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African Journal of Biotechnology

Full Length Research Paper

HBc antibody seroprevalence in HBs negative antigen blood donors at the Chad National Blood Transfusion Center in N'Djamena

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The aim of this study is to assess the risk of hepatitis B transfusion in negative HBsAg blood donors who test positive for anti HBcAb. From January to December 2019, an observational study of virological markers was carried out with blood donors (family and volunteers) at the National Blood Transfusion Center in N'Djamena. The donors included were also tested negative for the markers (Ab anti HCV, HIVAg/Ab and TPHA) according to standard methods of clinical microbiology. Of the 1106 donors included in this study, we determined a positivity rate of 41% of anti HBcAb. Significant differences were observed between the proportions of donors: Family (77.48%) and volunteers (22.51%), positive for anti HBcAb (41%) and negative (59.13%), male sex (72%) and female (28.09%) with probabilities of 0.01, 0.001 and 0.001, respectively. This survey made it possible to determine an elevated level of HBcAb in the study population and also to determine about ten cases of cirrhotic patients negative for HBsAg and positive for HBcAb. In view of this result, it is recommended to complete in blood donors with negative HBsAg and HBcAb carriers, screening for anti-HBsAb and, if possible, quantify the viral B DNA in order to minimize the risk of residual hepatitis B transmission among these blood donors in Chad.

Key words: Anti-HBc antibodies, seroprevalence, blood donors, hepatitis B, N'Djamena.

INTRODUCTION

The hepatitis B virus (HBV) is one of the leading causes of liver inflammation posing a serious public health problem worldwide (COMEDE, 2012; Meffre et al., 2010). According to the World Health Organization (WHO), 325 million people worldwide live with chronic infection with the hepatitis B virus (HBV) (Andreu, 2012; Vaux et al.,

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2019).

The prevalence of anti HBc antibodies has been estimated at 7.3% in the general population aged 18 to 80 years in Metropolitan France, which represented 3.5 million people who had been infected with HBV during their lifetime (MDM, 2011). Although vaccination against hepatitis B is an effective preventive measure, infection with hepatitis B virus remains a major public health problem (POC, 2018; Brouard et al., 2013; Syria et al., 2012).

The transmission mode is variable (HAS, 2012; WHO, 2015). In low endemicity areas (Percentage of HBsAg<2% in the population: North America, Australia and Western Europe), transmission is parenteral and sexual. In highly endemicity areas (Percentage of HBsAg>8% in the population: Africa, Southeast Asia, China, Japan), mother-to-child transmission is predominant. In Cameroon, also a highly endemicity country, markers of the hepatitis B virus are found in around 10% of the population (Brouard et al., 2016; Schweitzer et al., 2015; INSPQ, 2016). To date, Chad does not have national HBV seroprevalence data. However, the World Health Organization places Chad in the high endemicity area. An observational study was performed in cirrhotic patients with normal transaminase revealed the absence of HBs Antigen and the presence of anti-HBc Antibody (Registers, 2015-2020). Another study carried out on 2238 volunteers in N'Djamena by Bessimbaye et al. (2014) showed an HBsAg seroprevalence of 13.5% but it did not test for anti-HBc antibodies (Bessimbaye et al., 2014).

The search for HBV markers can be carried out either by enzyme-linked immunosorbent assays (ELISA) detecting direct markers in the blood (HBsAg, HBeAg, viral DNA), or even histological section of the liver (HBe antigen), or markers indirect (anti HBc IgM type antibodies and IgG type antibodies) of the infection, either by molecular tests detecting, quantifying or characterizing the DNA sequence of HBV. The advent of new high-performance tests (PCR RT, chemiluminescence, etc.) for detecting infectious risks on donations has made it possible to considerably reduce the risk of transmission of viral infections through blood products (ASPC, 2020; Larsen et al., 2005; Bottero et al., 2016). With a view to optimizing diagnostic strategies aimed at improving the quality of labile blood products, it is important to identify the serological profile of markers that may make it possible to avoid the risk of this condition in recipients of these products and their long-term consequence (Lefrere and Rouger, 2011; Pillonel et al., 2012).

Systematic screening for HBs Antigen on all donated blood has radically reduced the risk of infection by transfusion of the hepatitis B virus. However, there remains a residual risk of transmission of the hepatitis B virus; this risk could be four factors: a technical error; a viral variant not recognized by certain reagents; a blood donation from a recently infected subject; an infectious blood donation by a chronic seronegative carrier.

This study aimed to determine the percentage of positivity of anti-HBcAb in HBsAg negative blood donors. Also indicate the most appropriate HBV screening strategy(s) in blood transfusion (PA, 2014; WHO, 2016; Lange et al., 2017).

The results of this work could be a good awareness-raising tool with a view to actively participating in the fight against HBV in the search for maximum safety in the transfusion process and the establishment of a haemovigilance system in Chad.

MATERIALS AND METHODS

Study framework

This is a prospective observational study that took place in N'Djamena (Chad) for the recruitment of blood donors. Blood samples collected from donors for HBV markers were tested:

(1) At the laboratory of the National Blood Transfusion Center (CNTS) in N'Djamena (Chad);
(2) Laboratory Immunology Unit of the National Reference University Hospital (CHU-RN) of N'Djamena (Chad);
(3) Center for Study and Research in Applied Biology (CERBA) of Paris/France, within the framework of the agreements for the execution of examinations impossible to carry out on site, as for the quality control and evaluation of our results where all the electrochemiluminescence diagnostic steps were performed.

Selection of blood donors

The biological test strategy for screening donations blood for HBV can be summarized as four markers of transmissible diseases, one of which is bacterial (syphilis) and three viral (hepatitis B and C viruses, human immunodeficiency virus (HIV)). Blood donors were tested either by an Immunoserological test system with VIDAS or by Immunochromatographic tests for the detection of Ag/Ab.

Inclusion and exclusion criteria

Included were any blood donors who tested negative for the markers: HBsAg, HCV Ab, HIV Ag/Ab, and TPHA.

Not included were blood donors with clinical anemia, pregnant, postpartum or breastfeeding women, people with chronic pathology and subjects who tested positive for at least one of the markers: Ag HBs, HCVAb, Ag/Ab HIV, and TPHA.

The donors recruited at the CNTS were family donors and unpaid volunteers subject to residual risk of high contamination.
Family donors or replacement donors: Donors providing assistance to a sick relative;
Volunteers donors: Donors intervening in the context of assistance to the person in danger.

Sample collection

An anti-HBc antibody research was performed in 1218 informed consent negative HBsAg blood donors. In total, 1106 HBsAg blood donors were selected for the study. This is a population of

voluntary donors, aged at least 18, from all professions and social categories. The serum or plasma was collected from January to December 2019 in the CNTS laboratory either on EDTA-impregnated tubes or from bags of blood impregnated with citrate in preparation after separation of the plasma and the red blood cell concentrate. After centrifugation at 40,000 g for 5 mn, the serum was collected in 1.8 ml cryotubes and stored at -20°C.

Certain parameters were also collected on donors such as: sex, age, marital status, profession, type of donation, risky behaviors (previous transfusion, surgeries, multiple sexual partners, etc.), and the system used for serological screening, serological status, place of blood donation, etc. Analysis of these parameters has provided a better understanding of the profile of blood donors facing HBV infection and the risks of transmission of other viral diseases transmissible through blood transfusion.

Data processing

Data were entered and analyzed using Microsoft Word and Excel 2013. The Chi-square test (χ^2) was used to compare the qualitative variables with a significance level set at 5%.

Microbiological analysis

Presentation tests

Organic products reviewed were serum or plasma. These products have been tested in laboratories with the various screening kits and we have taken the electrochemiluminescence performed by the CERBA COBAS machine as a reference method.

Qualitative determination of total anti-HBc antibodies and HBsAg by the VIDAS machine (BioMérieux)

The BioMérieux VIDAS robot system was used for the detection of antigen-HBs and total anti-HBc antibodies (HBcT). A positive and negative quality control system for each run is available to validate a system test kit and internal control for each sample unit use. Barcodes ready to use reagents (reconstitution for some) were used.

The software supplied with the VIDAS system includes programs for analysis and data management. A two-way computer interface automatically transfers results to the user's Laboratory Information System (LIS) and to various product and patient reports. This avoids human errors in reading the results. A quality control system is available to validate a VIDAS system test kit.

As part of our work, VIDAS HBcT and HBsAg cartridges (BioMérieux) were used for the detection of anti-HBcT Ab and HBs Antigen. The VIDAS has 5 compartments. After collection of whole blood in a dry tube or lithium tube and centrifugation, 150 µl of serum was collected and transferred to the first well of the VIDAS HBcT cartridge and HBsAg. The cartridge is placed in the corresponding compartment of the VIDAS. The automaton is started by clicking on the appropriate compartment and the test result is expected within 1 h and 21 min.

Qualitative determination of total anti-HBc antibodies by the COBAS robot system (CERBA)

The immunological test for the qualitative determination of total anti-HBc antibodies is carried out with human serum or plasma. This electrochemiluminescence assay (ECLIA) is used on COBAS immunoassay systems (Roche, Germany). COBAS systems have powerful software including data analysis and management programs. A positive and negative quality control system for each run is available to validate a system test kit and internal control for each sample. Single-use, barcode, ready-to-use reagents (no freezing or reconstitution) was used.

The principle of COBAS is based on the pretreatment incubation of 40 µl of sample with a reducing agent. Then the HBcAg is added and incubated at 20 to 25°C. An immune complex is formed with the HBc antibodies in the sample. Biotinylated Antibodies, ruthenium labeled HBcAg specific antibodies and streptavidin coated microparticles were added and incubated. The complex has just become fixed on available sites of HBc antigens. The complex is attached to the solid phase by a biotin-streptavin bond. The reaction mixture is transferred to the measuring cell, the microparticles are held at the level of the electrode by a magnet. Removal of the free fraction is accomplished by passing ProCell or ProCell M. A potential difference applied to the electrode triggers the production of luminescence which is measured by a photomultiplier. The software automatically determines the results by comparing the electrochemistry luminescence signal generated by the reaction with the cutoff value that was obtained during a calibration. The CERBA results are returned to us as part of the collaboration of subcontractors between CERBA and the N'Djamena CHU-RN laboratory for the comparison of the two methods used for the analysis of the samples.

RESULTS

Study population

In total, 1218 blood donors were recruited. Among these, 112 blood donors with clinical anemia, pregnant, postpartum or breastfeeding women, people with HBV vaccination status or a chronic pathology and subjects who tested positive for at least one of the markers: HBs Ag, anti-HCV Ab, Ag/Ab-HIV and TPHA.

In total, we selected a population of 1106 HBsAg negative donors who were collected. The average age of the 1106 donors was 39 years with the extremes of 18 and 60. The 18 to 28 age group was the most represented with (521/1106) or a proportion of 47.10% of donors (Figure 1).

Blood samples (1050) were analyzed with the VIDAS machine at the immunology laboratory of the National Reference University Hospital (CHU-RN) of N'Djamena to search for HBcAb and 56 were selected at random and sent to the Research Center in Applied Biology (CERBA) from Paris, France to be analyzed by the electrochemiluminescence method. Indeed, the size of the samples sent to CERBA was small but the results obtained from these samples were used as quality control to evaluate the results obtained at the laboratory of the CHU-RN of N'Djamena.

Family donors were the most numerous 857 (77.48%), against 249 (22.51%) of voluntary donors (p = 0.01, significant difference). Female donors were 52.53% (581/1106) and 47.46% (525/1106) donors were male (p = 0.50, not significant difference). The male/female sex ratio in the present study is 1.11 (581/525) (Table 1). The donors were divided into categories according to the

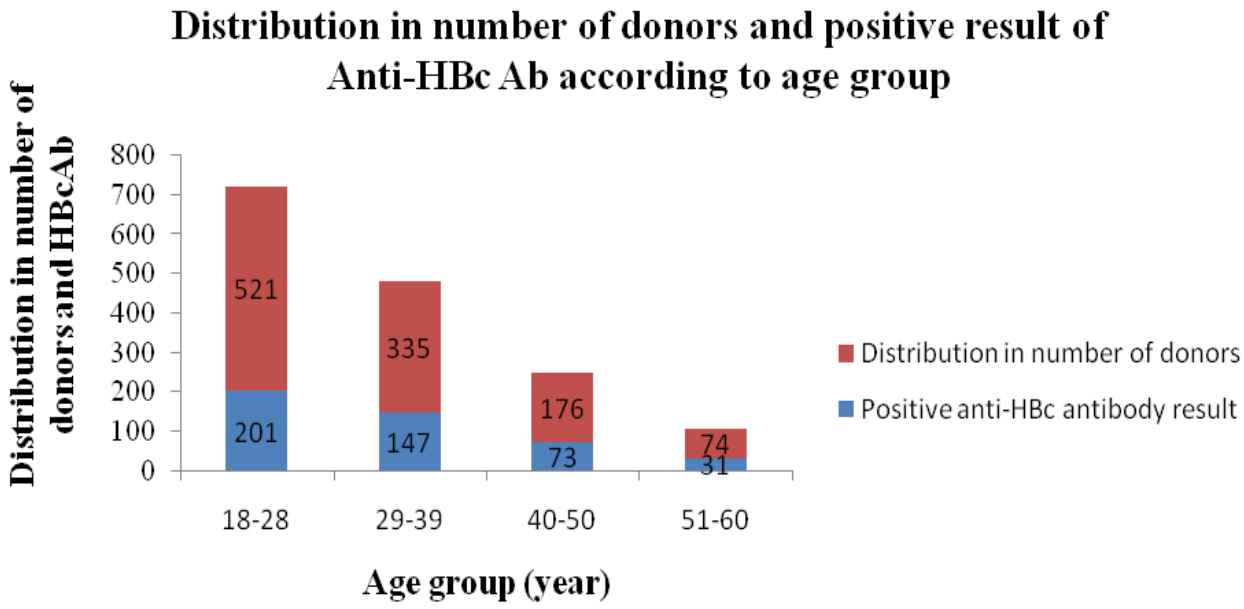

Figure 1. Distribution of 1106 donors and 452 positive results for anti-HBc Ab according to age group.

place of donation: MILITARY (all dressed bodies); SCHOOLS (pupils and students of universities and large schools); ASSOCIATION (Red Cross, footballers, functionary and company workers, etc.); CHURCH (places of worship: Protestants, Catholics…); MOSQUE (places of worship: Muslims…); CNTS (fixed collection at the national blood transfusion center: all professions and social categories combined).

Distribution of donors according to location of donation, types of donors, and positive HBc antibody results

The distribution of the results according to the place of donation gave for the positive anti HBcAb status of the donors: 61.53% (5/13) for the ARMY, 35.58% (305/857) at the CNTS, the ASSOCIATIONS 62% (40/64), SCHOOLS 44.23% (23/52), CHURCH 56.7% (34/60) and MOSQUE 70% (42/60), respectively (Table 1).

Analyses of 1050 samples from HBsAg negative donors carried out in Chad (Table 1), gave 417 samples positive for HBcAb (40%) and 633 (60.28%) were negative for HBcAb (x2 = 7.216> x20> 3.84, p = 0.01, dof = 1, significant difference).

In contrast, of the 56 specimens sent to CERBA in Paris, France, 35 (62.5%) were found positive for HBcAb and 21 (37.5%) were negative for HBcAb.

According to gender, it appears that 294 (28%) positive HBcAb donors were male versus 123 (12%) female donors positive for HBcAb (x^2 = 5.949> x^2_0> 3.84, p = 0.02, dof = 1, significant difference). The results of the CERBA analyzed in France were 31/49 (63.26%) for males and 4/7 (57.14%) for females (x^2 = 0.049 <x^2_0 <3.84, p = 0.50, dof = 1, non-significant difference).

The cumulative results of the two laboratories (CHU-RN, CERBA) revealed 452 HBcAb positive donors (41%) and 654 (59.13%) negative HBcAb donors (p = 0.001, significant difference). According to gender, the cumulative results of the two laboratories gave a seropositivity rate of HBcAb of 28.09% in women (127/452) and 72% in men (325/452) (p = 0.00, significant difference).

Distribution of samples according to socio-demographic group exposed to residual risk of transfusion and positive HBcAb status

The distribution of positive anti HBcAb was: 37% (198/499) for married, 30% (87/292) single, 45.28%, (48/106) divorced, 62% (90/146) sex workers, and 61.53% (8/13) military, respectively. High levels of positive anti-HBcAb were observed in sex workers and military personnel followed by divorced and celibates (Figure 2).

Distribution of donors and positive results for HBcAb according to profession

The most represented profession was functionary with 251/1106 (23%) donors of whom 94/251 (37.45%) were positive for HBcAb followed by 133/1106 housewives (12.02%), 112/1106 resourceful (10.12%), 105/1106 pupils (9.49%) and 102/1106 traders (9.22%) including 77/133 (58%), 54/112 (48.21%), 41/105 (39.04%) and

Table 1. Distribution of donors according to sex, types of donors, place of donation and status anti-HBcAb.

Parameter	Place of donation						
Sex	**Church (%)**	**Mosque (%)**	**CNTS (%)**	**Association (%)**	**Establishment school (%)**	**Military camp (%)**	**Total (%)**
Male (%)	30 (50)	60 (100)	362 (42.24)	40 (62.5)	20 (38.46)	13 (100)	525 (47.46)
Female (%)	30 (50)	0 (0)	495 (58)	24 (37.6)	32 (61.53)	0 (100)	581 (52.53)
Total	60	60	857	64	52	13	1106 (100)
Types of donors							
Volunteers (%)	29 (48.3)	14 (23.3)	190 (21.17)	8 (12.5)	6 (11.53)	2 (15.38)	249 (22.51)
Family (%)	31 (51.7)	46 (76.7)	667 (78)	56 (87.5)	46 (84.46)	11(85)	857 (77.48)
Total	60	60	857	64	52	13	1106 (100)
Status anti-HBcAb							
Négative (%)	26 (43.3)	18 (30)	552 (64.41)	24 (37.5)	29 (56)	5 (37.46)	654 (59.13)
Positive (%)	34 (56.7)	42 (70)	305 (35.58)	40 (62.5)	23 (44.23)	8 (61.53)	452 (41)
Total	60	60	857	64	52	13	1106 (100)

63/102 (62%) were positive for HBcAb, respectively (Figure 3).

Distribution of donors and anti-HBcAb positive results according to age group

The distribution by age group showed a predominance in the age group of 18 to 28 years with 521/1106 (47.10%) of donors of which 201/521 were positive for HBcAb (38.57%) followed by those aged 29 to 39, 40 to 50, 51 to 60 years with 335/1106 (30.28%), 176/1106 (14.46%), 74/1106 (7%) of donors of whom 147/335 (44%), 73/176 (41.47%) and 31/74 (42%) were positive for HBcAb, respectively (Figure 1).

DISCUSSION

At the end of this work, which focused on the proportion of anti-HBc antibodies, in HBsAg negative blood donors at the National Blood Transfusion Center (CNTS) of N'Djamena, it appears that 452/1106 HBsAg negative donors were positive for anti-HBcAb (41%). This rate is higher than those obtained by Villar et al. (2011). On the other hand, in terms of the number of cirrhotic patients positive for anti-HBcAb, the results of this study corroborate those of the other authors (Bottero et al., 2016; ECDC, 2018). This study showed a strong participation of female donors. Female donors were 581 (52.53%) and 525 (47.46%) donors were male. The massive participation of women in the study could be explained by the high level of compassion of women towards a sick relative and also by the proportion of women of 52% in Chad (RGPH 2, 2009). Blood safety therefore remains a major public health problem in the world. On the other hand, in terms of the number of cirrhotic patients positive for anti-HBcAb, the results of this study corroborate those of the other authors (Bottero et al., 2016; ECDC, 2018). According to the PHE, the residual risk is the risk of transmitting a virus through transfusion, despite the measures taken to select donors and screen for biomarkers of viral infection. It is represented almost exclusively by infectious donations, collected during the window of serological silence, which corresponds to the period between contagion and the appearance of the serological marker (antigen or antibody) sought in blood donations (EASL, 2012). The risk of contamination of hepatitis B by transfusion of blood tested for positive anti-HBcAb in a donor of HBsAg negative blood during a transfusion in a context of occult hepatitis is well known (ECDC, 2018; Gkouvatsos, 2017). Occult hepatitis B is defined by the presence of HBcAb in the blood of a subject tested for negative HBsAg, and characterized by the presence of HBV DNA in the serum and/or in the liver of a patient whose HBsAg is not detectable by the usual serological

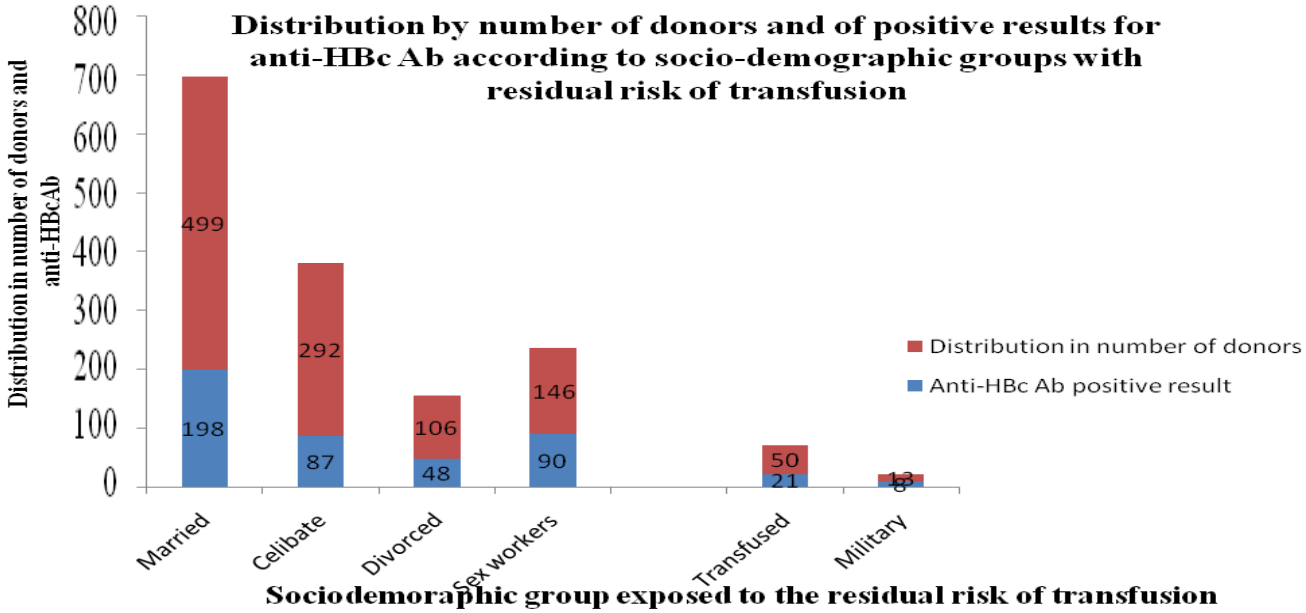

Figure 2. Distribution of 1106 donors and 452 positives HBc Ab results according to the socio-demographic group.

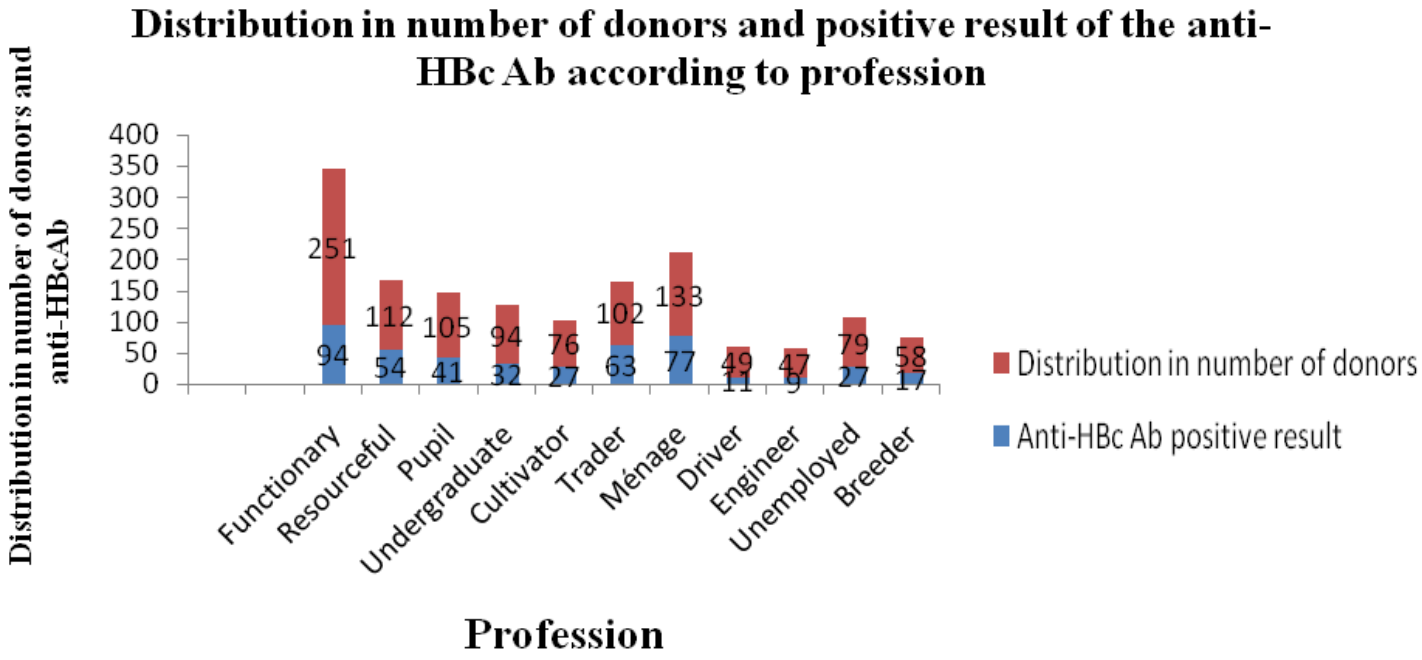

Figure 3. Distribution of 1106 donors and 452 positive results for HBc Ab according to profession status.

tests. In practice, two situations likely to mimic occult hepatitis should be excluded:

(1) In the incubation phase of an HBV infection characterized by an immunological window where HBsAg has not yet become positive, even though viral B DNA is present.

(2) During the healing period characterized by the disappearance of HBsAg, and when the HBV genome is still detectable with the corresponding antibodies (Laperche et al., 2012).

In fact, since Chad is located in an area of high endemicity for the carriage of hepatitis B markers, the problem is of definite interest in terms of blood transfusion

(Dhumeaux et al., 2014).

To do this therefore, it is important to assay the 3 hepatitis B markers: HBsAg, HBcAb, anti-HBsAb before any transfusion decision as recommended (WHO, 2016; Laporal et al., 2019) diagram, only people screened negative for HBsAg and HBcAb can be considered as safe donors since they have never been in contact with the hepatitis B virus. In contact subjects, that is to say with positive HBcAb, it is recommended to take into account the anti-HBsAb as best as possible to supplement with the viral load to know whether they are cured and thus limit the risk of transmission of occult viral hepatitis B (Papatheodoridis et al., 2016; Raffeti et al., 2016; Rahib et al., 2019). In Europe, the major interest in identifying these occult infections lies in the risk of transmission of HBV in hemodialysis patients, during organ or blood donation (Richard et al., 2017; SFM, 2015). In Africa, south of the Sahara, an area of high endemic for hepatitis B, the problem becomes even greater when we know that the blood transfusion systems are not efficient. In addition to the ethical aspects, this is a highly worrying issue falling within the framework of a blood safety policy (Meffre et al., 2010; Michele et al., 2011).

In Chad, data from the CHU-RN gastroenterology department register revealed around ten cases of cirrhotic patients negative HBsAg and positive of HBcAb (Registres, 2015-2020). In routine consultation, the proportion of contact cases is high and is responsible for most chronic negative HBsAg liver disease in our Chadian context. Admittedly, no study was done before even if the finding on the ground is indisputable, this study confirms it.

The seroprevalence of the carriage of anti-HBcAb is superimposed on the geographical distribution of the seroprevalence of markers of viral hepatitis B, and characterized in areas of high endemicity (sub-Saharan); an area of medium endemicity (Mediterranean) and an area of low endemicity (European) (Laperche et al., 2012; PA, 2014; EASL, 2017). In blood donation establishments, any blood product tested positive for HBcAb is excluded from donating blood and the donor is referred to a specialized center for biological monitoring and medical treatment even if international recommendations are not consensual on the management of isolated HBcAb (Michele et al., 2011; WHO, 2016). According to French recommendations, it seems to indicate to assay viral DNA in order to identify cases of occult hepatitis. If the DNA is negative, vaccination can be offered (HAS, 2012; MDM, 2011; Denis and Daddi, 2016; PHE, 2017).

Antibodies to the HBV capsid proteins (anti-HBc antibodies) are the best serological marker of contact with HBV. To this end, the initial search strategy for the detection of HBs Ag should be recommended in our region and in case of negativity, sought for anti- HBcAb and anti-HBsAb.

Anti-HBs antibodies also appear in the serum of patients vaccinated against HBV. In this case, their presence is not associated with that of anti-HBcAb IgM-type and is present in high titer during acute infection. They may also be present in a low and fluctuating titer during the immuno-elimination phase of chronic hepatitis B or recur with reactivation of chronic hepatitis B in an inactive HBV carrier. In some cases, the presence of anti-HBc antibodies is the only virological marker present in a subject infected with HBV. This situation can be observed:

(1) During acute hepatitis which follows the disappearance of HBsAg and precedes serological recovery characterized by the appearance of anti HBs antibody; in this case, the isolated presence of anti-HBc IgM and the subsequent appearance of anti-HBs antibodies allow the diagnosis;
(2) In “cured” patients who have lost their anti-HBs antibodies;
(3) In patients with occult hepatitis B infection, defined by the presence of HBV DNA in the liver, while HBsAg, produced in very small amounts, is undetectable by conventional commercial tests. In these patients, serum DNA may be detectable (generally <200 IU/ml) or undetectable (Dhumeaux et al., 2014; ECDC, 2018; INSPQ, 2014Raffetti et al., 2016).

The solution for Chad and the developing countries in areas of high endemics was to first recommend a low-cost screening algorithm which consists in detecting the majority HBsAg and secondarily detecting the anti-HBc antibody and better to supplement with the detection of viral HBV DNA.

Conclusion

Detection of isolated HBcAb remains very high in blood donors who tested negative for HBsAg in Chad. Transfusion safety depends on one hand, through better selection and retention of blood donors, and on the other hand, through the early diagnosis of the main viruses transmissible through the blood that may infect the recipient. The use of more sensitive and specific molecular techniques brings a decrease in the silent window and eliminates false positives, false negatives in the detection of viruses transmitted by blood. It would also be desirable to continue the study of the frequency of anti-HBcAb in blood donors in other regions of Chad, and also to promote vaccination against hepatitis B, especially in children under five years, and depending on gender.

Although localized, this study made it possible to determine a high level of HBcAb in the study population of blood donors and around ten cases of cirrhotic patients negative for HBsAg and positive for HBcAb in the gastroenterology department of the CHU-RN.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Prospective study of dental implantology related patents in Brazil

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Technological innovation and the development of new products is what currently boost the competitiveness between enterprises and moves the economy. The technical-scientific advances in Dentistry grow each day and there are great investments by research centers and universities in new materials, especially in dental implantology. The objective was to evaluate the patents deposited and granted in implantology at the National Institute of Industrial Property (INPI) in Brazil. This is a prospective study with searches performed during March 2020 on patents in dental implantology deposited and granted in the INPI. The research was carried out in titles and abstracts of reports on patent applications from the first deposit through to the most current using the search terms "dental implants" and "implantology". The information was extracted from documents and organized into tables and graphs. From 1988, the date of the first deposit, through to 2019 there were 139 patent application deposits. Among the deposited patents, 54% (75) were classified as utility model patents and 25% (35) as invention patents, the others 21% (29) have been in analysis. Brazil holds 80.6% (112) of deposits, and the others are from countries in Latin America, North America, Europe and Africa, and virtually all patents are of type A, which refers to products of human needs. Most deposits of patents for use in implantology in the INPI relate to implant and prosthetic component accessories, indicating greater investment by industry in Brazil in this sector. However, in 30 years there were only 134 deposited patents, suggesting that the importation of products in the area is still great, which consequently makes the products of this area more expensive. Thus, investments in patents play an important role in technological and economic development of a country.

Key words: Dental implants, intellectual property, patents.

INTRODUCTION

Technological innovation and the development of new products is what currently boost the competitiveness between enterprises and moves the economy (Dalmarco et al., 2011). The technical-scientific advances in dentistry

grows each day and there are great investments by research centers and universities in new materials, especially in dental implantology, which depends on the evolution of materials/instruments/processes to be an increasingly safer and accessible option as an alternative to removable prosthetics (Lotif et al., 2018).

The prognosis and long-term safety of prostheses in implants are already well documented in both laboratory and clinical-epidemiological studies. Like any area, materials and instruments may be subject to failures, making the search for safer materials and innovative methods one of the main interests of industries and scientific studies in Implantology, not only in relation to implants, prostheses and biomaterials, but also aseptic/antiseptic substances used to disrupt bacterial biofilm, improving long-term performance (Sandhu et al., 2017); thus, the scarcity and search to improve products increases industrial investment in production and development (John and Prates, 2015). The global biomaterials market in general is currently expanding, especially in the United States and Europe, being very expressive and profitable. It has two types of modalities: The first relates to compounds that constitute biomaterials such as metals, ceramics, and natural products, etc.; the second relates to the application form, which includes biomaterials used in dentistry (Pires et al., 2015).

Investing in technological innovation, especially in industries, is essential to keep up with the market. Patents are intellectual property that guarantees the inventor(s) the right over an invention or a utility model, also ensuring safety in profits in possible negotiations (Amadei and Torkomian, 2009). Therefore, the inventor(s) will have the exclusive right to commercialize the product for a certain time, varying according to the patent type and laws of the country, or even transfer rights to companies, which is very common practice when the inventor is not able to invest in the production of his/her invention (Furtado Júnior et al., 2018; Haase et al., 2005). In Brazil, for example, there is only one company among the largest depositors, as the others are individuals and universities (Pieroni et al., 2010).

The investments and the search for patent protection are increasingly disseminated in capitalism, where the whole system operation relates to scientific and technological advancement. Although patent law is known worldwide, most people, even researchers, do not partially or totally understand the process of preparation and deposit of patents (Tejedor and Romero, 2014).

After the deposit, intellectual property banks are responsible for analyzing documents and providing patents. Some of the major banks in the world are the National Institute of Industrial Property (INPI-Brazil), the Canadian Intellectual Property Office (CIPO-Canada), the German Patent and Trade Mark Office (DPMA-Germany), the Japanese Patent Office (JPO-Japan), the Intellectual Property Office (IPO-United Kingdom), the United States Patent and Trademark Office (USPTO-United States) and the World Intellectual Property Organization (WIPO-Europe) (Valadas et al., 2017). Therefore, the objective of this study was to evaluate deposited and granted patents regarding dental implantology at the National Institute of Industrial Property (INPI) in Brazil in order to assess the main materials and industrial production in implantology.

MATERIALS AND METHODS

This is a prospective study of exploratory analysis performed in March 2020, in which searches were conducted for Implantology patents deposited and granted in the National Institute of Intellectual Property (INPI), which is the bank responsible for intellectual property in Brazil.

For this, searches on the INPI bank, were performed in titles and abstracts of reports on patent applications related to dental Implantology, from the first deposit through to the most current. First, a search with the term "dental implants" was performed, which returned 44 patent deposits. Next, a search with the term "implantology", which returned 100 deposits. After reading the reports and deleting duplicate documents or those unrelated to implantology, 139 reports on patents were selected and have been read and analyzed. The information extracted from the reports, as year of the deposit, type of patent and products were organized in tables and figures in GraphPad Prism 6.

RESULTS AND DISCUSSION

From 1988, the date of the first deposit, through to 2019 there were 139 patent application deposits. Among the deposited patents 54% (75) were classified as utility model patents and 25% (35) as invention patents, the others 21% (29) have been in analysis.

Figure 1 shows the distribution of the reports at INPI along the years. There were no reports of deposits recorded between 1988 and 1996. Although the amount is modest, the number of deposits increased in 2007 and 2008.

Figure 2 shows the countries of the depositors. Brazil holds 80.6% (112) of deposits, and the other 19.4% are countries in Latin America, North America, Europe and Africa. After Brazil, the United States has the most deposited patents in the most recent years.

In Figure 3, when analyzing the applications for patents of companies/individuals in Brazil regarding the distribution of Brazilian regions, the Southeast region holds 74 deposits, which represents more than half of the

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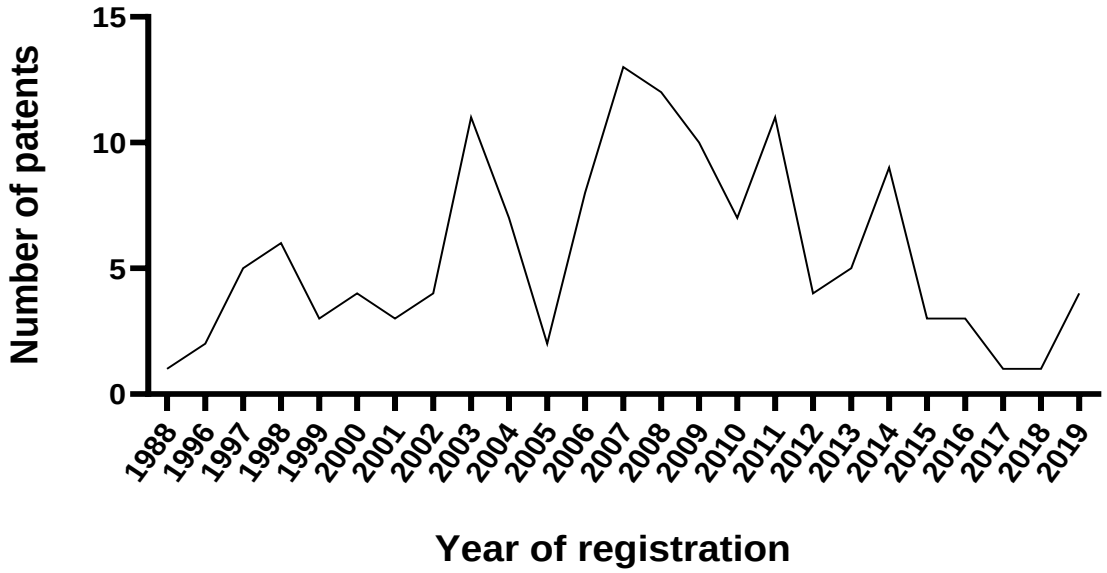

Figure 1. Amount of patents per year with the term "Implantology" and "Dental Implants" at *INPI*, Brazil, 2019.

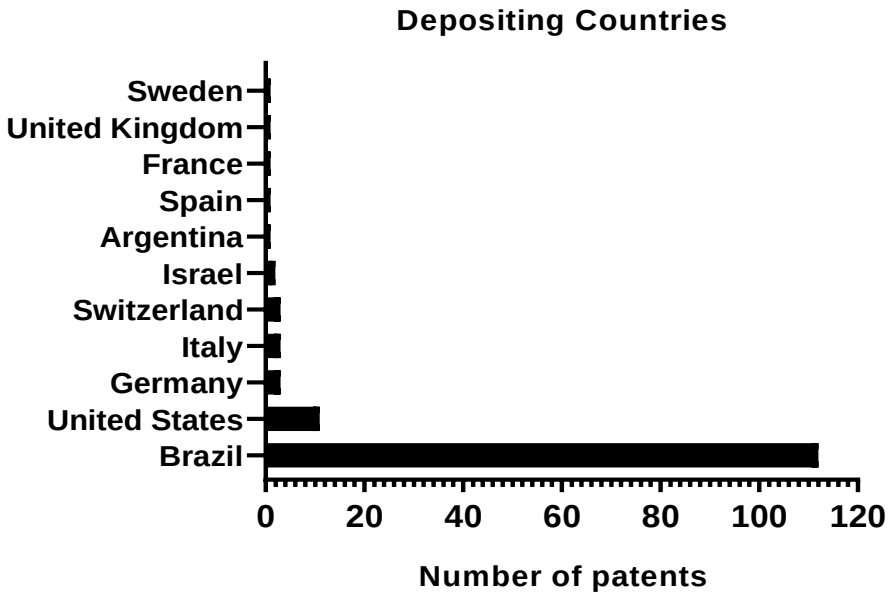

Figure 2. Depositors of patents by country with the term "Implantology" and "Dental Implants" at *INPI*, Brazil, 2019.

patent applications (68.5%), followed by the South region, with 26.8% (33) deposits. The North region has no record of deposits.

Table 1 shows the amount of products with application in dental implantology. The largest number of deposits relates to accessories of implants (28.78%); the lowest amount of deposits was related to accessories/ instruments for grafts (7.91%).

In order to standardize the deposits, every patent is classified in accordance with the IPC - International Patent Classification, which is the international classification system created in 1971, distributing patents by codes. The products are divided into technological areas of types A through H, and each type has its divisions and subdivisions (subclasses). Table 2 shows that virtually all deposits are type A (94.24%), which refers to products of human needs. Type C, related to chemicals/metallurgy, had only 2.16%, and type G, which refers to physical processes, had 3.6%. Types B, D, E, F and H had no deposits because they do not apply to the area.

Since the description of the osseointegration principle

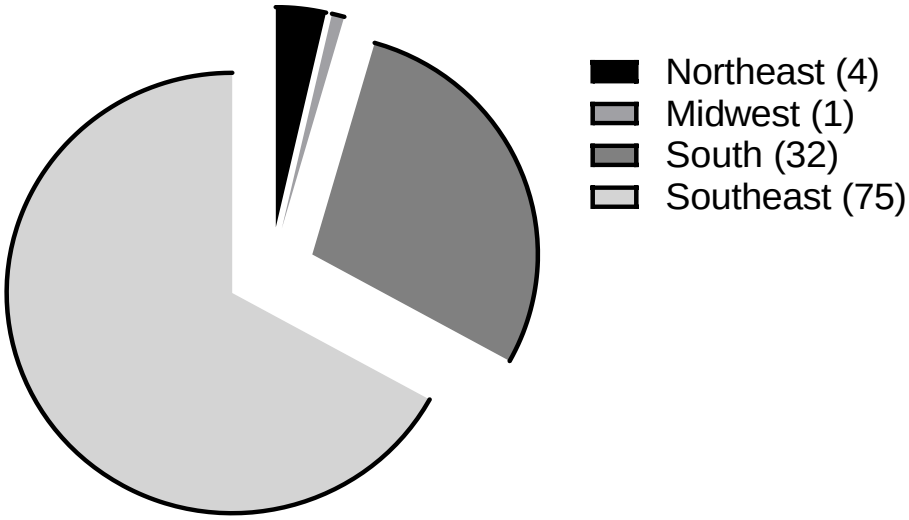

Figure 3. Distribution of patent deposits with the term "Implantology" and "Dental Implants" by regions of Brazil, 2019.

Table 1. Amount of deposited patents by type of material at *INPI*, Brazil, 2019.

Description of the patent material	Quantity (%)
Antimicrobial accessories/materials	7 (5.04)
Accessories/instruments for grafts	11 (7.91)
Implants	20 (14.39)
Accessories for implants	40 (28.78)
Protheses (materials/components)	29 (20.86)
Systems/methods	20(14.39)
Others (packaging, positioners, etc.)	12 (8.63)
Total	**139**

by Branemark and colleagues more than 45 years ago, a new era emerged in the research of materials for dental Implantology. Currently, there are approximately 1,300 different implant systems, each one with its particularities in relation to format, materials, surface treatment, prosthetic components, etc. Although rehabilitation with dental implants presents high rates of long-term success, new technologies are developed in order to promote long-term osseointegration in cases in which more failures occur, such as low quantity and bone quality and patients with comorbidities (Smeets et al., 2016).

In relation to scientific production in dental materials in the world context, Brazil has great expressivity. Studies in implantology in Brazil have been increasing, and dental materials had the highest number of published articles up until 2016 (13,708 articles). Nevertheless, the amount of patents is modest, as the process for a product to be patented is complex and the number of depositories is not proportional to the number of scientific studies in the area (Rosa et al., 2016).

Unlike developed countries, the government in Brazil is the main responsible organ for funding Research and Development (R&D), while the main source of investment in other countries comes from private capital/sources (Castro and Souza, 2012). However, in recent years, the trend to develop more complex studies by large companies and basic studies by universities has been increasing due to the costs (Dalmarco et al., 2011). Thus, the universities are responsible for most studies in Brazil, especially public ones, but the amount of products being patented by them is still small and a new phenomenon, indicating that the participation of Brazilian universities is modest, especially in the area of dental Implantology, probably because the country has few graduate programs in implantology (Valadas et al., 2017).

Regarding the Brazilian regions, the Southeast region has the highest amount of patent deposits in universities, especially being represented by the universities of São Paulo, followed by the South region in second place and the Northeast in third. These results result from a major investment of funding agencies, in addition to concentrating most studies in the country, which tends to decrease because of guidelines created for the decentralization of scientific and technological activity

Table 2. Classification of deposited patents with the term "Implantology" and "Dental Implants" at *INPI*, in accordance with the International Patents Classification - IPC.

Classification by section	Subclass of patent	Quantity (%)
Type A (human needs)	A61C	107 (76.98)
	A61K	12 (8.63)
	A61L	5 (3.6)
	A61B	4 (2.88)
	A61F	1 (0.72)
	A61N	1 (0.72)
	A41D	1 (0.72)
Type C (chemistry/metallurgy)	C01B	1 (0.72)
	C23C	1 (0.72)
	C23F	1 (0.72)
Type G (Physics)	G01B	1 (0.72)
	G01L	2 (1.44)
	G03B	2 (1.44)

A41D, External clothing; protective costumes; accessories; A61C, dentistry; oral or dental hygiene; A61K, preparations for medical, dental and hygienic purposes; A61L, methods or apparatus for sterilizing materials or objects in general; Disinfection, sterilization or deodorization of air; Chemical aspects of bandages, dressings, absorbent pads or surgical articles; materials for bandages, dressings, absorbent pads or surgical articles; A61F, Filters implanted in blood vessels; prostheses; devices that promote clearing or prevent collapse of tubular structures of the body, such as stents; orthopedic, nursing or contraceptive devices; fomentation; treatment or protection of eyes or ears; bandages, dressings or absorbent pads; first-aid cases; A61N, electrotherapy, magnetotherapy; radiation therapy; ultrasound therapy; A61B, diagnosis; surgery; identification; C01B, non-metallic elements; their compounds; C23C, coating of metallic materials; coating of materials with metallic materials; materials with surface treatment of metallic materials by diffusion, by chemical conversion or replacement; coating by vacuum evaporation, sputtering, implantation of ions or chemical deposition in vapor phase, in general; C23F, non-mechanical removal of metallic materials of surfaces; G01B, measuring of lengths, thicknesses or other similar linear dimensions; measurement of angles; measurement of areas; measuring surface irregularities, or outline; G01L, measurement of force, tension, torque, work, mechanical power, mechanical efficiency, or pressure of fluids; G03B, instruments or provisions to take photographs or to project them or view them; instruments or provisions that use similar techniques by means of waves different from optical waves; accessories for them.

(Oliveira and Velho, 2009). In the present study, only 3.73% (Pires et al., 2015) deposits were from public (federal) universities in the Southeast region.

This trend in depositing the products generated by searches will probably increase, because of Law 10,973 of 2 December 2004, which deals with measures to encourage innovation and scientific and technological research by the Federal Government, states and municipalities and development agencies to increase partnerships with companies and entities dedicated to scientific and technological researches, requiring an Information Technology Core (NIT) from each public university to strengthen this partnership (Dagnino and Silva, 2009; Mueller and Perucchi, 2014; Valadas et al., 2017).

Patents may be invention (IP) or utility model (UM). In the present study, 68.2% of deposits were UM and 31.8% were IP, suggesting more Brazilian investment in improving and adapting inventions than create original products. The number of accessories of implants was significantly higher than the number of implants patented in Brazil; thus, the country's industries are not investing in invention or adaptation of new implants. The same occurs regarding biomaterials, which have a significant participation in the market both quantitatively and qualitatively in generating profits; however, only two patents (UM 7700936-3; IP 1106507-9) were recorded in this area (Table 1) (Pires et al., 2015; Amadei and Torkomian, 2009).

Regarding prostheses and their components, the survey indicated that they had 29 (20.86%) of related deposits, an expressive number. For the development of new increasingly sophisticated prosthetic materials, aesthetics are not the only goal. Behind it, the industry uses engineering, physics and chemistry techniques, making the work not only increasingly more aesthetic but also functional and resistant, and this multiprofessional interaction is essential for this improvement (Hattori et al., 2011).

Studies indicate that the Brazilian dental industry used to focus on the production of restorative materials; however, the current trends are investments in technology, because there is a concern to improve the development of biomaterials (Rosa et al., 2016).

When observing the provision of deposits of patents over the years at the INPI bank, the last decade (2000-2010) had a lot more deposits than the current decade, indicating that technological innovation in the industry of dental implants and related materials decreased in the current decade. In 2017, for example, had only one

deposit record (BR 1020170069222) (Silva, 2017), but possible deposits can still be processing. Reports indicate that after the creation of the National Sanitary Surveillance Agency (ANVISA) in 1999, there was a greater demand of companies in providing products with guaranteed quality and increasingly more safety, thus influencing the development of new products in the last decade (Pieroni et al., 2010). Another explanation is that there were many mergers of companies in the health area in Brazil between 2005 and 2006, when more than 250 business transactions with international companies occurred (Burkhardt and Tardio, 2006).

Multinational companies commonly perform deposits in countries other than of their origin in order to increase the protection and disclosure of their products, which is normally done through the Patent Cooperation Treaty (PCT), known as the international patent. When depositing the invention in this way, the product is protected in all country members of the Treaty. Deposits on intellectual protection of products for application in dental Implantology are made by several countries, especially the United States, which is the country with the largest market movement of biomaterials (Pires et al., 2015). Although the data are modest, the existence of other countries as depositors indicates an interest in the Brazilian market, whose growth in the area and prospect of increased demand is attracting an increasing number of deposits by international companies (Tejedor and Romero, 2014).

There is a regional inequality of technical-scientific production in Brazil, in which the state of São Paulo and the Southeast region as a whole have a greater participation and enormous importance in the system of innovation in the country. Having the highest GDP (Gross Domestic Product) in the country, a large part of the universities, industries and scientific production are decisive factors for this scenario. An indicator of this scenario is deposits of patents, as in the present study, in which the Southeast and South regions together have almost 80% of the deposits (Albuquerque and Cassiolato, 2002).

The Northeast region has only four of the 112 Brazilian patent applications, where the state of Ceará has half the deposits, followed by Bahia and Pernambuco, with only one deposit each. Although the production is virtually non-existent, the development and protection of new products in the dental implantology market in Ceará may be considered an advance.

In Brazil, the industry of products for health services has large participation, in which dentistry is the only area that features a trade surplus and the second largest market in the world, only behind the United States. However, most of its products are low- and medium-technology. Regarding innovative products, the use of American products still prevails in Brazil. The current context requires new challenges and major investments in technology in this sector (Pieroni et al., 2010).

Conclusion

Records of patents are essential to evaluate the technological level of a given area. Analyzing the patent deposits for use in dental implantology at INPI provided a panoramic view and showed that the majority relate to accessories of implants and prosthetic components, indicating greater investment by industries in Brazil in this sector.

The number of patent applications in the years 2007 and 2008 increased, yet the number of deposits is small, since only 139 patents were deposited in 30 years, suggesting that the importation of products in the area is still great, which consequently makes the products of the area more expansive.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Biotreatment of water samples from Itakpe iron mining site, Kogi State, Nigeria using *Bacillus* species

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Four soil samples and water effluents each within the mining environment were tested in this study. Twenty six bacteria were isolated using nutrient agar and acidified nutrient agar. Gram staining and endospore staining were carried out to determine the *Bacillus* species used for bioremediation. Speciation of the microorganisms using 16S rRNA sequencing showed the organisms to be *Bacillus cereus* NK1, *Lysinibacillus* species TAI-282, *Lysinibacillus fusifomis, Bacillus aryabhattai* PM1 and *Bacillus megaterium* from the samples. The temperature of the water effluents ranged from 29.00 to 34.70°C. Sample C from beneficiation area had the highest temperature of 34.70°C. The pH ranged from 6.70 to 9.77 with effluent from primary crushing area two (PC2) having the highest of 9.77. Bioremediation of the water samples were carried out for 6 days using the identified *Bacillus* species from the mine site. For all the effluents treated, there was an increase in the concentration of magnesium with effluent from PC2 treated with *L. fusifomis* increasing to 7.453±0.004 from 4.278±0.003 and these values were significantly different at p≤0.05. Concentration of calcium in effluent from iron ore storage area increased from 1.350 ±0.002 to 15.450±0.004 after treatment with *B. aryabhattai* PM1, and the values were significantly at p≤0.05. *Bacillus* spp. from PC2 reduced the concentration of iron from 179.738 to 0.091 ppm on effluent from PC1, the concentration was significantly different at p≤0.05. The indigenous microorganisms from within the iron ore mining site had bioremediation potentials reducing the concentrations of most of the heavy metals present.

Key words: Iron ore, molecular analysis, bioremediation, *Bacillus* species.

INTRODUCTION

Heavy metals are difficult to remove from the environment and are ultimately indestructible unlike many other pollutants that can be chemically or biologically degraded (Ozaki et al., 2003). Severe damage is caused to aquatic life when such metals are present and microorganisms are killed during biological water purification process (Vinodhini and Narayanan, 2008). Contamination of metals is a major environmental problem and especially in the aquatic environment some of which at low concentration are toxic or carcinogenic. Metals remaining in contaminated sediments may accumulate in microorganisms which in return enter into the food chain eventually affecting human wellbeing (Shakeri and Moore, 2010).

The iron ore deposits in Nigeria are located at the middle belt geopolitical zone which is characterized by alternating layers rich in chart, a form of silica (SIO_2) and layers rich in iron minerals such as heamatite (Fe_2O_3),

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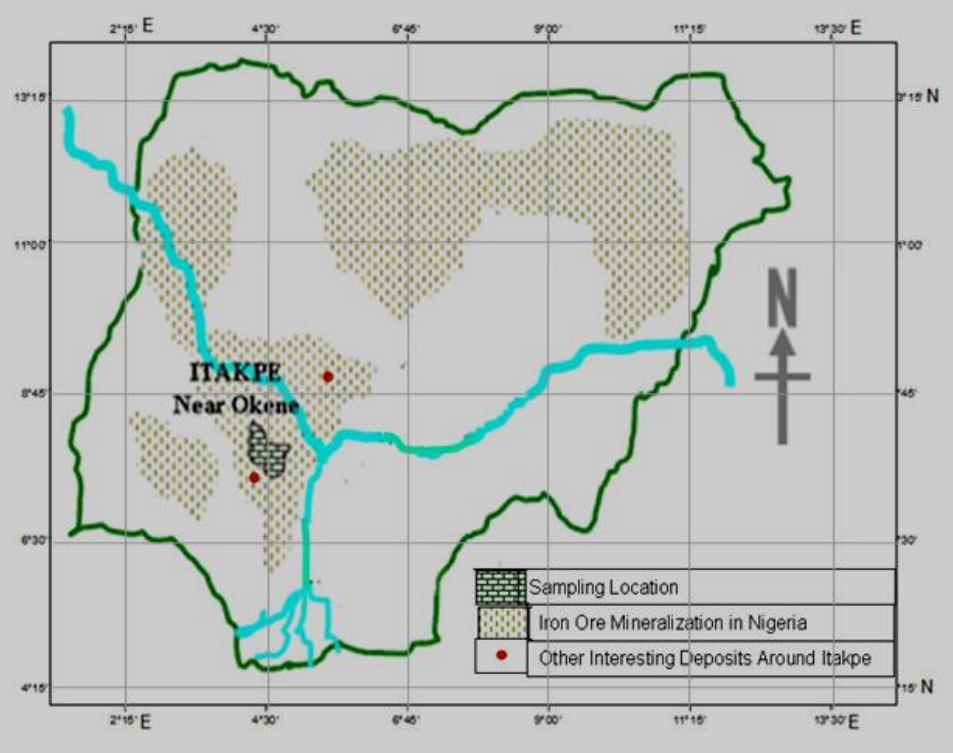

Figure 1. Map of Nigeria showing iron mineral formation in the country and Itakpe deposit (Onyemaobi, 2001).

magnetic (Fe_3O_4), iron silicate, chamosite and siderite (Fagade et al., 2010). These deposits are found in vast reserves of chemical and classic rocks such as sedimentary, igneous and metamorphic for over three thousand years (Morris, 2012). Itakpe iron ore is located in Latitude 07°36'20''N and longitude 6°18'35''E in Okehi Local Government Area of Kogi State, Nigeria (Ifeanyi et al., 2013).

The presence of contaminants in an environment leads to an increase in the numbers of microbes able to degrade such. The residues for the treatment are usually harmless products and include carbon dioxide, water and cell biomass (Sardrood et al., 2013).

The aim of this study was to isolate and identify *Bacillus* species from water effluents and soil samples from Itakpe iron ore mining site using Polymerase Chain Reaction (PCR) and bioremediate samples with some of the isolates.

MATERIALS AND METHODS

Study location

The study location was Itakpe iron ore mine site, in Kogi State, Nigeria. Four water samples and four soil samples were collected within the mining site at different locations aseptically in August, 2017 (Figure 1). Soil samples were collected using soil auger to a depth of 5 cm and put into polythene bags. Water samples were collected using Grab sampling method. The samples were transported to the laboratory for analysis within 12 h (Nafanda, 2005).

The temperatures of the samples were taken using mercury-bulb thermometer and the pH determined with Hanna pH 211 Model on site.

Determination of colour and presence of particles

Visual examination of the water samples was used to determine the colour and presence of particles.

Determination of odour

The water effluent containers were shaken vigorously for about 5 s. The covers were removed aseptically and the odour quickly determined by inhalation of air near the mouth of the plastic containers.

Determination of total dissolved solids (TDS) was according to the method of Jamal et al. (2015). Biochemical oxygen demand (BOD) was determined using the method of Thompson and Stevenson (1984) and the chemical oxygen demand (COD) was carried out by using the photometer method (Thompson and Stevenson, 1984).

Metal analyses

The Atomic Absorption Spectrophotometer (AAS) Buck Scientific model 210 was used to determine the metal and mineral

concentrations of the samples using the calibration plot method.

Isolation of bacteria

One gram each of the soil samples and 1 ml each of effluent from the primary crushing area one, primary crushing area two, beneficiation area and Iron ore storage area were used. Pour plate method according to Thompson and Stevenson (1984) was used. The samples were serially diluted using sterile distilled water as diluents according to the method of Murugalatha et al. (2018). Nutrient agar and acidified nutrient agar were used for the isolation of bacteria. Sub culturing was carried out until pure cultures were obtained.

Identification of bacterial isolates

Preliminary identification of the bacterial isolates was carried out. Gram staining and endospore staining were carried out using the methods of Bergey and John (2000).

DNA isolation and purification

This procedure was carried out using the QIAamp DNA mini kit (Qiagen, #51306). Isolates used were cultured in broth overnight, and DNA isolation was carried out according to manufacturer's instruction. Using a NanoDrop ND1000 (Thermo Scientific, USA) machine, DNA was quantified by calibrating the machine with 1 µl of water, followed by 1 µl blank (Tri-EDTA buffer) and then 1 µl of the DNA sample to be quantified (Kumar et al., 2016).

Polymerase chain reaction (PCR)

PCR procedure was carried out as described by Mullis et al. (1986), using a pair of primers, 16SF (GTGCCAGCAGCCGCGCTAA) and 16SR (AGACCCGGGAACGTATTCAC)**.** Denaturation was achieved at 94°C for 5 min, and subsequently for 30 s; annealing at 56°C for 30 s; extension at 72°C for 45 s. The processes occurred in 36 cycles and the final extension was at 72°C for 7 min. The products were further purified by adding 2 volumes (20 µl) of absolute ethanol, incubated at room temperature for 15 min, and then centrifuged at 10,000 rpm for 15 min. Supernatant was discarded and 2 v (40 µl) of 70% ethanol added, centrifuged again at 10,000 rpm for 15 min, supernatant was discarded and product air dried. Final product was held at 10°C for further analysis (Kumar et al., 2016).

Gene sequencing

The amplicons from the polymerase chain reaction were subjected to sequencing reactions using BigDye Terminator v3.1 Cycle Sequencing Kit, following manufacturer's guidelines. The products were loaded unto 3130xl Genetic Analyzer (Applied Biosystems, 2010) to generate the molecular sequences of each amplicon.

Base sequence analysis

The base sequences generated from each amplicon were analyzed by a combination of Basic Local Alignment Search Tool (BLAST) and Fast Alignment (FASTA) (Donkor et al., 2014). Sequences were submitted as query at http://www.ncbi.nlm.nih.gov/Blast.cgi for comparison with database sequences using the NCBI nucleotide BLAST. Isolates were identified based on DNA-DNA similarity at 99%.

Phylogenetic analysis

The analysis involved 7 nucleotide sequences. There were a total of 42 positions in the final dataset. All positions containing gaps and missing data were eliminated. Phylogenetic analysis was carried out by maximum parsimony (MP) method based on the partial sequences of 16S rRNA gene of representative isolates in this study. The best substitution model that described the sequence data set was obtained and 1000 bootstraps values were used to determine the confidence interval of the resultant tree. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Bioremediation of water samples using identified *Bacillus* spp.

The modified method of Fagade et al. (2010) was employed using seven isolates. These isolates were grown on nutrient broth for 48 h at 37°C and read on a spectrophotometer at 610 nm. The cultures were standardized at 0.00 optical densities. 1000 ml of each sample was used and 5 ml of identified *Bacillus* spp. was inoculated into the different samples. They were incubated at 28°C for seven days. Bioremediation of the samples were carried out using the following *Bacillus* spp. isolated from the soil samples and water effluents: *Bacillus cereus* NK1, *Lysinibacillus* species TAI-282, *Lysinibacillus fusifomis*, *Bacillus* spp. 1, *Bacillus aryabhattai* PM1, *Bacillus megaterium* and *Bacillus* spp. 2.

Statistical analysis

Data obtained were subjected to Analysis of Variance (ANOVA) using SPSS version 20 at P≤0.05 level of significance.

RESULTS

A total of eight samples from different locations were used for this study. Samples A, B, C and D were water samples and samples E, F, G and H were soil samples.

Physicochemical parameters of samples

Table 1 shows the physicochemical parameters of the effluents. The temperatures varied from 30.27 to 34.75°C and were within the standard limit for effluent which is <40°C. The temperature of Sample B from primary crushing area two (PC2) was 29.00°C and the lowest while that of Sample C from beneficiation area was 34.70°C and the highest. Particles were present in all samples. The color of sample A, effluent from primary crushing area (1) was brownish with no odor while sample B was colorless with an objectionable odor pH of 9.21. Sample D from the iron ore storage area had the highest pH of 9.77.

Biochemical characteristics of isolates

Table 2 shows the biochemical characteristics of isolates

Table 1. Physicochemical properties of effluent samples.

Sample	Color	Odor	Particles	Temp (°C)	pH
A	Brownish	Odorless	Has particles	30.27	8.79
B	Colorless	Odorless	Has particles	29.00	9.21
C	Colorless	Slightly offensive	Has particles	32.00	6.70
D	Brownish	Slightly offensive	Has particles	34.00	9.77
Limit	Nil	Odorless	Nil	<40	6-9

A= Primary crushing area (1), B= Primary crushing area (2), C= Beneficiation area, D= Iron ore storage area.

Table 2. Morphological and biochemical characteristics of selected isolates from soil and water samples using nutrient agar and acidified nutrient agar.

Nutrient agar	Isolate	Elevation	Edge	Shape	Chromogene	Color	Gram stain	Endospore stain
Acidified	H1*	Flat	Entire	circular	Opaque	Creamy	+ve rod	+ve
	S1*	Flat	Undulate	Circular	Opaque	Creamy	+ve rod	+ve
	S3*	Raised	Entire	Irregular	Opaque	Creamy	+ve rod	+ve
Non acidified	H1**	Flat	Entire	Circular	Opaque	Whitish	+ve rod	+ve
	H1	Raised	Entire	Circular	Opaque	Whitish	+ve rod	+ve
	H2	Flat	Entire	Circular	Opaque	Whitish	+ve rod	+ve
	S1	Flat	Entire	circular	Opaque	Creamy	+ve rod	+ve

from both soil and water samples. All selected isolates were Gram positive and endospore formers. Isolate S1 from soil around primary crushing area one (PC1) appeared flat with entire edge, circular and creamy in color. Isolate H1 from effluent around primary crushing area one appeared flat with entire edge, circular and greenish in color.

Phylogenetic tree of bacterial isolates

Figure 2 shows the evolutionary relationship of bacterial isolates in this study. The phylogenetic tree revealed the evolutionary relationships among the isolates in this study. The optimal tree with the sum of branch length = 3.68169715 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap tests (1000 replicates) are shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

The tree showed 2 sister clades sharing common ancestors; *Lysinibacillus* spp. TAI-282, *B. aryabhattai* PM1 and *Bacillus* spp.-*Bacillus* spp. (2) at 94 and 83%, respectively. Other isolates however showed weak relationships at <50% bootstrap replicates. *B. megaterium* Rhizo 2 and *B. cereus* NK1 had the lowest evolutionary relationship with 18% each.

Elemental and mineral composition of effluents from PCI before and after treatment with *Bacillus* isolates

There was a reduction by *Bacillus* spp. of iron concentration from 179.738 to 0.049 ppm. *Bacillus aryabhattai* PMI reduced lead concentration from 0.314 to 0.093 ppm and *L. fusifomis* reduced manganese from 1.255 to 0.007 ppm (Table 3).

Statistical analyses at P≤ 0.05 showed that calcium present in water was significantly different from other water samples after treatment with selected isolates. Control was 5.200^{f}±0.003 while the isolate with the highest amount of value was H2 *Bacillus* spp. from water sample from primary crushing area two, which indicated an increase in calcium after treatment. However, isolate S1 *B. megaterium* with a value of 7.600^{e} ±0.001 was statistically the same with isolate H1 *B. cereus* with value of 7.750^{e}±0.002 after treatment of mine water. The value for untreated lead was statistically different from other effluents after treatment with the selected isolates. The control had a value of 0.314^{a}±0.000 and was statistically different from water sample treated with isolate S1* *B. aryabhattai* with a value of 6.61^{h}±0.002. However, manganese present in water sample treated with isolate S1 *B. megaterium* was statistically the same with manganese present in water sample treated with isolate H1 *L. fusifomis* with values of 8.711^{a}±0.001 and 8.199^{a}±0.002, respectively.

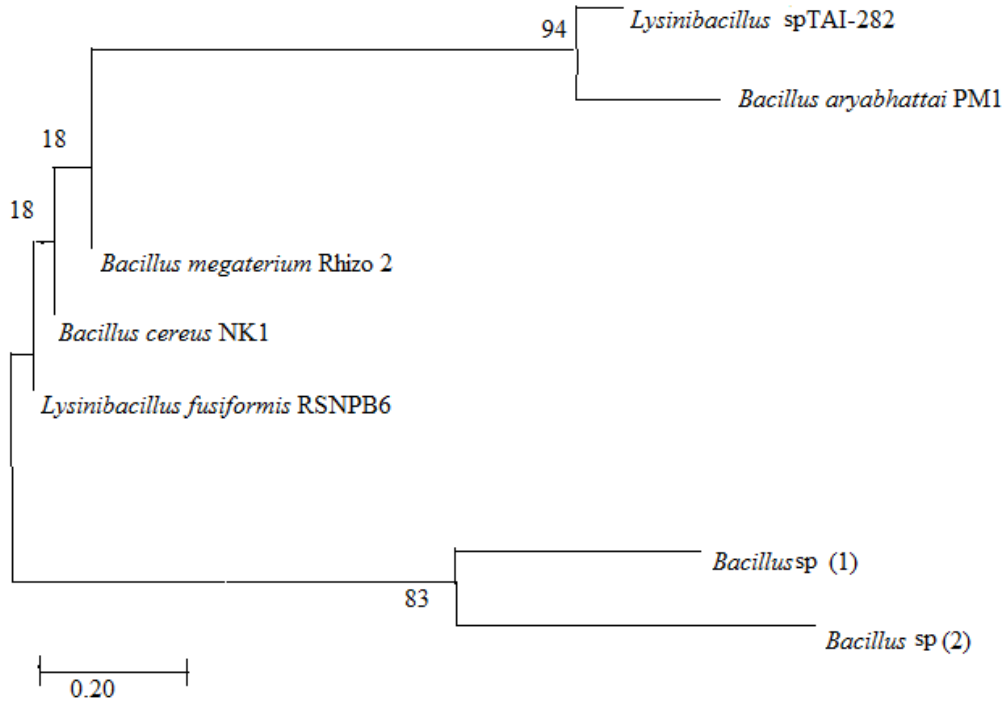

Figure 2. Evolutionary relationship of taxa.

Elemental and mineral composition of effluents from PC2 before and after treatment with *Bacillus* spp.

Statistical analysis showed that the concentration of iron before treatment was 70.368^{a}±0.118 and significantly different statistically after treatment with *Bacillus* spp. The concentration was reduced to 0.144^{c}±0.002. The concentrations of cadmium present in the effluent when treated with *B. aryabhattai* PM1 and *B. megaterium* were not significantly different at P≤0.005. The values recorded were 0.001^{e}±0.004 and 0.013^{e}±0.001, respectively (Table 4).

Elemental and mineral composition of effluents from Beneficiation area before and after treatment with *Bacillus* isolates

Statistical analyses at P≤0.05 showed that lead present in the control was not statistically different with that present in water sample treated with *Bacillus* spp. with values of 0.010^{b}±0.000 and 0.178^{b}±0.001. Iron present in water sample treated with *B. aryabhattai* PM1 was not different significantly from water sample treated with *B. megaterium* with values of 0.078^{b}±0.003 and 0.078^{b}±0.001. However, nickel present in water samples treated with *B. aryabhattai* PM1, *B. megaterium*, *L. fusifomis* and *Bacillus* spp. were all not statistically different (Table 5).

Elemental and mineral composition of effluents from Iron ore storage area before and after treatment with *Bacillus* isolates

Table 6 shows the elemental composition of effluent from Iron ore storage area before and after treatment. Statistical analysis at P≤0.05 shows that iron present in the control was significantly different from that present in water treated with *Bacillus* spp. with values of 2.176^{a}±0.0047 and 0.029^{f}±0.004, respectively. Also, arsenic present in the control was significantly different from that present in water treated with *B. megaterium* with values of 0.124^{a}±0.002 and 0.087^{d}±0.0021. In addition, cadmium present in water sample treated with *B. megaterium*, *L. fusifomis* and *Bacillus* spp. were all statistically not different with values of 0.015^{c}±0.002, 0.012^{c}±0.002 and 0.018^{c}±0.003.

Table 7 shows the physico-chemical constituents of effluent from primary crushing area two (PC2). There was a reduction in all the parameters after treatment for five days. Statistical analyses at P≤0.05 show that BOD for the control was statistically different from all samples treated with selected isolates; the value of the control was 125.80^{g} mg/l. The BOD value obtained when *B. cereus* NK1 was used was not statistically different from that obtained with the use of *L. fusifomis*. The values were 4.27^{d} and 4.50^{d} mg/l, respectively. The BOD value after treatment with *B. aryabhattai* PM1 was the lowest at 2.20^{a} mg/l against the control value of 125.80^{g} mg/l

Table 3. Elemental and mineral composition of effluent (ppm) from primary crushing area one (PC1) before and after treatment with isolates.

PC1	Calcium	Iron	Arsenic	Magnesium	Lead	Cadmium	Nickel	Manganese
Control	5.200^{f}±0.003	179.738±0.091	0.060^{c}±0.001	3.495^{g}±0.004	0.314^{a}±0.000	0.008^{e}±0.001	0.112^{c}±0.001	1.255^{a}±0.005
H2	17.360^{a}±0.007	0.049^{h}±0.000	0.011^{e}±0.001	7.109^{c}±0.002	0.130^{d}±0.001	0.004^{f}±0.001	0.019^{e}±0.001	0.005^{d}±0.001
S1*	9.750^{b}±0.005	0.579^{b}±0.004	0.006^{f}±0.001	6.129^{e}±0.005	0.61^{h}±0.002	0.054^{a}±0.004	0.122^{b}±0.003	0.000^{f}±0.000
S1	7.600^{e}±0.001	0.105^{f}±0.001	0.014^{e}±0.003	8.711^{a}±0.002	0.093^{g}±0.001	0.015^{c}±0.001	0.481^{a}±0.004	0.000^{f}±0.000
H1	7.750^{e}±0.002	0.085^{g}±0.004	0.021^{d}±0.003	7.521^{b}±0.002	0.123^{f}±0.001	0.045^{b}±0.004	0.078^{d}±0.005	0.003^{c}±0.001
H1**	7.700^{e}±0.000	0.395^{d}±0.004	0.020^{d}±0.001	6.055^{f}±0.001	0.192^{b}±0.004	0.012^{d}±0.001	0.001^{f}±0.000	0.016^{b}±0.001
H1*	8.350^{d}±0.002	0.122^{e}±0.006	0.665^{a}±0.000	8.199^{a}±0.002	0.189^{c}±0.001	0.006^{g}±0.001	0.081^{d}±0.004	0.007^{c}±0.000
S3*	8.650^{c}±0.010	0.412^{c}±0.004	0.430^{b}±0.001	6.389^{d}±0.003	0.127^{e}±0.003	0.016^{c}±0.001	0.099^{d}±0.001	0.003^{e}±0.001

Table 4. Elemental and mineral composition of effluent (ppm) from primary crushing area two (PC2) before and after treatment with isolates.

PC2	Calcium	Iron	Arsenic	Manganese	Magnesium	Lead	Cadmium	Nickel
Control	6.300^{g}±0.000	70.368^{a}±0.118	0.107^{b}±0.001	7.139^{a}±0.001	4.278^{h}±0.003	0.281^{a}±0.001	0.012^{e}±0.002	0.150^{a}±0.003
H2	12.900^{b}±0.011	0.144^{c}±0.002	0.018^{c}±0.004	0.126^{b}±0.004	6.439^{d}±0.003	0.112^{e}±0.002	0.016^{d}±0.003	0.021^{g}±0.005
S1*	7.750^{e}±0.002	0.138^{c}±0.001	0.365^{a}±0.002	0.000^{g}±0.000	5.020^{g}±0.125	0.015^{h}±0.003	0.013^{e}±0.004	0.129^{b}±0.002
S1	16.450^{a}±0.002	0.017^{f}±0.003	0.014^{c}±0.001	0.000^{f}±0.001	7.183^{b}±0.012	0.105^{f}±0.002	0.013^{e}±0.001	0.038^{f}±0.004
H1	9.550^{c}±0.001	0.007^{g}±0.001	0.009^{e}±0.001	0.051^{c}±0.002	7.453^{a}±0.004	0.119^{d}±0.004	0.011^{f}±0.000	0.075^{c}±0.000
H1**	6.350^{g}±0.002	0.257^{b}±0.011	0.005^{g}±0.001	0.041^{d}±0.001	5.341^{f}±0.001	0.020^{g}±0.002	0.054^{c}±0.002	0.047^{e}±0.002
H1*	8.550^{d}±0.001	0.066^{e}±0.002	0.006^{f}±0.002	0.028^{e}±0.003	6.185^{e}±0.007	0.139^{c}±0.004	0.089^{b}±0.004	0.059^{d}±0.004
S3*	7.250^{f}±0.002	0.057^{d}±0.005	0.011^{d}±0.000	0.000^{g}±0.000	6.473^{c}±0.026	0.178^{b}±0.001	0.124^{a}±0.004	0.041^{e}±0.003

H1*= *Lysinibacillus fusifomis* from effluent from primary crushing area one; S1*= Bacillus *aryabhattai* PM1. from soil from primary crushing area one; S3*= *Bacillus* sp from beneficiation area; H1**= *Lysinibacillus* sp TAI-282. from effluent from primary crushing area one, H1= *Bacillus cereus* NK1.from effluent from primary crushing area one; H2= *Bacillus* sp from primary crushing area two; S1= *Bacillus megaterium* from soil from primary crushing area one.

making *B. aryabhattai* PM1 the most effective in reducing BOD in the water sample from Primary crushing area two (PC2). The COD for the control was 260.17^{h} mg/l and statistically different from sample treated with *B. aryabhattai* PM1 with a value of 4.40^{a} mg/l. *B. megaterium* has the highest COD value after treatment with different isolates with a value of 26.23^{g} mg/l and statistically different to *Bacillus* spp. with a COD value of 17.30$^{f.}$

Statistical analyses at P≤0.05 showed that the control of PC2 had the highest TDS value of 1780.00^{g} mg/l and statistically different from other samples treated with selected isolates. *B. megaterium* with TDS value of 314.67f mg/l was statistically different from *B. cereus* NKL1 with value of 113.00^{b} mg/l. In addition, *Lysinibacillus* spp. TAI-282 with value of 235.00^{e} mg/l was statistically different to *B. megaterium* with a value of 314.67^{f} mg/l and the highest value after treatment.

DISCUSSION

Isolation of *Bacillus* spp. from soil samples and water effluents from Itakpe iron mining site was carried out. Eight different locations were used for sampling namely with soil and effluents collected; Primary crushing area 1 (PC1), Primary crushing

Table 5. Elemental and mineral composition (ppm) for Beneficiation area before and after treatment with isolates.

PC2	Calcium	Iron	Arsenic	Manganese	Magnesium	Lead	Cadmium	Nickel
Control	6.300^{c}±0.003	0.204^{a}±0.002	0.007^{e}±0.000	0.053^{b}±0.002	1.501^{g}±0.006	0.010^{b}±0.000	0.001^{c}±0.001	0.014^{a}±0.007
H2	2.550^{g}±0.001	0.004^{f}±0.002	0.023^{c}±0.003	0.126^{c}±0.004	2.319^{c}±0.001	0.003^{d}±0.001	0.016^{c}±0.003	0.001^{c}±0.000
S1*	2.950^{e}±0.001	0.078^{b}±0.003	0.001^{f}±0.001	0.0000^{e}±0.000	1.990^{f}±0.057	0.002^{e}±0.000	0.012^{b}±0.002	0.002^{c}±0.001
S1	10.450^{b}±0.001	0.078^{b}±0.001	0.077^{a}±0.001	0.000^{d}±0.001	2.153^{d}±0.018	0.097^{a}±0.002	0.013^{a}±0.001	0.002^{c}±0.001
H1	2.700^{f}±0.0.003	0.013^{e}±0.0001	0.016^{d}±0.001	0.051^{a}±0.002	2.078^{e}±0.010	0.006^{c}±0.001	0.021^{a}±0.002	0.005^{b}±0.002
H1**	17.400^{a}±0.001	0.045^{d}±0.003	0.048^{b}±0.004	0.041^{f}±0.001	3.053^{b}±0.012	0.001^{e}±0.000	0.001^{c}±0.001	0.047^{e}±0.002
H1*	2.250^{h}±0.002	0.062^{c}±0.002	0.006^{c}±0.002	0.034^{d}±0.014	3.103^{b}±0.002	0.002^{e}±0.000	0.089^{a}±0.004	0.001^{c}±0.000
S3*	4.800^{d}±0.002	0.057^{e}±0.006	0.011^{a}±0.000	0.079^{a}±0.003	4.194^{f}±0.023	0.178^{b}±0.001	0.002^{c}±0.001	0.002^{c}±0.001

Table 6. Elemental and mineral composition of effluent (ppm) from iron ore storage area before and after treatment with isolates.

Iron ore	Calcium	Iron	Arsenic	Manganese	Magnesium	Lead	Cadmium	Nickel
Control	1.350^{h}±0.002	2.176^{a}±0.005	0.124^{a}±0.002	0.208^{a}±0.004	3.202^{e}±0.004	0.487^{a}±0.002	0.006^{d}±0.001	0.112^{f}±0.005
Bacillus spp.	8.250^{d}±0.001	0.029^{f}±0.004	0.027^{f}±0.000	0.007^{e}±0.002	3.115^{g}±0.004	0.189^{c}±0.005	0.042^{a}±0.001	0.139^{c}±0.002
Bacillus aryabhattai	8.750^{c}±0.001	0.155^{c}±0.001	0.092^{d}±0.001	0.014^{c}±0.002	4.191^{c}±0.008	0.105^{f}±0.101	0.020^{b}±0.000	0.153^{b}±0.005
Bacillus megaterium	15.450^{a}±0.04	0.101^{e}±0.004	0.087^{d}±0.002	0.011^{d}±0.002	4.456^{b}±0.008	0.079^{c}±0.006	0.015^{c}±0.002	0.127^{d}±0.005
Bacillus cereus	6.700^{g}±0.000	0.023^{f}±0.003	0.047^{e}±0.002	0.039^{b}±0.002	4.813^{a}±0.004	0.239^{b}±0.003	0.008^{d}±0.001	0.074^{g}±0.003
Lysinibacillus spp. TA1-282	7.150^{f}±0.001	0.120^{d}±0.001	0.119^{b}±0.002	0.016^{c}±0.001	3.192^{f}±0.003	0.147^{d}±0.004	0.006^{d}±0.000	0.119^{e}±0.004
Lysinibacillus fusiformis	10.750^{b}±0.002	0.253^{b}±0.009	0.105^{c}±0.001	0.019^{c}±0.004	3.818^{d}±0.003	0.123^{e}±0.002	0.012^{c}0.002	0.135^{c}±0.003
Bacillus spp.	7.900^{e}±0.0.003	0.002^{g}±0.001	0.021^{g}±0.002	0.005^{e}±0.003	4.816^{a}±0.005	0.011^{f}±0.005	0.018^{c}±0.003	0.171^{a}±0.003

area 2 (PC2), Beneficiation area and Iron ore storage area. The temperatures of the samples varied from 29.00 to 34.70°C. Jamal et al. (2015) recorded similar temperatures of 29.80 to 39.00°C from acid mine drainage. The pH of samples varied from 6.70 to 9.77 with effluent from Iron ore storage area having the highest pH value of 9.77 while that from Beneficiation area (Sample C) had the lowest pH of 6.70. A study carried out by Jiang and Xu (2017) on process water in iron flotation of Yuanjiacum iron mine site recorded a pH of 9.12 for the tailings wastewater. This is similar to that recorded in this study. The temperature and pH values obtained in this study were within the Federal Environmental Protection Agency (FEPA) limits for discharge of effluents into water bodies.

Total Dissolved Solids (TDS) recorded for all water samples were below the FEPA limit of 2000 mg/l; values between 45.00 and 1780.00 mg/l. This was different from the study conducted by Jamal et al. (2015) on heavy metals from acid mine drainage (AMD) where TDS values ranged between 2213 and 2908 mg/l. *B. aryabhattai* PM1 and *B. megaterium* were effective in reducing the TDS in beneficiation (BENEF) sample from the initial TDS of 181.67^{f} to 45.00^{a} mg/l and 45.00^{a} mg/l, respectively, the values were not significantly different statistically from each other. According to APHA (1998), high levels of TDS were as a result of the presence of potassium, chlorides and sodium in water. However, these ions have been found to have little or no effect but in the presence of toxic ions such as lead, cadmium, nitrate and arsenic in water, the result will be more hazardous to the ecosystem (APHA, 1998).

Biochemical oxygen demand (BOD) value ranged between 2.20 and 125.80 mg/l. The initial BOD of effluent from Primary crushing area

Table 7. Physico-chemical constituents of effluent from primary crushing area two (PC2) (mg/l) before and after treatment with isolates

Isolate	BOD	COD	TDS
Control	125.80^{g}	260.17^{h}	1780.00^{g}
H2	8.33^{e}	17.30^{f}	104.67^{a}
S1*	2.20^{a}	4.40^{a}	122.33^{c}
S1	12.30^{f}	26.23^{g}	314.67^{f}
H1	4.27^{d}	8.50^{d}	113.00^{b}
H1**	3.60^{c}	7.47^{c}	235.00^{e}
H1*	4.50^{d}	9.63^{e}	104.67^{a}
S3*	2.87^{b}	6.73^{b}	135.33^{d}

two (PC2) was 125.80 mg/l and reduced to 2.20 mg/ml by *B. aryabhattai* PM1 and this result was different from the report on study conducted by Hammer and Hammer (2004) on water and waste water technology where BOD values between 130 and 200 mg/l were recorded.

The chemical oxygen demand (COD) values of the effluents were between 4.40 and 260.17 mg/l. *B. megaterium* reduced the COD value from the initial reading of 260.17 to 4.40 mg/l. According to Nafanda (2005), high COD value indicates the presence of high organic matter in effluent, so with the reduction in COD value, the microorganisms were effective in bioremediation. This result was similar to that obtained from study carried out by Fagade et al. (2010) where decrease in COD from initial 714.05 to 281.60±49.78 mg/l was recorded. Also, Jiang and Xu (2017) reported a reduction in COD of tailings wastewater from131 to 21 mg/L in their study. There was a decrease in TDS in this study with values of 181.67 to 45.00 mg/l for sample obtained from Beneficiation area but an increase in the TDS was reported by Fagade et al. (2010) with initial TDS value of 54.1 to 160.6 mg/l.

The bacteria isolated from the soil and water samples were Gram positive rods. These microorganisms included *B. megaterium*, *L. fusifomis*, *B. aryabhattai* and *Bacillus* spp. The absence of Gram negative bacteria in the environment is supported by Edwards et al. (1999), who reported less than 50% of Gram negative bacteria out of the total viable population in an acidic mine environment. This result is also in accordance with Fagade et al. (2010), where Gram positive bacteria were those mainly isolated.

Molecular characterization of organisms showed all isolates were *Bacillus* spp. and included *B. cereus* NK1, *Lysinibacillus* spp. TAI-282, *L. fusifomis*, *B. aryabhattai* PM1, *B. megaterium* and *Bacillus* spp. The results obtained were similar to those reported by Mohamed and Farag (2015) who carried out 16S rDNA gene sequencing on isolates and identified *B. fusiformis,* two species of *Lysinibacillus*, and three species *B. cereus*.

Bioremediation was carried out using the seven selected *Bacillus* spp. identified, majority from water samples for five days. There was a significant drop in the level of iron (Fe) present in the water samples.

Result of elemental and mineral analysis showed that the iron content of sample from Primary crushing area one (PC1) which was high with value of 179.738±0.091 mg/l. Nickel from Primary crushing area two has a mean value of 0.150±0.003 mg/l. Beneficiation area had the lowest initial mean values for all selected elements and minerals, calcium 6.300±0.003 mg/l, iron 0.204±0.002 mg/l, arsenic 0.007±0.000 mg/l, manganese 0.053±0.002 mg/l, magnesium 1.501±0.006 mg/l, lead 0.010±0.000 mg/l and nickel 0.014±0.007 mg/l. This result is close to a study carried out by Kakulu and Mathews-Amune (2012) where they recorded low mean values for metallic contents while working on heavy metal pollution from Itakpe mine Kogi State. The recorded mean values were 0.16±0.02 for cadmium, 0.15±0.03 for copper, 0.04±0.02 for magnesium, 0.11±0.02 for nickel, 0.07±0.01 for lead and 0.04±0.03 mg/ml for zinc, respectively.

Bacillus cereus NK1 used in bioremediation of effluents from Primary crushing area one (PC1) and Primary crushing area two (PC2) reduced the arsenic concentration from 0.060±0.001 to 0.021±0.003 mg/l and 0.107±0.001 to 0.009±0.001 mg/l, respectively. In a study carried out by Mohamed and Farag (2015) on arsenic removal from aqueous solutions using different *Bacillus* and *Lysinibacillus* spp, a reduction in arsenic concentration from 0.50 to 0.01 mg/l was reported using *B. cereus* EA5. This is similar to the report of this present study. Also, *B. megaterium* were reported to remove arsenic through adsorption (Miyatake and Hayashi, 2009).

Bacillus spp. used in bioremediation of effluent from primary crushing area two (PC2) reduced manganese from 7.139^{a}±0.001 to 0.126^{b}±0.004 mg/l. A study on isotherm equilibria of manganese biosorption in drinking water treatment by locally isolated *Bacillus* spp. and sewage activated sludge carried out by Hasan et al. (2012), produced similar result. Here, they used *Bacillus* spp. as biosorbent for manganese with initial metal ion concentration of 25 to 300 mg/l and achieved maximum biosorption capacity of 43.5 mg/g indicating the ability of

Bacillus spp. to absorb and utilize manganese.

Bacillus spp. reduced the concentration of lead present in sample from Iron ore crushing area from initial concentration of 0.487±0.002 mg/l. This result is related to a study on bioremediation of heavy metals from sewage discharge canal bank by Guo et al. (2010) where they reported reduction in lead concentration after 24 h from 825±25 to 200±80 mg/l. The investigation showed the multi-metal resistance and hormesis of endophytic bacterium (EB) L14 exhibited excellent adaptation abilities for practical *in-situ* bioremediation of heavy metals by *Bacillus* spp. L14.

Conclusion

Bioremediation of water samples from Itakpe iron mine site using *Bacillus* spp. from this environment identified through molecular analysis was effective. These organisms were two *Bacillus* spp., *L. fusifomis*, *B. aryabhattai* PM1, *Lysinibacillus* spp. TAI-282, *B. cereus* NK1 and *B. megaterium.* These indigenous microorganisms can be used to reduce heavy metals concentrations in soil and water thereby bringing about reduction of contaminants within the mining site and effluents that will be discharged into water bodies and leached into the environment.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Screening of maize germplasm for resistance to maize lethal necrosis disease in Zambia

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Maize is an important staple crop for humans and livestock feed in Zambia. A total of 473 maize germplasms were screened under maize lethal necrosis (MLN) artificial inoculation and disease pressure using an alpha lattice design to identify potential tolerant varieties as sources of resistance. The analysis of variance (ANOVA) based on the MLN score range of 1-9 showed that the levels of MLN resistance in the maize germplasms were very low, with almost all the hybrids widely grown in Zambia and germplasms drawn from the genebank found to be highly susceptible. However, the pro vitamin A varieties, GV662A and IICZ3085 showed a moderate level of resistance/tolerance, with disease scores of 4.7 and 5.3, respectively. There is need to enhance development of MLN tolerant maize cultivars by the national maize breeding programme and seed companies to avert the possible calamity posed by the threat of the effects of MLN. In addition, intensive awareness creation among various stakeholders in the maize value chain, systematic monitoring and surveillance of MLN, practicing integrated disease management approaches such as avoiding maize monoculture and continuous cultivations of susceptible maize throughout the year, and practicing maize crop rotation with compatible crops especially legumes that do not serve as hosts for maize chlorotic mottle virus (MCMV)/MLN are recommended.

Key words: Maize lethal necrosis, screening, maize germplasm, Zambia.

INTRODUCTION

Maize is an important staple crop for humans and livestock feed accounting for 60% of national calorie consumption and serves as the dietary mainstay in Zambia (Dorosh et al., 2009). The crop has a consumption level of over 130 kg per capita annually (Shiferaw et al., 2011). It is grown throughout the country of which about 80% produced by the majority small holder farmers is estimated at 2,394,907 metric tonnes and cultivated on an area of 1,086,000 ha with average yields of 2.2 t/ha (FAOSTAT, 2018). According to Kassie

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et al. (2012), most of the cultivated maize area is covered with hybrid maize (65.49%) followed by local varieties (28.5%) and improved open-pollinated varieties (6.0%). The maize seed industry in Zambia is highly developed and regarded as one of the major commercial maize-seed producer in southern Africa (Boddupalli et al., 2020).

The increasing global spread of transboundary pests and diseases poses a threat to the food security, income and livelihood of numerous resource-poor farmers particularly in southern Africa whose 85% of the maize produced is for food consumption (Boddupalli et al., 2020). The occurrence and outbreak of maize lethal necrosis (MLN), first reported in Kenya (Wangai et al., 2012) and later on in Rwanda (Adams et al., 2014), D.R. Congo (Lukanda et al., 2014), Taiwan (Deng et al., 2014), Uganda (Boddupalli et al., 2020), Ethiopia (Mahuku et al., 2015a), Ecuador (Quito-Avila et al., 2016) and Spain (Achon et al., 2017), stand as the major current risk and danger to the maize and seed production efforts in southern Africa (Boddupalli et al., 2020). The cause of MLN is as a result of the synergistic co-infection of maize by *maize chlorotic mottle virus* (MCMV; genus *Machlomovirus*, family Tombusviridae) and with any one of the viruses from the family Potyviridae, such as *sugarcane mosaic virus* (SCMV), *maize dwarf mosaic virus* (MDMV) or *wheat streak mosaic virus* (WSMV) (Uyemoto et al., 1980; Redinbaugh and Stewart, 2018). Coupled with abiotic stresses, a number of other potyviruses can similarly cause synergistic reactions in co-infections with MCMV and can aggravate MCMV infection to cause MLN (Redinbaugh and Zambrano-Mendoza, 2014). While the maize-infecting potyviruses are generally common; the outbreak of MLN in Africa has been driven by the emergence and spread of MCMV (Mahuku et al., 2015b). There is experimental evidence pointing to the possibility of thrips and other vectors to be playing a major role in MCMV movement, thus MLN, within and between fields in the affected countries in Africa (Mahuku et al., 2015b). The MCMV can also be transmitted by seed contamination, a mechanism which can contribute to speedy and long-range spreading of the MLN disease (Jensen et al., 1991; Zhang et al., 2011).

The impact of MLN on maize production in eastern Africa has been reported (De Groote et al., 2016; Marenya et al., 2018) with estimated maize yield losses ranging between 23 and 100% in Kenya (Boddupalli et al., 2020). The disease remains a key hazard to the maize crops in eastern Africa (Isabirye and Rwomushana, 2016), and therefore, poses as the biggest threat to its emergence in other sub regions especially southern Africa (Boddupalli et al., 2020). The recent survey data in the southern Africa shows the sustained absence of MCMV/MLN in Tanzania, Malawi, Zambia and Zimbabwe (Boddupalli et al., 2020). Zambia Agriculture Research Institute (ZARI) has been developing maize varieties tolerant to biotic stresses. However, breeding for MLN was not included in the breeding programme since the disease does not occur in the country. The outbreak of MLN in east and central Africa has brought up measures to curb the spread of the disease by restricting germplasm exchange, seed trade, grain export or imports (Boddupalli et al., 2020).

This study evaluated the response of maize varieties from the private and public seed institutions as well as maize accessions from the National Plant Genetic Resources Centre (NPGRC) of Zambia to MLN. The results would guide the maize breeding programs in Zambia to identify germplasm that is tolerant and/or resistant to MLN and help to prepare and respond to possible outbreak of the disease in Zambia.

MATERIALS AND METHODS

Assembly of maize varieties and genebank accessions

The establishment of the KALRO _ CIMMYT MLN Screening Facility at the KALRO-Naivasha Dairy Research Center, Kenya in September 2013 has allowed for screening of a large number of maize germplasms against MLN under artificial inoculation from different countries (Boddupalli et al., 2020). For this study, a total of 473 maize germplasms (Table 1) were collected from the major seed companies, the national maize breeding team and the NPGRC and were sent to Naivasha, Kenya for MLN field evaluation. The collected maize germplasms comprised accessions and landraces from the NPGRC, hybrids and open pollinated varieties released in Zambia commonly cultivated from seed companies (Seedco, Zamseed, Pioneer, Pannar) and ZARI.

Trial design, artificial inoculation and disease scoring

The trials were established under MLN artificial inoculation at Naivasha using an alpha lattice design. The entries were planted in two-row plots, 3 m long, with rows spaced at 0.75 m between rows. Two seeds per station were planted at 0.25 m intervals and the stands were thinned to one plant per station 3 weeks after emergence to obtain a final plant population density of 53,333 plants per hectare. All recommended agronomic management practices such as land preparation, weeding, fertilization and pest control were followed.

The maintenance of MCMV and sugarcane mosaic virus (SCMV) in susceptible host plants and preparation of MLN inoculum for artificial inoculation was done using the optimized protocols as described by Gowda et al. (2015) and Sitonik et al. (2019). The inoculum for the MLN field trial was prepared by following an optimized combination of the SCMV and MCMV viruses (ratio of 4:1). The infected leaves, verified prior by the Enzyme-Linked Immunosorbent Assay (ELISA), were weighed, chopped and homogenized in 0.1 M potassium phosphate buffer in a 1:10 dilution at pH 7.0. The inoculum was sieved through a nylon mesh paint strainer and 0.02 g/ml of Carborundum was added. The MLN inoculum was applied to the maize seedlings mechanically by using a motorized, back-pack mist blower (Solo 423 Mist Blower, 12 L capacity). An open-nozzle (2-in. diameter) was used to deliver inoculum spray at a pressure of 10 kg/cm^2. To reduce any possibility of escapes and ensure uniform inoculation, the inoculation on the maize seedlings was carried out twice at one-week intervals with the first at 4-6 leaf stage and a second 7 days after the first inoculation. Across all entries, any symptomatic plants observed before inoculation were discarded. The presence of both

Table 1. Maize germplasms used in this study

Group	Germplasm	Source	Remarks	Reference(s)
1	ZM 4623, ZM 4380, ZM 4631, ZM 5191, ZM 4429, ZM 4342, NPGRC 4267, ZM 6815, ZM 8173, ZM 5017, ZM 5698, ZM 4444, NPGRC 4311, ZM 7035, ZM 4451, ZM 4440, NPGRC 4352, ZM 4764, ZM 7353, ZM 8224, ZM 4760, ZM 4455, ZMB 8181, ZM 4618, ZM 4418, ZM 4763, ZM 4632, ZM 8231, ZM 4414, ZM 4419, ZM 4417, ZM 4748, ZM 4369, ZM 8259, ZM 5649, ZM 4753, ZM 4416, ZM 8265, NPGRC 4361, ZM 4625, ZM 4630, ZM 5173, ZM 8115, ZM 5645, ZM 8172, ZM 4249, NPGRC 4363, ZM 8235, ZM 5385, ZM 5387, ZM 4368, ZM 5190, ZM 4616, ZM 4384, ZM 4453, ZM 4629, ZM 4367, ZM 4352, ZM 5034, ZM 5196, ZM 4431, ZM 5490, ZM 4764, ZM 4610, ZM 5462, ZM 4381, ZM 8180, ZM 4773, ZM 4342, ZM 4432, ZM 8298, ZM 5210, ZM 4735, ZM 2375, ZM 4762, ZM 4307, ZM 4359, ZM 5631, ZM 4348, ZM 8211, ZM 4423, ZM 4736, ZM 4754, ZM 4347, ZM 8232, ZM 6593, ZM 4350, ZM 5189, ZM 4733, ZM 5043, ZM 8239, ZM 4613, ZM 4433, ZM 4313, ZM 4413, ZM 4772, ZM 4413, ZM 4339, ZM 4756, ZM 4415, ZM 4041, ZM 4375, ZM 4749, ZM 4266, ZM 5200, MMV 405, MMV 415, MMV 420, MMV 530, POP10, POP25, ZM421, ZM521, ZM621, OBATANPA, MMV 409	ZARI	ZARI Maize accessions and ZARI released varieties	NPGRC; Badu-Apraku et al. (2006)
2	ZM 4262, NPGRC 4309, ZM 6777, ZM 4369, NPGRC 4269, ZM 4236, ZM 4617, ZM 4373, ZM 6863, ZM 5207, ZM 5686, ZM 8208, ZM 4367, ZM 4238, ZM 4422, ZM 4456, ZM 5021, ZM 4619, ZM 4239, ZM 6593, ZM 4366, ZM 8188, ZM 8162, ZM 4261, ZM 5191, ZM 4747, ZM 5667, ZM 4442, ZM 4439, ZM 4622, ZM 4376, ZM 4327, ZM 4626, ZM 4264, ZM 4314, ZM 6967, ZM 4458, ZM 4374, ZM 5209, ZM 4430, ZM 4628, ZM 4382, ZM 4378, ZM 5020, ZM 5383, ZM 7418, ZM 4255, ZM 4612, ZM 7148, ZM 4611, ZM 4263, ZM 8252, ZM 4448, ZM 4370, ZM 5705, ZM 4240, NPGRC 4318, ZM 5207, ZM 4454, ZM 6964, ZM 4237, ZM 4627, ZM 6017, ZM 5047, ZM 4308, ZM 4347, ZM 4436, ZM 8243, ZM 4424, ZM 7010, ZM 4240, ZM 4768, ZM 4744, ZM 5195, ZM 5643, ZM 4739, ZM 4360, ZM 4447, ZM 6984, ZM 4766, ZM 4624, ZM 5666, ZM 4735, ZM 4376, ZM 6981, ZM 4428, ZM 4622, ZM 4457, ZM 5208, ZM 4450, ZM 4761, NPGRC 4356, ZM 4613, ZM 4447, ZM 4245, ZM 4315, ZM 4605	ZARI	ZARI Maize accessions	NPGRC
3	ZM 7120, ZM 7397, ZM 7285, ZM 8262, ZM 8238, ZM 4256, ZM 4335, ZM 8236, ZM 3641, ZM 7396, ZM 7435, ZM 5196, ZM 4253, ZM 6653, ZM 7476, ZM 8219, ZM 4329, ZM 7456, ZM 8213, ZM 8254, ZM 4325, ZM 5197, ZM 5039, ZM 7285, ZM 4254, ZM 7235, ZM 4268, ZM 8214, ZM 4259, ZM 6860, ZM 4383, ZM 6656, ZM 8253, ZM 8226, ZM 7380, ZM 7373, ZM 4242, ZM 8215, ZM 8256, ZM 8183, ZM 7267, ZM 4358, ZM 4233, ZM 8195, ZM 8190, ZM 6639, ZM 7324, ZM 5194, ZM 4757, ZM 4385, ZM 8219, ZM 4758, ZM 7355, ZM 4234, ZM 4745, ZM 4232, ZM 7374, ZM 8171, ZM 8184, ZM 4750, ZM 8207, ZM 6706, ZM 7145, ZM 4353, ZM 5708, ZM 4622, ZM 8201, ZM 4425, ZM 6594, ZM 7146, ZM 4771, ZM 5680, ZM 4321, ZM 4257, ZM 6846, ZM 4609, NPGRC 4260, ZM 4420, ZM 4434, ZM 7019, ZM 8230, ZM 4365, ZM 6785, ZM 4614, ZM 4615, ZM 4235, ZM 5675, ZM 7275, ZM 4737, ZM 4606, ZM 4344, ZM 4438, ZM 8163, ZM 4334, ZM 5697, ZM 5710, ZM 4765, ZM 6957, ZM 4452	ZARI	ZARI Maize accessions	NPGRC
4	ZM 8248, ZM 7236, ZM 4426, ZM 4755, ZM 8185, ZM 4748, ZM 7421, ZM 4742, ZM 8187, ZM 4246, ZM 4752, ZM 7002, ZM 7441, ZM 7422, ZM 7120, ZM 5214, ZM 7153, ZM 7141, ZM 8228, ZM 8192, ZM 7151, ZM 8251, ZM 7348, ZM 5215, ZM 4437, ZM 7336, ZM 7147, ZM 5205, ZM 7237, ZM 8157, ZM 5213, ZM 7007, ZM 7254, ZM 6639, ZM 8225, ZM 4250, ZM 5045, ZM 5216, ZM 7038, ZM 6815, ZM 7171, ZM 7446, ZM 4768, ZM 4429, ZM 6968, ZM 7233, ZM 8237, ZM 8157, ZM4756, ZM 7142, ZM 7305, ZM 5642, ZM 4445, ZM 5984, ZM 7318, ZM 6965, ZM 4607, ZM 4741, ZM 7305, ZM 6843, ZM 8197, ZM 7427, ZM 8260, ZM 7263, ZM 8244, ZM 7246, ZM 4421, ZM 4323, ZM 6843, ZM 7315, ZM 6790, ZM 4746, ZM 6623, ZM 6611, ZM 5194, ZM 6846, ZM 4745, ZM 8223, ZM 5203, ZM 6866, ZM 4771, ZM 5210, ZM 4734, ZM 8196, ZM 5210, ZM 7143, ZM 4335, ZM 8174, ZM 7433, ZM 7012, ZM 5198, ZM 5042, ZM 6628, ZM 8165, ZM 7354, ZM 4251, ZM 7114, ZM 8220	ZARI	ZARI Maize accession	NPGRC
5	MM 441, ZMS 405, ZMS 528, ZMS 606, ZMS 616, ZMS 620, ZMS 623, ZMS 638, ZMS 652, ZMS 720, ZMS 721, ZMS 722, ZMS 607Y, ACCROSS 917, DKC 8053, DKC 8033, DKC 9053, DKC 8031, DKC 9089, DKC 8073, P2859W, PHB 30G-19, P3812W, PHB 3253, PAN 8M-93 (LOT #: 1412-ZEM-27366) - R2, PAN 12 (LOT #: N/174504/DAD) - F3, PAN 8M-91 (LOT #: 14911-ZEM-20204) - R3, PAN 413 (LOT #: M/199904/JAD) - F3, PAN 6777 (LOT #: 14911-ZEM-20297) - F3, PAN 4M-21 (LOT #: 13911-ZEM-20876B) - F3, PAN 7M-83 (LOT #: N/198504/IAD) - R4, PAN 4M-19 (LOT #: N/177304/EAD) - F3, PAN 6227 (LOT #: K/575401/DAA) - F3-60K, PAN 14 (LOT #: M/144903/FAC) - F3, PAN 53 (LOT #: 14911-ZEM-20266) - R2, PAN 7M-81 (LOT #: N/194404/HAD) - R2-50K, PAN 69 (LOT #: K/260901/IAA) - SFS, 14CZ405, SC 647, SC 637, SC 627, SC 719, 14CZ404, SC 727, 11CZ3087, 11CZ3085, MRI 734, MRI 514, MRI 614, MRI 624, MRI 744, MRI 594, MRI 634, MRI 724, ZM 421, ZM 521, ZM 621, OBATANPA, POP10, POP25, GV 662A, GV 664A, GV 665A	ZAMSEED, Monsanto, MRI-Syngenta, Pannar Seed, Pioneer, ZARI	Popular maize hybrids in Zambia, ZARI Maize varieties	Badu-Apraku et al. (2006), Masole and Gumbo (1994), Howard and Mungoma (1996), Smale et al. (2013), and Mubanga et al. (2014; 2018)

NPGRC-National Plant Genetic Resources Centre (http://www.fao.org/pgrfa-gpa-archive/zmb/nfp.html)/Zambia; ZARI-Zambia Agricultural Research Institute; MRI-Syngenta Maize Research Institute-Syngenta seed company.

Table 2. Analysis of variance and F-statistic tests for stand count (SC) and 10-days interval four MLN severity scores when assessing 473 maize germplasms.

Source of variation	Df	Measured parameters					
		SC	MLN 1	MLN 2	MLN 3	MLN 4	AUDPC
Germplasm	435	2289.7	74.4	41.4	92.4	90.8	34859.1
Residual	37	131.5	6.8	2.8	3.2	2.6	1492.2
Total	472	2421.2	81.2	44.2	95.7	93.4	36351.3
Mean		12.4	5.5	6.1	6.6	7.5	191.6
CV %		15.2	7.8	4.5	4.5	3.5	3.3
P		0.072	0.6	0.2	0.001	0.001	0.006
Significance		ns	ns	ns	***	***	***

ns = non-significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$, Df = degrees of freedom, SC = stand count; MLN 1 to MLN 4 denote Maize Lethal Necrosis severity score (1-9) recorded at 10, 20, 30 and 40 days after the second inoculation; AUDPC = Area under disease progress curve; CV = Coefficient of variation; *p* = F probability test.

viruses (MCMV and SCMV) in the field trial was confirmed by ELISA. The MLN disease severity (DS) was visually scored on each plot in an ordinal scale of 1 (completely clean plants with no visible MLN disease symptoms); 2 (fine or no chlorotic specks, but no loss of plant vigor); 3 (mild chlorotic streaks on emerging leaves); 4 (moderate chlorotic streaks on emerging leaves); 5 (chlorotic streaks and mottling throughout the plant); 6 (intense chlorotic mottling throughout the plant, with necrosis of leaf margins); 7 (severe chlorotic mottling, mosaic, and leaf necrosis all through the plant); 8 (severe chlorotic mottling, leaf necrosis, dead heart and premature death of plants) and 9 (complete plant necrosis, and dead plants) (Boddupalli et al., 2020).

In order to ensure that MLN was determined at the low, medium and high levels in all germplasm, data were recorded at 10-day intervals, beginning from 14 days after the second inoculation for up to four observations. For the DS analyses, after analyzing each time score, a third score was used (40 days post-inoculation) which also had high heritability compared to other scores. The analysis of variance for each trial was performed using Genstat for Windows 20th Edition, VSN International (2019). The area under the disease progress curve (AUDPC) was calculated for each plot to provide a measure of the progression of MLN severity across time using the method described by Jones et al. (2007).

RESULTS

Analysis of variance (ANOVA) for stand count, maize lethal necrosis severity and area under disease progress curve (AUDPC)

The average plant stand count was 12.4 with the lowest and highest being 1 and 13, respectively. The analysis of variance for the four MLN score-sets showed that the germplasms were only significantly different at MLN 3 and MLN 4 (Table 2). Highly significant differences ($p < 0.001$) were also obtained for the AUDPC scores of the germplasms. Germplasms mean of Turkey's multiple comparison range tests at significant levels of 5% denoted by letters A to M showed the lowest and highest MLN scores of 4.7 and 7.5, being GV 662A and ZM 4384, respectively (Table 3). Typical leaf MLN leaf symptoms of infected plants observed in the evaluations are as shown in Figure 1A to D.

Area under disease progress curve (AUDPC) for the MLN

The mean AUDPC values of the germplasms showed progressive increase to MLN infection with time (Figure 2). The final MLN infection and the AUDPC values were indicative of the variable reaction of the test germplasm to MLN.

DISCUSSION

This study evaluated 473 diverse maize germplasms in order to explore their genetic variation as sources of resistance, generate information on their response to MLN and as a rapid response to the serious threat to the food security, income and livelihoods of many smallholder farmers and their families in Zambia. The validity of the study, undertaken under artificial inoculation at Naivasha, is shown by the efficiency of MLN phenotyping under artificial inoculation supported by the heritability values across various trials undertaken at Naivasha, ranging from 0.71 to 0.95 (Boddupalli et al., 2020).

The levels of MLN resistance in the maize germplasms evaluated in this study were very low, with almost all the maize hybrids widely grown in Zambia and accessions held in the national genebank, found to be highly susceptible. In studies consisting of nearly 200,000 germplasms and development of MLN-tolerant/resistant hybrids undertaken by CIMMYT at the MLN screening facility in Naivasha under artificial inoculation, high levels of susceptibility to MLN were shown (Boddupalli et al., 2020; Prasanna, 2015; Semagn et al., 2015). A list of CIMMYT maize inbred lines and pre-commercial hybrids with potential resistance to MLN disease can be obtained

Table 3. Germplasms mean (Top 15 and Bottom 15) of Turkey's multiple comparison range tests at significant levels of 5% denoted by letters A to M. Means with the same letter are not significantly different at 5% level.

Germplasm (Top 15)	MLN score	Germplasm (Bottom 15)	MLN score
GV 662A	4.7^{a}	ZM 4307	$7.0^{cdefghijklm}$
11CZ3085	5.3^{ab}	ZM 6981	$7.0^{cdefghijklm}$
PAN 4M-19 (LOT #: N/177304/EAD) - F3	5.5^{abc}	ZM 5643	$7.0^{cdefghijklm}$
ZM 621	5.6^{abcd}	ZM 7354	$7.0^{cdefghijklm}$
ZMS 722	5.6^{abcd}	NPGRC 4356	$7.0^{cdefghijklm}$
DKC 9053	5.7^{abcde}	ZM 7348	$7.0^{cdefghijklm}$
SC 719	5.7^{abcde}	ZM 5189	$7.0^{cdefghijklm}$
11CZ3087	5.7^{abcde}	ZM 4631	$7.1^{cdefghijklm}$
NPGRC 4309	5.8^{abcdef}	ZM 8225	$7.1^{cdefghijklm}$
MRI 514	$5.9^{abcdefg}$	ZM 4424	$7.1^{cdefghijklm}$
MRI 744	$5.9^{abcdefg}$	ZM 4455	$7.2^{defghijklm}$
DKC 8031	$5.9^{abcdefg}$	ZM 4348	$7.3^{efghijklm}$
DKC 8033	$5.9^{abcdefg}$	ZM 4373	7.4^{fgiklm}
DKC 8073	$5.9^{abcdefg}$	ZM 4628	7.5^{giklm}
ZM 8226	$5.9^{abcdefg}$	ZM 4384	7.5^{ikm}

Figure 1. Variable MLND reaction types of maize germplasm at mid-whorl growth stage observed during evaluation; fine chlorotic streaks and mottling throughout plant (A); excessive chlorotic mottling and leaf necrosis (B); near complete plant necrosis (C) and field level infection (D).

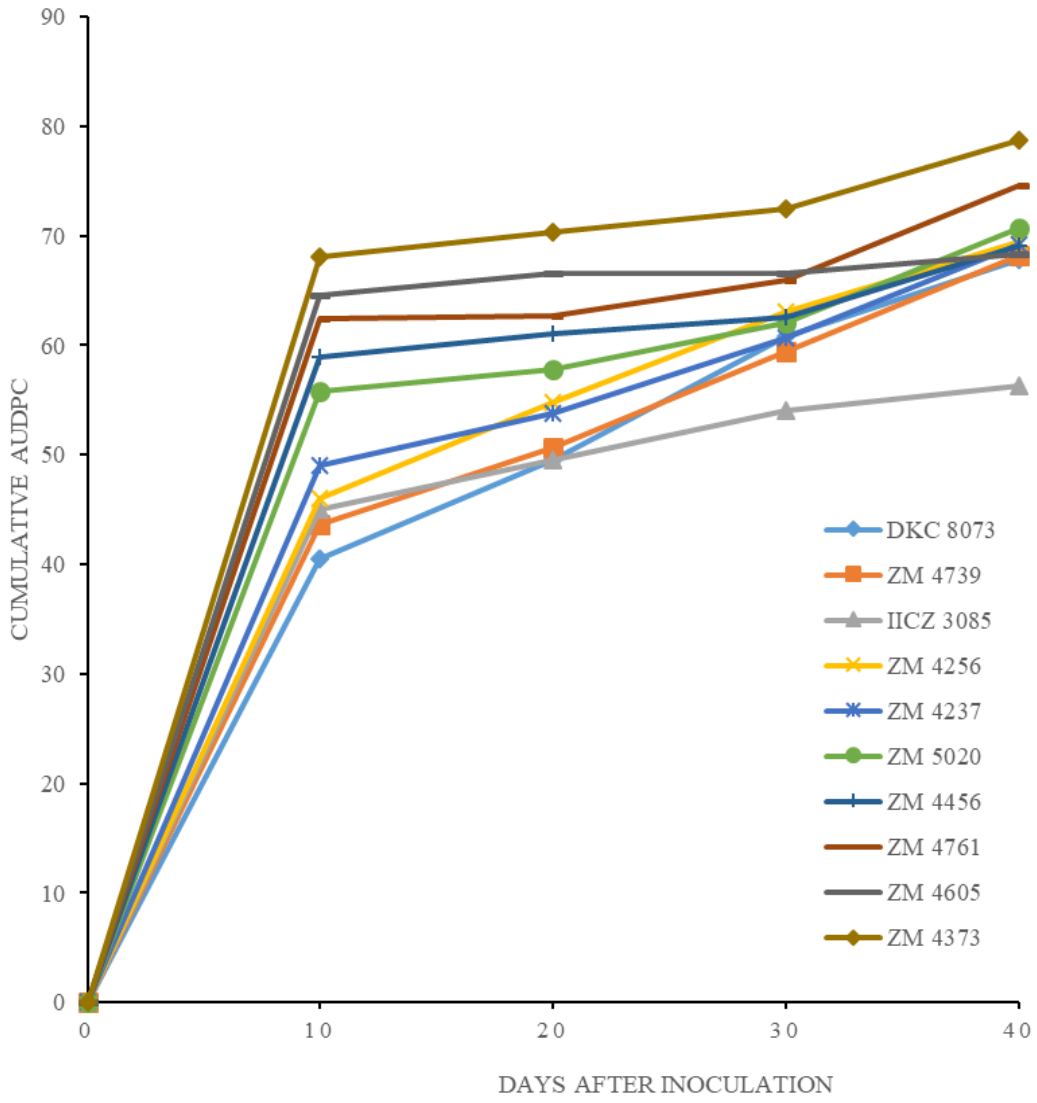

Figure 2. The cumulative AUDPC of MLN on selected top and bottom five germplasm inoculated at 10 day intervals.

at http://www.cimmyt.org. Our study had only two maize germplasms, GV 662A and IICZ 3085 showing a moderate level of resistance, with disease scores of 4.7 and 5.3, respectively. According to Boddupalli et al. (2020), for germplasm classification, mean MLN severity scores between 7 and 9 are considered “susceptible”, scores of 5 or 6 as “tolerant”, and scores between 1 and 4 as “resistant”. The current objective of the MLN conversion pipeline is to deliver improved versions of the elite African lines having 0.6 to 1.0 point lower MLN severity score (Semagn et al., 2015). Several attempts have been made to introgress MLN resistance into adapted germplasm, using conventional backcrossing and marker-assisted backcrossing (MABC) leading to a number of first- and second-generation CIMMYT-derived MLN-tolerant hybrids being released on the market in East Africa (Boddupalli et al., 2020).

In the recent regional MLN surveillance using standardized protocols, current survey data indicates the continued absence of MCMV/MLN in the southern highlands of Tanzania, Malawi, Zambia and Zimbabwe (Boddupalli et al., 2020; https://mln.cimmyt.org/). This study provides the first documented experimental evidence undertaken to identify sources of tolerance/ resistance to MLN in elite maize germplasms in Zambia and provides information on their status. The development of virus-resistant crops has been shown as an economically viable and environmentally sustainable approach for disease control which requires identification and evaluation of resistant plants and incorporation of favorable alleles into agronomically desirable genetic backgrounds (Semagn et al., 2015).

The on-going strategy for ZARI and other partners will be to continue re-confirming the potential resistance of pre-commercial hybrids and inbred lines that may have the least susceptibility to MLN and work urgently to develop resistant varieties. There is need to look for donor lines that are very tolerant to MLN as well as resistant varieties from CIMMYT and evaluate them for adaptation and release as a short-term measure while long term breeding program incorporating introgressions be put in place. Both the public and private sectors should intensify breeding for tolerance to MLN.

The study has shown that the commonly cultivated varieties in Zambia are susceptible to MLN with exception of GV 662A and IICZ 3085 which were moderately resistant. There is need to enhance the development of MLN tolerant maize cultivars by the national maize breeding programme and seed companies to avert the possible calamity posed by the threat of the effects of MLN. Furthermore, there is need for more intensive awareness creation among the stakeholders in maize value-chain, systematic monitoring and surveillance of MLN, practicing integrated disease management approaches such as avoiding maize monoculture and continuous cultivations of maize throughout the year and practicing maize crop rotation with compatible crops especially legumes that do not serve as hosts for MCMV/MLN.

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CONFLICT OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Use of mineral salts to remove recalcitrance to somatic embryogenesis of improved genotypes of cacao (*Theobrama cacao* L.)

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Some improved genotypes of cocoa tree (*Theobroma cacao* L.), have shown in standard study conditions of absence or a very low response to the induction of somatic embryos. This is the case of the recalcitrant genotypes C8 and C15, partially recalcitrant genotypes C14 and C16. This study aims to improve the production of somatic embryos of recalcitrant genotypes. Staminodes and petals excised from the immature buds of the five genotypes C1, C8, C14, C15 and C16 were used as plant material. These floral explants were cultured on different media containing two type of mineral salts such as potassium sulphate (K_2SO_4) at concentrations 18, 27, 36 and 45 mM and magnesium sulphate ($MgSO_4$) whose concentrations used are 5, 10, 15 and 20 mM. Calli induction was obtained in the five (05) genotypes at percentages ranging from 50 to 100% with staminodes and from 61 to 100% with petals on all media. The transfer of the callogenic explants on the developmental medium allowed the induction of embryogenic calli and somatic embryos after 84 days only with petal explants. The most important PEC and NSE were obtained with two mineral salts concentrations, 27 mM K_2SO_4 and 15 mM $MgSO_4$ for all genotypes and varied to 20 to 40%. Potassium sulphate (K2SO4) at 27 and magnesium sulphate ($MgSO_4$) at 15 mM are the concentrations of the most suitable mineral salts to overcome recalcitrance of cocoa genotypes.

Key words: Recalcitrant, *Theobrama cacao*, improved genotypes, mineral salts, somatic embryogenesis.

INTRODUCTION

The cocoa tree (*Theobroma cacao* L.), a species of the humid tropics, is mainly grown for its beans, which produce the cocoa powder used to make chocolate (Kone et al., 2019).

Global demand for cocoa continues to increase due to economic growth and public awareness of the benefits for

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chocolate consumption.

The work done on the cocoa bean has shown that it has virtues on the stimulation of the immune system, the improvement of digestion and cellular detoxification. Cocoa products also have antimicrobial and antioxidant properties beneficial to human health (Sarmadi et al., 2012; Yapo et al., 2013).

Cocoa production is important for many cocoa-producing countries in order to maintain export stability and ensure the continuity of supply of industrial raw materials for the chocolate industries. Cocoa plays an important economic role as a source of foreign exchange for many tropical countries, including Côte d'Ivoire. It is grown by about 6 million farmers globally, and livelihoods of more than 40 million people depend on cocoa (ICCO, 2012; Conseil Café-Cacao, 2014; Beg et al., 2017).

However, demand remains unfulfilled despite the fact that its world production has been increasing considerably for several years (ICCO, 2016). This is due to the fact that in the agricultural sector, cocoa farming is affected by several pathogens, to which is added the aging of orchards, high cost of inputs, use of poor quality plant material, inappropriate agronomic techniques. To overcome these constraints and increase production, elite genotypes, high producers have been created for extension to allow the creation and renewal of plantations (Sonwa, 2002). However, the mode of reproduction of the plant, sexed and allogane, does not make it possible to obtain a homogeneity of the improved material. Since cocoa is naturally pollinated, seed cocoa planting material generally has a very heterogeneous genetic background. As a result, the agronomic performance of seed-derived cocoa plantations is highly variable (Li et al., 1998). To overcome these difficulties, traditional vegetative propagation techniques of plant material, such as cuttings and grafting, have been advised.

These techniques are not very competent (Figueira and Janick, 1993) because of the poor root and air systems of plants associated with slow processes. One of the appropriate solutions to all these problems is somatic embryogenesis. This technique is used to carry out the propagation of desirable cocoa genotypes. However, this method presents some difficulties in the cocoa tree because of the recalcitrance of certain genotypes. This recalcitrance is expressed by the variation of the somatic embryo rate from one genotype to another, often with very low or zero somatic embryo levels. The lifting of this recalcitrance requires the development of a new protocol. Moreover, several studies on the somatic embryogenesis of cacao have revealed a variation of callogenic and embryogenic responses according to genotypes. The work on somatic embryogenesis developed by Li et al. (1998), without being exhaustive, have shown a variation of the responses of 19 cocoa genotypes to somatic embryogenesis. To be used as explants, the response of staminodes to callogenesis ranged from 1 to 100% and the number of somatic embryos per explant ranged from 1 to 46 in this work.

Another somatic embryogenesis protocol was developed by López-Báez et al. (2001) using an induction medium comprising the macro and microelements of Murashige and Skoog (1962) (MS) supplemented with different types and concentrations of phytohormones (2,4-dichlorophenoxyacetic acid or 2,4-acid, 5-trichlorophenoxyacetic, and kinetin), out of 12 different genotypes. Once again, a variation of the responses according to the genotypes was found.

The elite genotypes such as C1, C8, C14, C15 and C16 are characterized by good genotypic performance (high productivity) and resistance to diseases. However, low levels of embryogenic calli genotypes C8 (0.03%), C14 (14.32%) (Kouassi et al., 2018) and C16 (8.85%) (Kouassi et al., 2017b) were obtained with somatic embryogenesis. Therefore, it is highly important to develop a protocol allowing maximum high embryo production even in recalcitrant genotypes. The objective of the present study is to improve the production of somatic embryos in the recalcitrant genotypes used for cocoa farming. To achieve this goal, two types of explants (petals and staminodes) will be tested on different media with different concentration of mineral salts.

MATERIALS AND METHODS

Plant

The plant material consisted of staminodes and petals excised from immature flower buds of the cocoa genotypes coded C1, C8, C14, C15 and C16. The flower buds were taken from the field of cocoa experimentation of the International Center for Agroforestry Research (ICAFR) of Abidjan (Côte d'Ivoire). Five cocoa genotypes were chosen according to their response to somatic embryogenesis. The genotype coded C8 originated from Trinidad and Tobago is recalcitrant to somatic embryogenesis. Genotypes coded C15, recalcitrant, C14 and C16, partially recalcitrant and C1 which is embryogenic, are from Côte d'Ivoire.

Collection and disinfection of flower buds

In the morning before 9 am, 4 to 5 mm long flower buds were collected and placed in jars and stored in a cooler containing ice and sent to the laboratory. Then, buds were disinfected under a laminar flow hood in sterile conditions, first by soaking them in a 1% (w/v) calcium hypochlorite solution, followed by three rinses in sterile distilled water. After that, they were re-dipped in 70% alcohol solution for 30 s and rinsed thoroughly three times with sterile distilled water. Finally, they were immersed a second time in the same solution of calcium hypochlorite 1% (m/v) with three drops of Tween 20 for 10 min and then rinsed thoroughly three times with sterile distilled water.

Isolation and culture of explants

Petals and staminodes were isolated from disinfected flower buds after dissection with a scalpel blade. Petals and staminodes were placed on the calli induction medium with 15 samples per explant

(15 petals and 15 staminodes) and per Petri dish under a laminar flow hood for sterile conditions.

Composition of culture media

The media used callus induction medium (I) and their development into somatic embryos (ED medium) are composed of macro and micro elements of DKW (Driver and Kuniyuki, 1984). For testing their effect on calli induction and their ability to induce embryogenic calli, two mineral salts: potassium sulphate (K_2SO_4) and magnesium sulphate ($MgSO_4$) were added to DKW medium at different concentrations 18, 27, 36 and 45 for potassium sulphate (K_2SO_4) and 5, 10, 15 and 20 mM for magnesium sulphate ($MgSO_4$). Thus a total of eight induction media varying by the nature and concentration of mineral salts were prepared. Cytokinin used was kinetin at 1.162 µM. The control medium contained 9 mM potassium sulphate (K_2SO_4) and 3 mM magnesium sulphate ($MgSO_4$). Four (4) weeks after culturing in calli induction medium, explants were transferred to embryo development medium (ED medium) described by Li et al. (1998). The transfer of the explants on this medium was done every 28 days.

Culture conditions

The pH of the media was adjusted to 5.8 for callus induction medium (I) and 5.7 for embryo development medium (EDM) using 1 N NaOH or HCl solutions. Media were solidified with 2 g/L phytagel before being sterilized by autoclaving for 20 min at 121°C and 1 bar. After sterilization, these culture media were dispensed due to 15 ml in sterile Petri dish of 90 mm diameter under a laminar flow hood. Cultures were incubated on continuous darkness conditions in the culture chamber at 24 ± 1°C with a relative humidity of 70%. Petri dishes were arranged on rows according to a completely randomized experimental.

Variables used to assess the success of somatic embryogenesis

Twenty eight (28) days after induction, percentage of callogenic explants (PCE) was evaluated. Eighty four (84) days after induction, percentage of embryogenic calli (PEC) and average number of somatic embryos (NSE) were evaluated on ED medium. These three parameters were calculated according to the following formulas:

(1) induction evaluation was made on medium induction of calli (IC), after 28 days of culture;
(2) somatic embryos were assessed on ED medium by the percentage of embryogenic calli 84 days after explants induction.

The percentage of callogenic explants (PCE), the percentage of embryogenic calli (PEC) and the mean number of somatic embryos (NSE) per explant were given respectively by the following formulas:

$$PCE = \frac{\text{Number of explants that induced calli}}{\text{Total number of explants cultured}} \times 100$$

$$PEC = \frac{\text{Number of calli that induced embryos}}{\text{Number of explants that induced callis}} \times 100$$

$$NSE = \frac{\text{Number of induced embryos}}{\text{Number of calli that induced embryos}}$$

Statistical analysis of the data

Results were subjected to analysis of variance (ANOVA) with Statistica 7.1 software. For unequal numbers, analysis of variance across the generalized linear model (GLM) was adopted. When a significant difference was observed between averages, the Newman-Keuls multi-range test at a 5% threshold was used to separate the averages.

RESULTS

Evaluation of calli induction in the various media after 28 days

Table 1 shows the percentage of calli induction from staminode and petal explants after 28 days of culture on the various induction media.

The analysis of the results of this table after the statistical analyses shows that with the C1 and C14 genotypes, there is no significant difference between the calli induction percentages from the staminode and petal explants on the medium supplemented with different types and concentrations of mineral salts. These calli induction percentages were between 90 and 100% for C1 and between 92 and 100% for C14. Moreover, these percentages are identical statistically to that of the control medium. The contribution of mineral salts did not improve or reduce the responses to the callogenesis of genotypes C1 and C14.

With the C8, C15 and C16 genotypes, mineral salts improved the response to callogenesis. A beneficial effect of using potassium sulfate mineral salts and magnesium sulfate was clearly observed on the induction of calli for genotype C15.

However, the addition of $MgSO_4$ mineral salt at a concentration of 5 mM resulted in a reduction in calli induction percentages of genotypes C8 (staminodes 75.08% ± 0.17 and petals 84%) and C16 (the staminodes 74.33% ± 1.01). For all genotypes, calli percentages ranged from 74 to 100% with staminodes and from 92 to 100% with petals. The petals yielded calli at slightly higher percentages than staminodes in all genotypes (Figure 1).

Evaluation of calli and somatic embryos in the various media after 84 days

The calli obtained, transferred to the embryonic development medium (EDM medium), gave embryos 84 days after the initiation of the cultures. The percentage of embryogenic calli (PEC) and the average number of embryos (NSE) produced per explant are shown in

Table 1. Percentage of calli produced from staminode and petal explants as a function of mineral salt concentrations and genotype used.

Genotype	Mineral salt	Concentration (mM)	Percentage of callogenic explant (PCE) (%)	
			Staminodes	Petals
C1	Control (K_2SO_4 + $MgSO_4$)	(9 +3)	90.80 ± 0.01^{ab}	93.34 ± 0.06^{ab}
	K_2SO_4	18	93.76 ± 0.02^{ab}	94.43 ± 0.08^{ab}
		27	100^{a}	100^{a}
		36	98.10 ± 02.00^{a}	100^{a}
		45	94.94 ± 0.37^{ab}	94.02 ± 0.07^{ab}
	$MgSO_4$	5	92.00 ± 0.00^{ab}	93.80 ± 0.08^{b}
		10	93.64 ± 0.46^{ab}	93.00 ± 0.00^{ab}
		15	100^{a}	100^{a}
		20	94.00 ± 0.00^{ab}	95.00 ± 0.00^{ab}
C8	Control (K_2SO_4 + $MgSO_4$)	(9 +3)	93.42 ± 4029^{ab}	94.29 ± 01.23^{ab}
	K_2SO_4	18	94.09 ± 1.22^{ab}	93.29 ± 01.03^{ab}
		27	100^{a}	100^{a}
		36	100^{a}	100^{a}
		45	94.87 ± 0.72^{ab}	94.87 ± 0.72^{ab}
	$MgSO_4$	5	75.08 ± 0.17^{c}	84.00 ± 0.00^{b}
		10	92.66 ± 1.02^{ab}	94.66 ± 0.00^{ab}
		15	100^{a}	100 ^{a}
		20	100^{a}	100 ^{a}
C14	Control (K_2SO_4 + $MgSO_4$)	(9 +3)	92.33 ± 0.04^{ab}	92.33 ± 0.04^{ab}
	K_2SO_4	18	92.00 ± 0.00^{ab}	94.55 ± 0.04^{ab}
		27	100^{a}	100^{a}
		36	93.40 ± 0.10^{ab}	100^{a}
		45	93.39 ± 0.08^{ab}	94.85 ± 0.02^{ab}
	$MgSO_4$	5	93.88 ± 0.11^{b}	92.00 ± 0.00^{ab}
		10	92.21 ± 0.87^{ab}	94.00 ± 0.00^{ab}
		15	100^{a}	100 ^{a}
		20	94.00 ± 0.00^{ab}	94.55 ± 0.04^{ab}
C15	Control (K_2SO_4 + $MgSO_4$)	(9 +3)	94.64 ± 0.06^{ab}	94.57 ± 0.02^{ab}
	K_2SO_4	18	93.16 ± 0.68^{ab}	94.68 ± 0.20^{ab}
		27	100^{a}	100^{a}
		36	100^{a}	100^{a}
		45	94.00 ± 00.00^{ab}	100^{a}
	$MgSO_4$	5	92.00 ± 00.00^{ab}	94.95 ± 0.03^{ab}
		10	93.95 ± 0.09^{ab}	94.00 ± 00.00^{ab}
		15	100^{a}	100^{a}
		20	100^{a}	100^{a}
	Control (K_2SO_4 + $MgSO_4$)	(9 +3)	75.38 ± 20.02^{b}	100^{a}

Table 1. Contd.

C16	K_2SO_4	18	90.88 ± 21.80^{ab}	100^{a}
		27	100^{a}	100^{a}
		36	100^{a}	100^{a}
		45	100^{a}	100^{a}
	$MgSO_4$	5	74.33 ± 1.01^{b}	92.44 ± 1.92^{ab}
		10	100^{a}	100^{a}
		15	100^{a}	100^{a}
		20	100^{a}	100^{a}
Statistical tests		P	<0.001	<0.001
		F	30.31	29.68

In the same column, the averages followed by the same letter are statistically equal (test of Newman-Keuls to the threshold of 5%).

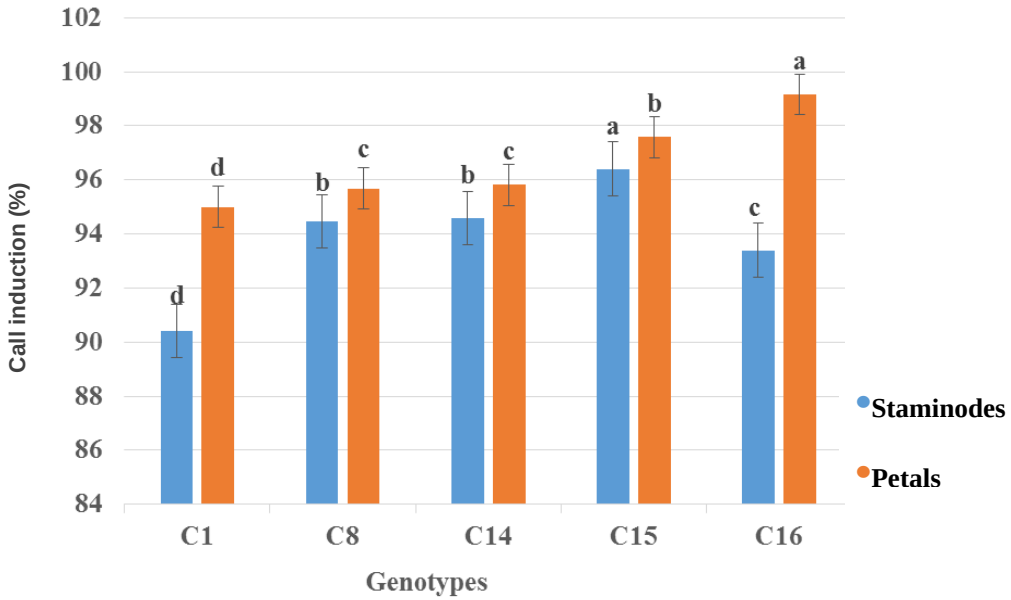

Figure 1. Callogenic potential of genotypes C1, C8, C14 C15 and C16 with staminodes and petals explants on the calli induction medium.

Table 2. Only the petal explants induced somatic embryos. The percentage of embryogenic calli (PEC) of the different genotypes ranged from 0 to 42.63% ± 0.19 and the average number of embryos (NSE) from 0 to 23.53 ± 0.09 with all of the salts minerals. No embryogenic or embryo calli was induced on the media containing 5 and 20 mM $MgSO_4$ and 45 mM K_2SO_4 for the genotype C8 and on media containing 5 mM $MgSO_4$ and 45 mM K_2SO_4 for the genotypes C14, C15 and C16. Only the genotype C1 produced embryogenic calli and embryos on all media used.

However, the percentage of embryogenic calli (PEC) and the average number of embryos (NSE), were improved in the presence of 27 mM K_2SO_4 (41.53% ± 2.59 and 21.76 ± 2.50), 10 mM $MgSO_4$ (39.97% ± 0.40 and 19.87 ± 0.04), 15 mM $MgSO_4$ (42.63% ± 0.19 and 23.53 ± 0.09) and 20 mM $MgSO_4$ (40, 03% ± 0.09 and 20.53 ± 1.04) for the genotype C1.

For the genotype C8, the highest PEC and NSE were produced on media enriched with 27 mM K_2SO_4 (35.50% ± 1.09 and 19.10 ± 1.89) and 15 mM $MgSO_4$ (36.63% ± 0.59 and 19.15 ± 0.89).

For the C14 genotype, high PEC and NSE are obtained with media containing 27 mM K_2SO_4 (39.03% ± 1.59 and 19.53 ± 0.39) and 10 mM $MgSO_4$ (39.78% ± 0.64 and 19.67 ± 1.63), 15 mM $MgSO_4$ (40.68% ± 0.93 and 20.08

Table 2. Somatic embryos induction as a function of mineral salt concentrations and genotypes used.

Genotype	Mineral salt	Concentration (mM)	Somatic embryo induction by petals explants	
			Induction rate embryogenic calli	Mean number of somatic embryos
C1	Control (K_2SO_4 + $MgSO_4$)	(9+3)	30.90 ± 0.40^{ab}	15.46 ± 0.40^{ab}
	K_2SO_4	18	32.97 ± 0.44^{ab}	16.49 ± 0.44^{ab}
		27	41.53 ± 0.59^{a}	21.76 ± 0.50^{a}
		36	30.68 ± 0.64^{ab}	15.35 ± 0.61^{ab}
		45	18.39 ± 0.14^{bc}	9.90 ±1.01^{bc}
	$MgSO_4$	5	25.00 ± 00.00^{b}	12.40 ± 0.22^{b}
		10	39.97 ± 0.40^{a}	19.87 ± 0.04^{a}
		15	42.63 ± 0.19^{a}	23.53 ± 0.09^{a}
		20	40.03 ± 0.09^{a}	20.53 ± 1.04^{a}
C8	Control	(9 + 3)	10.97 ± 0.04^{bc}	5.22 ± 0.22^{bc}
	K_2SO_4	18	08.07 ± 0.14^{bc}	4.22 ± 0.52^{bc}
		27	35.50 ± 1.09^{a}	19.10 ± 1.89^{a}
		36	12.00 ± 0.00^{bc}	6.00 ± 0.00^{bc}
		45	00.00 ± 00.00^{c}	00.00 ± 00.00^{c}
	$MgSO_4$	5	00.00 ± 00.00^{c}	00.00 ± 00.00^{c}
		10	20.00 ± 00.00^{b}	11.40 ± 0.22^{b}
		15	36.63 ± 0.59^{a}	19.15 ± 0.89^{a}
		20	00.00 ± 00.00^{c}	00.00 ± 00.00^{c}
C14	Control	(9 + 3)	30.05 ± 0.02^{ab}	15.01 ± 0.37^{ab}
	K_2SO_4	18	32.00 ± 0.00^{ab}	15.68 ± 1.20^{ab}
		27	39.03 ± 1.59^{a}	19.53 ± 0.39^{a}
		36	30.58 ± 1.64^{ab}	14.97 ± 3.64^{ab}
		45	00.00 ± 00.00^{c}	00.00±00.00^{c}
	$MgSO_4$	5	14.97 ± 0.14^{bc}	7.22 ± 0.20^{bc}
		10	40.68 ± 0.93^{a}	20.08 ± 0.14^{a}
		15	39.78 ± 0.64^{a}	19.67 ± 1.63^{a}
		20	39.83 ± 1.02^{a}	19.87 ± 0.19^{a}
C15	Control (K_2SO_4 + $MgSO_4$)	(9 + 3)	31.97 ± 0.44^{ab}	15.27 ± 0.04^{ab}
	K_2SO_4	18	30.73±0.03^{ab}	14.90±0.16^{ab}
		27	40.50±0.04^{a}	20.70±0.21^{a}
		36	32.88±0.05^{ab}	14.68 ± 3.64^{ab}
		45	00.00±00.00^{c}	00.00±00.00^{c}
	$MgSO_4$	5	00.00±00.00^{c}	00.00±00.00^{c}
		10	39.01±0.04^{a}	19.98±0.08^{a}
		15	40.81±0.03^{a}	20.10±0.15^{a}
		20	18.48±0.05^{bc}	09.50±0.40^{bc}
	Control (K_2SO_4 + $MgSO_4$)	9 + 3	30.07±1.44^{ab}	14.07 ± 1.04^{ab}

Table 2. Contd.

C16	K_2SO4	18	32.73±0.03[ab]	15.80±0.16[ab]
		27	39.50±0.04[a]	19.96±0.21[a]
		36	33.01±0.07[ab]	15.28±0.08[ab]
		45	00.00±00.00[c]	00.00±00.00[c]
	$MgSO_4$	5	00.00±00.00[c]	00.00±00.00[c]
		10	39.18±0.05[a]	19.80±0.45[a]
		15	40.01±0.06[a]	20.98±0.02[a]
		20	26.88±0.05[b]	13.80±0.45[b]
Statistical tests		P	<0.001	<0.001
		F	9.11	11.01

In the same column, the averages followed by the same letter are statistically equal (test of Newman-Keuls to the threshold of 5%).

± 0.14), and 20 mM $MgSO_4$ (39.83% ± 1.02 and 19.87 ± 0.19).

Concerning the genotype C15, the high values of PEC and NSE were obtained on media supplemented with 27 mM of K_2SO_4 (40.50% ± 0.04 and 20.70 ± 0.21), of 10 mM (39.01% ± 0.04 and 19.98 ± 0.08) and 15 mM $MgSO_4$ (40.81% ± 0.03 and 20.10 ± 0.15).

For the genotype C16, high PEC and NSE were obtained on media containing 27 mM K_2SO_4 (39.50% ± 0.04 and 19.96 ± 0.21), 10 mM $MgSO_4$ (39.18% ± 0.05 and 19.80 ± 0.45) and 15 mM $MgSO_4$ (40.01% ± 0.06 and 20.98 ± 0.02).

The highest PEC and NSE were obtained with media supplemented with 27 mM K_2SO_4 in all genotypes with C1 (41.53% ± 2.59 and 21.76 ± 2.50), C8 (35.50% ± 1.09 and 19.10 ± 1.89), C14 (39.03% ± 1.59 and 19.53 ± 0.39), C15 (40.50% ± 0.04 and 20.70 ± 0.21), C16 (39.50% ± 0.04 and 19.96 ± 0.21) as well as with the medium enriched with 15 mM $MgSO_4$: C1 (42.63% ± 0.19 and 23.53 ± 0.09), C8 (36.63% ± 0.59 and 19.15 ± 0.89), C14 (40.68% ± 0.93 and 20.08 ± 0.14), C15 (40.81% ± 0.03 and 20.10 ± 0.15) and C16 (40.01% ± 0.06 and 20.98 ± 0.02).

Effect of mineral salts on the development of somatic embryos

After transfer of the callies to the embryo development medium (EDM), some continued to proliferate while others died. From the 84th day after the initial culture, embryogenic calli and embryos were observed for all genotypes. Figures 2 and 3 illustrate an example of embryogenic calli and embryos developed from petal explants of genotypes C1, C14 and C15.

DISCUSSION

The response of explants of *T. cacao* to somatic embryogenesis is dependent on genotype.

In order to develop a protocol applicable to certain genotypes deemed recalcitrant to somatic embryogenesis, two types of explants, staminodes and petals of five (05) genotypes, from the most embryogenic (C1) to at least embryogenic, C14, C16, C15 and C8 were used as plant material. A total of eight (08) induction media with four (04) concentrations of two (02) inorganic salts that are potassium sulfate (K_2SO_4) at concentrations 18, 27, 36 and 45 mM and magnesium sulfate ($MgSO_4$) at concentrations 5, 10, 15 and 20 mM were carried out for the avoidance of recalcitrance in the studied cocoa genotypes.

The use of mineral salts in the induction medium did not have a negative impact on the callogenesis responses of staminode and petal explants in the five (05) genotypes C1, C14, C15, C8 and C16 in the most cases. The various mineral salts used, improved the responses to callogenesis, particularly in the most embryogenic C1, the partially recalcitrant C14 and the recalcitrant C15 genotypes. A reduction of the rate of calli induction was however observed when the concentration of 5 mM $MgSO_4$ was added to the culture medium with the two types of explant (staminodes and petals) for the most recalcitrant genotype C8 and the partially recalcitrant genotype C16. These results show that the responses to callogenesis varied according to the genotype of cacao, the type and concentration of the mineral salt used.

The petal and staminode explants of the genotypes tested allowed, however, the induction of calli with levels higher than 74% on all media. These results are similar to those obtained by Minyaka et al. (2008) who showed that petal and staminode explants respond to calli production with the mineral salts K_2SO_4 and $MgSO_4$.

In addition, the petals had higher callogenesis percentages than staminodes for the five (05) genotypes evaluated. This response shows that petals respond better to callogenesis than staminodes with mineral salts. Similar results have been obtained by Da Silva et al. (2008) who found that petals are better adapted to calli

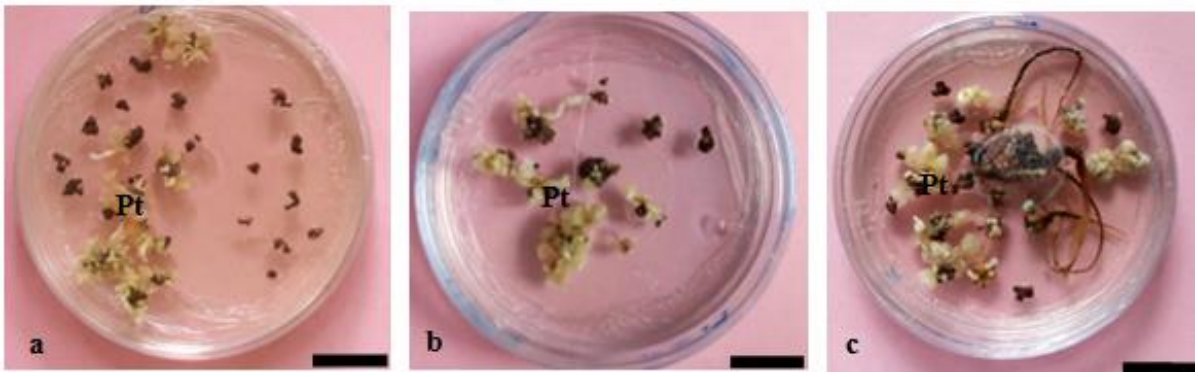

Figure 2. Embryogenic calli derived from petal explants of the C1, C14 and C15 genotypes after eighty-four (84) days in media culture. a - embryogenic calli of genotype C1 on EDM medium supplemented with 27 mM of K_2SO_4; b - Embryogenic calli of genotype C14 on EDM medium supplemented with 20 mM of $MgSO_4$; Embryogenic c -calli of the C15 genotype on medium supplemented with 15 mM $MgSO_4$. Bar = 1 cm.

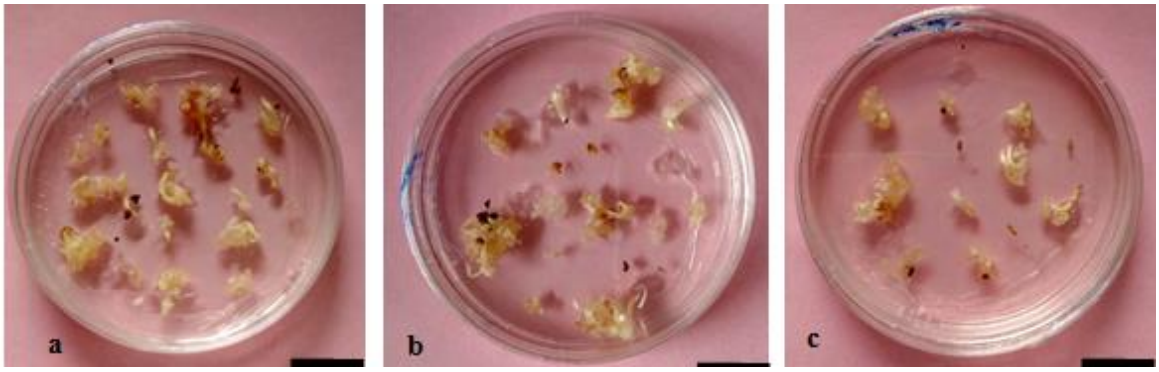

Figure 3. Development of embryos induced from petal explants of genotypes C1, C14 and C15 after ninety (90) days of culture: a - embryos of genotype C1 in medium supplemented with 27 mM K_2SO_4; b - embryos of the C14-genotype in medium supplemented with 20 mM $MgSO_4$; c - embryos of genotype C15 in medium supplemented with 15 mM $MgSO_4$. Bar = 0.5 cm.

formation than staminodes in *T. cacao*. Staminodes and petals could therefore indistinctly of genotype provide high percentages of callogenic explants with a maximum for the petals. These results, however, contradict those of Bahoya (2012). Indeed, these authors have shown that staminodes of genotypes named genotype 1, genotype 2 and genotype 3 used during their work were more conducive to callogenesis than petals. This shows that the response to callogene in addition to being dependent genotype is also a function of the explant. These authors have worked on genotypes that are different from those used in the present study. In the study, the percentage of embryogenic explants and the average number of somatic embryos were obtained with the calli derived from the petal explants in the five (05) genotypes C1, C8, C14, C15 and C16 of cacao and not with staminode explants. This result shows that the protocol developed in this study is more adapted to petal explants and the response to somatic embryogenesis also depends on the genotype. These results are consistent with those of Issali et al (2008), Kouassi et al. (2017a) and Eliane et al. (2019) which revealed that petals are better adapted to somatic embryo production than staminodes.

With mineral salts, inhibition of embryo production was observed with the concentration of 45 mM K_2SO_4 for the genotypes C8, C14, C15 and C16 and with 5 mM $MgSO_4$ for the genotypes C8, C15 and C16. This shows that too low concentrations are sometimes insufficient to lift the recalcitrant however when they are too high they cause toxicity. These concentrations of mineral salts are too high for K_2SO_4 and too low for $MgSO_4$ to allow the lifting of recalcitrance. This shows that the lowest concentrations are sometimes insufficient to overcome the recalcitrant ; however, when they are too high they cause toxicity regardless of the compound. Inhibition of somatic embryo production of *T. cacao* would be due, in addition to the insufficiency or toxicity of certain compounds used, to a strong secretion of ethylene and polyphenols by the explants of certain genotypes according to Fang et al. (2014) and Minyaka et al. (2017).

Polyphenols by their oxidation act as inhibitors of metabolic or antagonistic reactions of growth substances. The work of Alemanno et al. (1996) and Boutchouang et al. (2016) conducted on cacao flowers showed that they would synthesize a significant quantity of phenolic compounds. Indeed, these compounds intervene in the defense of plants (Kouassi et al., 2017a; Minyaka et al., 2017). When the plant is subjected to mechanical injury, simple phenols are synthesized and the peroxidase activity characteristic of the lignifying tissues is stimulated. Phenolic secretions and other exudates in plant tissue culture systems inhibit the development of the callogenic explant in embryos (Kouassi et al., 2017a). Among the inorganic salts used in the induction medium, the concentration of 27 mM potassium sulfate (K_2SO_4) and of 15 mM magnesium sulfate ($MgSO_4$) taken individually, gave the the most important percentages of embryogenic calli and of average numbers of embryos for all genotypes C1, C8, C14, C15 and C16 regardless of their level of recalcitrance. This response shows that concentrations of 27 mM potassium sulphate (K_2SO_4) and 15 mM magnesium sulphate ($MgSO_4$) compensate the deficiencies in mineral salts (potassium sulphate (K_2SO_4) and of magnesium sulphate ($MgSO_4$)); in the calli induction of these genotypes and making them able to produce embryos. According to Minyaka et al. (2010), a deficiency of potassium sulphate (K_2SO_4) or magnesium ($MgSO_4$) has a negative influence on the production of somatic embryos, resulting in a gradual loss of embryo production during culture and thus explains the fact that these metabolites, K_2SO_4 or $MgSO_4$, are essential for the good development of plants. The use of such concentrations allows compound to fill the deficit of minerals and avoid the development of medium often complex with different minerals.

Conclusion

Results of the current study showed that somatic embryogenesis of *T. cacao* genotypes are genotype and explant dependent. This study set up an improved protocol compared to previous works in terms of embryo production for cocoa.

The results obtained also reveal that the elimination of recalcitrance even for recalcitrant genotypes is possible with certain concentrations of mineral salts. This lifting of recalcitrance was carried out with 27 mM potassium sulfate (K_2SO_4) and 15 mM magnesium sulfate ($MgSO_4$).

These two concentrations gave the best percentages of embryogenic explants and the highest average numbers of embryos for the five genotypes tested. These two concentrations produce embryogenic callies with the recalcitrant genotypes C8 and C15, partially recalcitrant C14 and C16 at the same level as the most embryogenic C1 genotype.

It can therefore be concluded that the dependence of the genotype on somatic embryogenesis has been mastered for *T. cacao*.

These different defined concentrations constitute the optimal concentrations, which supplement the data of the literature for the lifting of the recalcitrance of the genotype for this species.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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