultana et al. [27] reported that roots induction of *Momordicacharantea* L. shoots on MS medium supplemented with NAA. Prolific rooting of *in vitro* grown micro shoots is critical for the successful establishment of these shoots in the greenhouse and field.

4. Acclimatization and Field Transfer

Plantlets with well-developed roots were successfully acclimatized and eventually established in green house. Acclimatization of these *in vitro* plants was maintained with high humidity which gave the better survival percentage (98%). Among the various hardening media used for acclimatization of rooted plants, they were transferred to pots containing a steam sterilized soil mix (peatmoss:loam:styrofoam, 1:1:0.5, v:v:v) under tightly controlled atmosphere of the greenhouse.

5. Conclusion

In the present study growth of *Dahlia* sp. is greatly influenced by different carbon sources supplemented in the media. The present study performs to the conclusion that *Dahlia* cultures have quite selective carbohydrate needs. These results could contribute to the improvement in the micropropagation of this economically important ornamental plant on commercial scale.

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Development of School Methodology for Field Study of a Botanical Pineland

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Abstract: The major importance of forests is critical to the survival on planet Earth. Forests are threatened by a lot of dangers and all of us have to contribute to the fight for their survival. The methodology of school research on pine woods which is described below, aims to cultivate more sensitive, educated and activated students, by accordingly specialized teachers who are going to organize the educational background, also to decide techniques and materials for the easiest and most effective learning, for the cultivation of values, attitudes and friendly behaviors towards forests. The suggested methodology is based on the findings of a European Comenius Program and two Programs of Environmental Education. The research usually takes place in a pine land, by using a worksheet as experimental tool, and focuses on the vitality of the trees, the biodiversity, the effects of pollution and other cultural elements related to the pine bio-community. There is also a lot of informative discussion and research over cultural, social and economical factors with locals and specialists.

Key words: Pineland, bio-community, field research, school teaching.

1. Introduction

The importance of forests is enormous and critical to our survival on Earth. The dangers that threaten them are many and require the contribution of us all in the fight to rescue them. Greece is the poorest country in Europe in forest cover due to the bad management of the past. The forests, extending from the sea level to 1,900 m height, are bio-communities with a wide variety of plants and animals. The trees of the canopy (top layer) are pine, cypress, fir, oak, chestnut, beech, elm, poplar. Cultivated trees are citrus fruits, olives, bananas [1]. The sub-canopy consists of brushwood shrubs, bushes, heather, ladanies, skina, sedges, ferns, ivy, moss, lichen. The protection of forests from fires, logging, farming, agriculture and building should be the first concern of every healthy society along with the protection from their natural enemies. Alternative tourism and agro-tourism can give breath to the rural areas, as Greece has significant requirements for this type of development, such as the great variety of scenery, good weather, sense of the virgin, the history and tradition that is a unique value for the nature lovers. Students need to get to know the forests on the spot and not only through books and documentaries, in order to be aware, and be prepared to undertake action, by appropriately trained teachers, who will organize the educational environment and choose methods, techniques and materials for the most convenient and effective learning. Jerom Bruner [2] believes that in the dialogue about the education a minor meaning and importance to the deeper nature of teaching and school learning has been given, neglecting the means that a teacher utilizes to teach and the students have developed to learn. A detailed scientific study may easily and enjoyably prompt students to love and protect forest in the future, contributing to the overall planning and strategy of their schools and local community on the protection of the forest wealth [3]. According to a holistic

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investigation within target levels, students, at the level of ecological foundation, will become acquainted with the forest ecosystems, key ecological concepts and terms, the interconnections and interactions between biotic and abiotic factors [4].

At the level of ecological consciousness, students will realize the contribution of forests to the life and economy of the local communities, the value of the equilibrium and the consequences of their disorders (reduction of biodiversity, climate change, degradation of land, displacement of people, loss of jobs and economic resources, reduction of the natural resources in general). At the level of life skills, in addition to the cultivation of their general education, skills of observation, formulation of hypotheses, searching for alternative solutions, data interpretation, communication, collaboration and participation in jointed activities will be developed. For the emotional and psychomotor development, the students will experience the value of a collaborative team spirit based on equality, understanding, mutual support and open communication. They will also come closer and work with other students, teachers and institutions, enjoying innovative pedagogical methods and procedures significantly different from the usual traditional that are followed at the school curriculum [5].

Below, a methodology of School Teaching Research of a pine-forest plant community is presented, based on the findings of the European Program Socrates/Comenius *European Forests Network* which was materialized by the Lyceum of Soroni Rhodes in the period 1996-1999 in collaboration with schools of Italy, Germany, Ireland and Sweden and on the Environmental Education Programs The Forest of the Epanosifis Monastry and The Forest of our Schoolyard, that were materialized by the Lyceum of Meleses during the school years 2000-2003 and 2009-2010. This is carried out in the context of the Greek Pedagogic Institute on teaching about forests [6] following the guidelines of the Greek Ministry of Education [3] on the design and implementation of the school Environmental Programs, leveraging ideas from the educational programs and networks that apply the Greek Centres for Environmental Education, in a comprehensive overview. The particular reference on the pine forests is dew to the studied pinewoods, common on the Mediterranean islands.

2. Materials and Methods

The field study is based on innovative educational methods and techniques concentrated on the Methodology of Projects [7]. Students are being motivated to discover by themselves the new knowledge and solve complex local environmental problems, with teachers' monitoring and guiding [8]. The students have to work as scientists exploring nature. They work alone or in groups on approaching the subject in a multi-faced way, to collect data, to formulate it and draw conclusions which can be publicized to the school and local community [8, 9]. The teacher avoids intervene, only if it is necessary. The guided discovery is a constructive teaching strategy because students involved in this process, develop and restructure their knowledge and conceptual patterns through the gained experience and the exploratory discussion, supported by the teachers [10]. Team work helps them to tolerate and respect the others' opinions and attitudes and to form an integral part of the procedure of their socialization [5]. It is necessary, all the participators in this study to concentrate, apply and spread the acquired knowledge, focusing on biodiversity, which is linked both to the study of the ecosystem and to the impact of human societies on its maintenance, according to the analysis of school textbooks in a various description of biodiversity [11, 12].

The frequency of visits to the forest depends on the school planning, but visits during different seasons give the possibility of noticing and recording the changes of the nature during the year. All the

observations and measurements are recorded on a worksheet, individually for each tree. The worksheet contains questions concerning the weather, the soil, the animals seen either in transit or hidden among plants or into the soil, the trees, the woody, herbaceous and flowering plants, weeds and floor covering, the human activities, cultural features etc. [6, 13-15]. Emphasis is given to the particular characteristics of the dominant species and to the differences among the *Pinus* species such as *Pinus brutia*, *Pinus halepensi*s, *Pinus nigra*, etc. [14]. The statistical analysis accompanied with the discussion of results of similar researches, leads to conclusions for the type of forest, the density and synthesis of the botanical community, in what condition the pine forest is, etc.. The results can be presented by maps which illustrate the diversity regions or be uploaded at the school website or specific data bases. The interdisciplinary approach of the studied object is achieved through courses of history, literature, biology, chemistry, physics, ICTs, technology, music, theater, folklore, also through the collaboration of different specialty teachers, scientists, organizations and authorities. Questionnaires, concept maps, interviews from experts and locals, laboratory experiments, field observations and measurements, drama, scenarios can be the educational techniques for the students' acquaintance and awareness [6]. Educational scenarios, based on the synthesis of the collected material and information, can be developed on the protection of the forest from fires and the economic future management of the forest.

Environmental research differs from most formal curricula of Science in Secondary Education because of its emphasis on the sociological nature of science [16].

Below is the tool of pineland research, a worksheet for field work, a guide for the students before, during and after the visit to the pineland. In an added column on the right of the table, students write observations and measurements, as shown in Table 1.

Table 1 Tool for pineland school research.

I	Before the visit to the pineland (at School)	
A	Decision on the methodology (interdisciplinary approach)	
1	Connections to the curriculum	Courses of history, literature, biology, chemistry, physics, ICTs, technology, music, theater, folklore, etc.
2	Collaboration with	Different specialty teachers, scientists, organizations and authorities
3	Selection of Innovative educational techniques	Questionnaires, concept maps, interviews from experts and locals, laboratory experiments, field observations and measurements, drama, scenarios, games
4	Frame of the team work	Coordinators, roles and the ways of communication
B	Discovering the subject matter	
1	Inquiry in libraries, internet, and archival material of Media	About the life in/close a forest, threats, usefulness, consequences and results from the destruction of forests
2	Clarification of key environmental terms of biotic and non-biotic factors of a forest ecosystem	Flora, endemic plants, spontaneous plants, fauna, food chains, soil, water, weather etc.
3	Discussions on the forests' value	In the confrontation of climate changes, global warming, pollution and halting the decline of biodiversity
		In the life of local community in the past and present
4	Selection of the study area	Describe the close around
		Set up the boundaries, calculate the slope, the dimensions, the altitude, the distance from the main road on a Google map
		Hills, mountains, roads, rivers, lakes, farmlands, villages, towns, cities, houses, churches, cemeteries, hospitals, industries, sheepfolds, disturbing units etc of the vicinity of the pineland plan how to access the study area.
5	Investigation of the existing rights and ownership of the forest	Along with the local social and economic management and development with emphasis on the protective legal status
6	Collection of cultural features	Literature, songs, myths, stories, traditional and historical elements

Table 1 continued

II	Visit to the pineland	
A	Weather conditions during the day of visit and the day before	
1	Atmospheric phenomenon	Atmospheric fallout, fog, clouds, humidity, winds speed
2	Signs taken into account for the weather forecasting	Signs in the behaviour of animals for upcoming good or bad weather, observations to shape, height, colour and movement of clouds, barometer indications, the direction and intensity of wind, visibility and humidity of the air
B	Soil observations and sampling	
1	Different soil types concerning the composition and properties	Sandy, clay, humus, mixed etc
2	Features	Color, rocks, fallen needles
3	Possible types of erosion that may have occurred during the creation of the territory of the studied forest (discussion on hypothesis)	Physical, chemical, biological
4	Escavation and soil sampling	Samples for chemical analysis
C	Plant community	
1	Species of the canopy trees	Identification by key usage(Latin or/and Local names)
2	Species of sub-canopy trees and plants	Identification by key usage (Latin or/and Local names)
3	Age of the canopy trees	Average
4	Observations of the trees	The tree belongs to the species
		Position/class standing alone, in cluster, dominating, tallest, repressed
		Slope caused by the wind
		Trunk damages
		Broken branches
		Type of foliage (needle) loss (bottomless, edgeless, topless, middle, window, otherwise)
		Discoloration of the needles
		Tree grazing
		Vitality of the tree top
		Presence of cones (a few, many)
		Clinging plants, fungi, mosses and lichens on the trunk
		Attack of noxious insects/caterpillar co-coons
		Resin outflow
5	Measurements of the trees	Tree height (m)
		Trunk perimeter (cm)
		Central branch stem length (cm)
		Percentage of lost tree needles (approximately)
		Needle length (cm)
6	Specific characteristics of the dominant species	Tree form (pyramidal, spherical)
		Needles sprout (singleton, in pairs)
		Needles (smooth, rough on one side, rough)
		Needle color (dark, light)
		Needle cross section (semicircular, triangular)
		Cones outgrowth (stem-less, short stem, vertically to the branch)
D	Animals seen	
1	Positions of animals seen	Passing, on the ground, in the air, on the trees and plants, hidden among plants, hidden into the soil
2	Description of the interaction of animals-plants	Grazing, shelter, crawling, none
E	Feel the forest through the senses	
1	Vision	Colors, shapes, natural creations

Table 1 continued

2	Hearing	Noises, sound, silence
3	Touch	Leaves, branches, flowers, plants
4	Smell	Leaves, flowers, plants
5	Balance	Walk on the rocks, trees, wood logs
F	Human activity	
1	Human interventions in the vicinity of the study area	Roads, fields of grazing, farmland, industries/crafts, churches, cemetery, houses, schools, hospital, dam, electricity network, water network, public service, disturbing units, other
2	Pollution hot spots	Pesticides, litter, debris, smog, else
III	After the visit to the pineland (at school)	
A	Laboratory work	
1	Chemical analysis	Of the soil samples
2	Microscopy	Microscopic and stereoscopic observation of collecting material by using specialized software (Statistical Package)
B	Statistical analysis of quantitative data	
1	Conclusions on the	Type of forest (coniferous, mixed, dense-sparse, deciduous, etc)
		Density and synthesis of the botanical community
C	Synthesis of the results	
1	Energy flow	Design of trophy pyramid, food/energy chains and webs
2	Estimation of forest condition	Correlation of human activity with tree condition and recorded biodiversity
D	Dissemination	
1	Results illustrated on maps	Map containing information of the study area: boundaries, slope, orientation, dimensions, altitude, the distance from the main road
		Map of hills, mountains, roads, rivers, lakes, farmlands, villages, towns, cities, houses, churches, cemeteries, hospitals, industries, sheepfolds, disturbing units etc of the vicinity of the study area
		Maps of the biodiversity of the study area
		Maps of pollution hot spots (pesticides, garbage, debris, else)
2	Publications	Upload the collected material at the school website or specific data bases
		Publications in printed or digital form (guides, reports, albums, else)
3	Social activity	Open events in the school and local community
		Formulation proposals for the future management and exploitation of the forest in the frame of the sustainable development and the Local Agenda to local authorities and government
		Tree planting in burned areas
		Campaign for forest rescue

3. Results and Discussion

The protection of forest wealth is social priority of great importance which requires informing, knowledge, updating, awareness and activation of the society [1]. In local level, priority for each municipal authority and strategic goal for all schools are to raise awareness of youth in contemporary environmental issues, to undertake initiatives and actions to solve them, for the environmental and cultural upgrading of their place. According to Francis Bacon, the aim of Science is to improve the people's destiny by collecting events via systemic observation and the extraction of theories from them [17]. The program "Investigating & Evaluating Environmental Issues & Actions", [18] reports that since students have a potent interest in the natural environment, helping them to make independent research on environmental issues and take action then as responsible citizens, they can achieve effective environmental knowledge. The

students, by learning and applying the methodology of biological science, progressively learn how to face the real life situations because the life is, actually, experimentation, a survey [19].

3.1 Executive Investigation of the Subject before the First Visit to the Pineland

In the beginning, students and teachers discuss about the subject, the research methodology, the frame of the team work, the roles and the ways of communication. Students search in libraries, internet and archival material of media about the life in/close a forest, threats, usefulness, consequences and results from the destruction of forests [6]. Teachers clarify key environmental terms of biotic and non-biotic factors of a forest ecosystem, particularly flora, endemic plants, spontaneous plants, fauna, food chains, soil, weather, et al. and introduce the students to the experimental research [14, 15]. The soil is dealt as support instrument and plant nutrition, as living space of large number of animal species. In a wider acquaintance with the multi-factorial subject of forest study, discussions are developed on the contribution of forests in the confrontation of climate changes, global warming, pollution and halting the decline of biodiversity. Also, is inquired the value of the forest for the life of local community in the past and present. Concluding the results, students formulate proposals for the future management and exploitation of the forest in the frame of the sustainable development and the Local Agenda.

After this, chosen the study area of the pineland and describe the close around. By using the Google Earth Map, the students work in teams, set up the boundaries, calculate the slope, the dimensions, the altitude, the distance from the main road and they plan how to access it. Hills, mountains, roads, rivers, lakes, farmlands, villages, towns, cities, houses, churches, cemeteries, hospitals, industries, sheepfolds, disturbing units etc of the vicinity of the study area are noted, also. Progressively a map is built up containing all the related information. The existing rights and ownership of the forest are investigated along with the local social and economic development in connection with the forest with emphasis on the protective legal status. A collection of local literature, songs, myths, stories, traditional and historical elements concerning the forest unravel the thread, connecting the past with the present.

Data about weather of the day of visit to the pineland area is recorded, regarding the atmospheric fallout, fog, clouds, humidity, winds speed [13-15]. Searched signs in the behaviour of animals for upcoming good or bad weather and take into account various data for the weather forecasting, such as observations to shape, height, colour and movement of clouds, the barometer indications, the direction and intensity of wind, visibility and humidity of the air [15, 20].

3.2 Field Work at the Pineland

During the first visit, students bound the study area and give numbers to the trees, facilitating the following visits, monitoring each tree individually or in total. They can also put a label which informs that this section is part of a forest school study. They practice in the orientation by using compass and recognition of signs of ecological sequence and forest regeneration.

Recognizing different soil types (e.g., sandy, clay, humus and mixed), the composition and properties, discussed the possible types of erosion (physical, chemical, biological) that may have occurred during the creation of the territory of the studied forest [21]. Students excavate and take samples of the soil from different sites to make chemical analysis in the school laboratory.

The measuring of the height of the trees proposed to be done by an approach based on the proportions of the sides of similar triangles, by two students who use a meter and a stripe. A student puts the strip parallel to the tree in front of his face in appropriate distance in order to the strip to be able to cover the entire tree

height and then rotates it 90 degrees. Another student starts to walk from the tree, perpendicularly to the straight jointing the first student with the tree until to coincide with the second edge of the strip, the first is on the base of the tree. The distance between the two students is the height of the tree [14].

The annual growth of the trees can be calculated by measuring the trunk circumference, by using a plastic meter, or by the length of the central stem of a tree branch. The first way needs some years mediate for any noticeable change, while the edge part of a branch corresponds to the recent growing period [21]. Growth measurements which have been materialized for many years can lead to conclusions on the age structure and growth of the forest.

The age of the trees can be calculated by the growth rings of some dead or cut down trees. The mode and the shape of deposition tell the story of each tree. Their density shows the rapid or slow growth of the tree due to favorable or non-environmental conditions during the previous years. The transverse cracks are indication of large frost. A small carrot from the periphery towards the centre of the trunk can also be excavated, with a special tool which does not cause significant damage to the tree [22].

Studying the special characteristics of the dominating species within pineland, students make observations relating to the height and shape of the trees, the truck thickness, the type of needles, the resin outflow, the sprout, length, color, cross section of needles, the cones outgrowth, et al. [13, 14].

As for the constitution of the botanical community (flora) regarding the trees of canopy and the trees and plants of sub-canopy, students seek the local and/or Latin name and classify them as deciduous, evergreen, annuals and perennial, woody plants which are shrubs and saplings, herbaceous plants which are grasses, grains and small flowering plants, weeds. Their trunks and stems can characterized as juicy, fleshy, smooth, rough, and their leaves as wide, narrow, toothed, oval, palmate, oblong, etc. [15]. The floor covering made up of the fallen leaves, stems, mosses and lichens, etc..

As for the fauna, animals seen either in transit (e.g., insects, birds and hares), hidden among plants (e.g., snakes, lizards and spiders) or into the soil (e.g., worms, arthropods and mice), some of them noticed by magnifying glasses, are recorded and recognized by using niche keys. Students emphasise on the type of mobility and interaction of animals and plants, for example passing or crawling on the ground or plants, flying in the air, jumping on the trees and plants, hidden among plants, hidden into the soil, shelter, grazing, etc..

In a synthesis of the forest bio-community, students put the identified organizations in energy pyramid, in food and energy chains and nets, not ignoring the saprophytes and decomposers that feed on dead organic material and developing discussion on the energy flow, the biogeochemical cycles, the trophy relations, the causes and consequences of possible fluctuations of the referred populations.

Students, also, look for indications and elements of human interventions, such as housing development, grazing, farming, opening of roads, logging, pollution coming from pesticides, garbage, debris or other signs of soil degradation, giving explanations of the causes and the needs dictated them [15].

In order to assess the tree and forest condition, are checked the thinning of the crown/defoliation, the type of needle loss, the crown vitality, the tree discoloration, the presence of fungi, the clinging of plants, mosses and lichens, caterpillar co-coons, air pollution affection, natural or stress of human activity, etc. [13, 21].

During the visits to the forest, students try to realize, to feel the forest through their senses by concentrating elements which reveal and highlight the colors of each season, staying alone concentrated to themselves and eavesdropping of the sounds, feeling the silence, touching and smelling of leaves, broken branches, flowers, plants, collecting material for works of arts or for observation under the microscope and

stereoscope [14, 23]. Experiential games and various innovative teaching techniques can be applied inside the forest or the classrooms acting additively to the scientific field work [21].

3.3 Actions and Interventions

After detailed observations, records and discussions about the forest habitats, capitalizing the research results, the students can establish an environmental pathway for the visitors of the pineland, consisting of special signing and publish a guidebook, containing geological, ecological, historical, cultural information about the forest, maps and special guidelines. Wooden labels placed on signs at issue can prevent hunting and disturbance, drawing the attention and sensitivity of the visitors to the protection of the forest communities. Students can also give concerts, to organize photo and visual artistic exhibitions, speeches, round tables, contributing in the better awareness, management and caring of the forest by the locals, with emphasis in fires and hunting. In consultation with the local forestry, they can plan tree planting for burned areas or enrich others. Also, students can attend letters to government and organizations for the management and protection of the forest, in collaboration with their parents' union, local authorities, NGO, et al. and start a campaign for the forests rescue.

3.4 Evaluation

The proposed framework of the design and implementation of a school field study of pine forests promotes much improvisation and self-motivation in the educational process to sensitize initially hesitant and uninvolved students and work together with enthusiasm, joy and pleasure, yielding the maximum. This allows continuous, formative assessment, with the possibility of feedback in the developing programs. The final evaluation is accomplished by synthesis and discussion of the results, methods, processes and materials which can be presented at educational conferences and discussions. The evaluation of the educational process can be done by observing how students work or by answering some short questions which were attended to the students. The program's evaluation can be carried out by a committee established with the participation of the school teachers' association, local municipality, the association of parents and students or by questionnaires and discussions in the teams in a formative or final evaluation [6, 15, 24].

The study of a forest area, from the prism of the organization of the plant and zoo community, the factors that affect them, the impact of human interventions on their balance can be modified in order to correspond to each age knowledge base, occupations and pursuits. Choosing forest area near the school facilitates the access to the sites of study offering the opportunity of frequent and extended visits, reducing costs and organizational difficulties. The development of the projects gives emphasis on the research dimension of the acquisition of the knowledge and exercise students to the scientific method by introducing them to the scientific mindset. They are practicing on observation, experimentation, analysis and synthesis of opinions, data and results, improving the skills of communication, collaboration and partnerships.

4. Conclusion

As a growing number of decisions depends on the developments in Science and Technology, in order for the citizens of the future democratic societies to be able to decide as thoroughly as possible, they should receive the best scientific and technological knowledge, without being appropriate specialists. This is a challenge for the Science Education and Environmental Education and for the Sustainable Development, to provide the framework for the development of these skills and competences. Experiential learning is proposed in the place of traditional teaching because it provides students with opportunities to experience issues and investigate

phenomena, affecting the deeper understanding of the concepts and the laws that define them and their appreciation to values, feelings and attitudes towards the nature [25].

The children have an innate curiosity about the nature and the world around which is stifled by the traditional teaching, causing negative effect on their positive attitude towards Natural Sciences [26]. By teaching with inquiry, students explore ideas and perceptions about various environmental concepts and they can achieve conceptual change with the implementation of systemic practice and the use of innovative teaching techniques [27]. The proposed Tool of Pineland Research, which was drawn to meet the requirements of the students-researchers, contains appropriate field exercises for the study of a forest plant community and can be supported by schools in terms of knowledge and materials, completing the teaching of courses such as geography, biology, chemistry, physics and mathematics, in the frame of the School Curriculum and the Projects of Environmental Education. The research is being developed into the school nearby forest area focusing on the vitality of trees, biodiversity, the particular characteristics of the dominant species, the implications of the pollution and other cultural elements associated with the forest. The exercises of the worksheets have been designed to connect with axes on community, environment, economy, introducing education for the sustainable development [28, 29]. As Mahatma Gandhi said, “an ounce of action is more worth than a ton of teaching. The books offer the knowledge, but the action translates it into understanding.”

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