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Review

Molecular epidemiology of foot and mouth disease, bluetongue and pest de petites ruminants in Algeria: Historical perspective, diagnosis and control

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Molecular tools have become an increasingly important part of studying the epidemiology of infectious agents. These tools have allowed the aetiological agent within a population to be diagnosed rapidly with a greater degree of efficiency and accuracy than conventional diagnostic tools. They have enhanced understanding into the pathogenicity and virulence of the aetiological agent and subsequent deployment of appropriate control strategies. This paper reviews the contribution of molecular epidemiology to the diagnosis and control of some animal diseases such as foot and mouth disease (FMD), buetongue and peste des petites ruminants (PPR) in Algeria. Molecular epidemiology has helped in the characterization of FMDV type O circulating in Algerian cattle in 1999 and in 2014; in 1999, the sequencing analysis showed that the Algerian viruses belong to the West-African topotype with 99% similarity to a strain isolated in Côte d'Ivoire. In 2014, the virus was identified as 0/ME/SA/Ind-2001d lineage which was 99.69% identical to the field strains isolated from earlier Tunisian outbreaks. In a related development, two episodes of bluetongue outbreaks were reported in Algeria; the first with serotype II in 2000 that showed no significant difference with the Tunisian strain reported two months earlier and the second episode involving serotype I epidemiologically linked to South Africa (with 94.3% not similarity) indicating an origin from sub-Saharan Africa. Molecular techniques have also described the PPRV strain implicated in an outbreak in Ghardaïa district, in the centre of Algeria. The strain clustered with lineage IV of PPRV and shared 97 to 99% similarity with the strain implicated in neighboring Morocco and Tunisia.

Key words: Algeria, control, diagnosis, molecular epidemiology, Foot and Mouth Disease (FMD), bluetongue (BT), Peste des Petites Ruminants (PPR).

INTRODUCTION

Molecular tools have increasingly become an integral part of studying the epidemiology of infectious agents globally

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(Van Belkum et al., 2007). Epidemiology, the study of factors determining the occurrence of disease/health outcome in a population, aims to describe the health status of a population, aetiology of disease, predict disease occurrence and to help control the distribution of disease. Molecular biology provides one of the many diagnostic tools that can be utilized to strengthen understanding of the epidemiology of a disease, both infectious and non-infectious (Grenfell et al., 2004). Molecular epidemiology has recently been defined as "a science that focuses on the contribution of potential genetic and environmental risk factors, identified at the molecular level, to the aetiology, distribution and prevention of disease within families and across populations" (Maslow et al., 1993). This science has emerged through integration of the disciplines of epidemiology and molecular biology. Molecular tools often provide additional dimension to the epidemiology of disease that would otherwise be impossible using conventional diagnostic tools. The objectives of molecular epidemiology for the study of infectious diseases include: (i) descriptive and analytical studies to evaluate host/environmental interactions in disease, and (ii) the development of strategies for the control of bacterial, parasitic and viral disorders through molecular diagnosis (Grenfell et al., 2004; Van Belkum et al., 2007).

Recently, the use of molecular tools has been helpful in unraveling the epidemiological relationships between infectious diseases, particularly emerging and re-emerging transboundary animal diseases (TADs). These emerging TADs are those that are newly recognized and/or newly evolved or that have occurred previously but show an increase in incidence or expansion in geographical, host or vector range (Thompson et al., 1998; Riley, 2004). Multiple host species are sometimes involved in the transmission of diseases including wildlife, companion animals, livestock and fish, as well as several human behavioral risk factors that increase exposure to diseases. An integral part of prevention and control of these diseases is based on early and rapid detection, epidemiological investigation, multidisciplinary collaboration and the development of advanced diagnosis and surveillance tools, including the use of molecular biological methods such as PCR and phylogenetic analysis (Levin et al., 1999; Riley, 2004).

These molecular tools have been helpful inaccurately diagnosing diseases within a population with greater sensitivity and specificity compared to conventional methods. It has contributed to the increased understanding of the pathogenicity and virulence of aetiological agents, identified genes of the aetiological agent that are responsible for virulence, drug resistance, evasion of the immune system, provided information on the population structure, taxonomy, source and transmission dynamics of aetiological agents and identified genes that increase host susceptibility to disease (Thompson et al., 1998; Levin et al., 1999).

Adequate application of molecular epidemiological principles requires a working knowledge of both molecular biological and epidemiological methods. This review describe how the application of molecular tools can help elucidate aspects of the epidemiology and transmission patterns of foot and mouth disease (FMD), bluetongue (BT) and pest de petites ruminants (PPR) in Algeria using data available in research papers and reports of international organizations and databases (OIE, FAO).

Algeria is the largest country in Africa. It is located between latitudes 19° and 37°N and longitudes 9°W and 12°E. It is bounded by the Mediterranean Sea to the north, Tunisia to the east, Morocco to the west, Mali and Niger to the south. It has a long coastline at the Mediterranean Sea (1600 km); Most of the coastal areas (northern region) are hilly and sometimes even mountainous. South of the northern region is a steppe; farther south, there is the Sahara Desert. For reasons of animal health, transportation of animals is forbidden between Sahara and Northern Algeria. Administratively, Algeria is divided into 48 districts with a superficies of 2,147,570 km^2 and with about 40 million people. More than 80% of the people live in coastal areas (MADR, 2015).

In Algeria, livestock farming represents a significant financial income of an important part of the Algerian population with ≈ 2 million cattle and 31 million small ruminants all reared under traditional extensive husbandry system, although intensive husbandry systems have recently been introduced in the country. By virtue of its geographical location and its borders with the North African and Sahel countries, Algeria is vulnerable to several trans-boundary diseases, including FMD, BT and PPR (MADR, 2015).

MOLECULAR EPIDEMIOLOGY OF FMD IN ALGERIA

The 1999 epidemic

The livestock population (cattle, sheep and goat) at risk for FMD during the 1999 epidemic in Algeria was approximately 78 million heads because most of these livestock were not vaccinated against any of the circulating FMD serotype. Subsequently, on the 20th and 21st February 1999, two cases of foot-and-mouth disease (FMD) were suspected in cattle in Algiers district, Algeria (FAO, 1999). The vesicular material collected and sent to the World Reference Laboratory (WRL) for FMD in Pirbright confirmed type O circulating and OIE and FAO were informed appropriately (FAO, 1999). Sequence analysis of the virus revealed a genetically different type O virus from the strains that were currently circulating in the Meddle East and North Africa (MENA) between 1989 and 1997. Sequence analysis showed that the Algerian viruses (O/ALG/1/99) belong to the West-African

topotype with 99% similarity to a strain isolated in Côte d'Ivoire (O/CIV/8/99) and Guinea (O/GNA/6/99) in 1999 (Samuel et al., 1999; Samuel and Knowles, 2001).

However, this confirmed the suspicion about the origin of the disease. Indeed, zebu cattle introduced illegally across the Algerian southern frontiers during the month of February 1999, were intercepted within the southern borders of the country. At the time of capture, these zebu cattle did not present any clinical signs of FMD (Samuel and Knowles, 2001). However, their presence demonstrated that transboundary animal movements took place on the southern frontier with Niger and Mali which are endemic for FMD. From the beginning of the epizootic up to the 22th June 1999; 179 outbreaks were recorded from 36 districts out of 48 infected by the disease. On the 22nd February 1999, cases of FMD were declared in Souk-Ahras district 50 km from the Tunisian border and Tlemcen at the west border of the country with Morocco (FAO, 1999).

Following this FMD epidemic, an appeal for vigilance was launched throughout national territories in Algeria, Tunisia and Morocco with active surveillance in all farms and all veterinary professionals were mobilized and biosafety measures observed. The media was used to sensitize and disseminate information on the benefits of farmer's participation in disease prevention and control program to protect their livestock. All cattle within affected farms were destroyed and the owners compensated, along with intensification of surveillance on a 10 km radius from the diseased area. The vaccine used for this campaign contains the O Manisa strain in accordance with the recommendations of the WRL, Pirbright (FAO, 1999; Thomson, 2002).

The 2014 to 2015 epidemics

On 24 April, 2014, two cows with clinical signs suggestive of FMD were reported in Nabeul district, Tunisia (OIE, 2016). The disease was confirmed by real-time RT-PCR and the phylogenetic analysis identified topotype O/ME-SA/Ind 2001d which is closely related (99%) to recent viruses isolates from Libya (LIB/2/2013 and Saudi Arabia (SAU/3/2013). OIE report suggested that the source of the outbreak was due to the illegal movement of animals from Libya. During the first month (May, 2014), 32 new outbreaks were reported in domestic sheep, goats and cattle in 11 different districts. In June, new cases were subsequently declared in Jendouba districts 50 km to the western border of the country with Algeria (OIE, 2016).

According to OIE report, the source of the outbreak was due to illegal movements of ruminants from Libya into other Maghreb countries (OIE, 2016). Oueslati (2012) reported that most uncontrolled movements of ruminants in Maghreb countries occurred by land transport; given that the region is characterized by very long borders that stretch into the desert; 460 km (Tunisia-Libya), 520 km (Tunisia-Algeria) and 1040 km (Algeria-Libya). This remained a major constraint for border control services; the flow of uncontrolled movements of animal across the border that occurs along the east-west axis between Maghreb countries is difficult to estimate and depends on several factors (price changes, religious festivities, etc.). Furthermore, political unrest in Libya increased the potential risk of transboundary diseases spreading into neighboring border countries especially Tunisia and Algeria; this is mainly driven by the disruption of public health services, insecurity and massive displacement of refugees across the borders (OIE, 2016).

On the 23rd of July, 2014, FMD outbreak was detected in Setif district at the East of the Algeria, 260 km from border with Tunisia. The first outbreak occurred on a fattening cattle farm, the source of the outbreak was attributed to illegal introduction of animals from Tunisia. Clinical signs of the disease at the time of diagnosis included fever, blisters, lameness and mammary lesions (OIE, 2016). Samples were forwarded to the Experimental Zooprophylactic Institute (IZSLER), Brescia (OIE's Reference Laboratory) and the virus isolated and identified as O/ME-SA/Ind-2001d lineage with identity of 99.69 and 99.37%, to field strains O/TUN/1031/2014 and O/TUN/1054/2014, respectively isolated during the current outbreaks in Tunisia (WRLFMD, 2016). Outbreaks were reported in the first week in six different districts. Again the second week also witness new outbreaks in 13 new districts, and by the end of August more than 350 outbreaks were recorded since the epidemic started in 33 different districts. Cases were subsequently declared in Oran districts 160 km to the western border of the country with Morocco. All the cases recorded were from cattle and there were no clinical signs of FMD in small ruminants. However, in March 2015, twelve FMD outbreaks involving sheep were reported in El Bayadh and El Oued districts ending nearly five months of absence of the disease in the Algeria (OIE, 2016).

Following the FMD epidemic in Tunisia in April 2014, several measures were implemented in Algeria (OIE, 2016)]. Crisis cells centres at national and regional levels were instituted; disinfection of vehicles leaving affected or suspected district; vaccination points of susceptible species at the entrance of livestock markets, Peri-focal vaccination in 5 km radius; epidemiological investigation to determine the origin of the infection; closing of livestock markets, ban on movement of animals within the infected districts. Treatment of animals was not carried out. In the affected farms, all cattle infected were destroyed and their owners compensated. Further control measures were; stamping out, screening, vaccination in response to outbreaks, disinfection of infected premises/establishments. Vaccination campaign throughout Algeria and Tunisia was performed. The vaccination was carried out with the same vaccine (O Manisa) used in Libya and Tunisia. In Algeria, the

vaccination campaign rate by June 2014 was 85% in cattle, however and despite the vaccination coverage FMD outbreaks has occurred in Algeria with a wide spared in all the country (OIE, 2016; WRLFMD, 2016).
A summary of vaccine matching data generated at the WRLFMD for representative member countries for the O/ME-SA/Ind2001d lineage showed results for 22 field virus samples sent to WRLFMD to contain data for viruses from Algeria and Tunisia. In general, three vaccine antigens (O/TUR/5/09, O-3039 and O/TAW/98) were matched against these viruses, while the in-vitro test indicated a poorer match for O-Manisa and O-BFS; the vaccine strains used in Algeria and Tunisia. Arguably, this may be the reason why the FMD epidemic continued in Tunisia and Algeria despite the vaccination efforts applied by the two countries, until August 2014 where the vaccine strain O/TUR/5/09 was used that allowed the control and resolution of the episodes (WRLFMD, 2016).

MOLECULAR EPIDEMIOLOGY OF BT IN ALGERIA

The 2000 epidemics

For the first time in history, Algeria reported 28 outbreaks of BT between July and September 2000 in the north-eastern part of the country. Circulation of BTV serotype 2 was confirmed by the Institute of Animal Health (IAH) in Pirbright (Hamida, 2000). The disease spread after the first cases were reported to affect 24 localities in the district of Jijel. Of the 21,175 susceptible sheep, 2,661 (12.6%) were clinically affected. The disease continued to spread and by the end of the epidemic, six more districts in the eastern and central parts of the country were also affected (Skikda: 1,277 cases; Souk Ahras: 430 cases; Annaba: 500 cases; Guelma: 2,871 cases; Oum El Bouaghi: 5 cases; Tebessa: 35 cases; and Jijel: 18 cases) (OIE, 2016). Molecular studies comparing genomic segments 2 and 7 of the virus isolated in Algeria to those isolate in Tunisia in May 2000 showed no significant difference between them (segment 2: 99.4% homology; segment 7: 100% homology). Therefore, the two isolates were probably of the same origin (Ben Fredj et al., 2003).

Since the incursion of BT into Tunisia in June 2000, the Algerian veterinary authorities implemented surveillance programmes to control BT in the country and to detect new clinical cases by serological diagnosis and determine the presence and distribution of known vectors of the disease. Once BT had been confirmed in Tunisia, the national veterinary authorities implemented a series of control measures also. Premises where outbreaks were recorded, flocks were isolated and dead animals buried. Sick animals, animal holdings and surrounding areas were sprayed with insecticide. Surveillance for the detection of new clinical cases in the nearby flocks was initiated but no vaccination was carried out (Hamida, 2000).

The 2006 to 2007 epidemics

In July 2006, an outbreak of BTV serotype 1 occurred in central Algeria and a total of 28 outbreaks were officially confirmed in the whole of Algeria between July 19 and August 30, 2006. BTV-1 was isolated in two regions, and was clinically identified and confirmed by real-time PCR with high level of seroconversion. A total of 5245 sheep were considered as susceptible with 263 cases. Thirty-six (36) deaths were reported during the outbreak with an apparent morbidity and mortality rates of 5.01 and 0.69%, respectively (Cêtre-Sossah et al., 2011; Madani et al., 2011).

BTV was isolated from three different samples, derived from two different provinces (El Bayadh and Médéa). Amplification and sequencing of different genome segments (Seg-2, -7, -8, -9 and -10) was successfully carried out for identification of three isolates (Cêtre-Sossah et al., 2011). In depth analysis of BTV-1 isolates from around the world, have identified two introductions of BTV-1 into the Mediterranean region, in 2001 and 2006 with a separate introduction of an African strain of BTV-1 into Oman in 2009 (www.reoviridae.org/dsRNA_virus_proteins/ReoID/BTV-mol-epidem.htm):

1. BTV-1in Greece in 2001 was thought to have entered Europe from the east, possibly via Turkey, although there was no previous evidence of BTV-1 during a serological survey of Turkey in the early 1980s. Unlike other western strains of BTV-1, the Greek type 1 strain did not spread to other European countries (Mellor et al., 2008).
2. In 2006, BTV-1 appeared in Algeria, and then spread to Morocco. Analyses of the Seg-2 from BTV strains belonging to the outbreak that started in Algeria during 2006 were (collectively) most closely related to the reference strain of BTV-1 from South Africa (94.3% not similarity) indicating an origin from sub-Saharan Africa. The Algerian strains were only distantly related to BTV-1 from Greece 2001 (GRE2001/01) or BTV-1 from India (IND1992/01) (74.4 and 74.9% identity, respectively) although the Indian and Greek isolates were themselves closely related (95.9% not similarity). BTV-1 has also been isolated in South Africa, India, China, Honduras, USA and Australia giving it a global distribution (Cêtre-Sossah et al., 2011; Madani et al., 2011).

In 2007, BTV-1 belonging to the same western virus lineage was again identified, North Africa, in Algeria, Tunisia, Libya and Morocco and in the south of the Iberian Peninsula (Spain, Portugal and Gibraltar). This movement into Europe may have been caused by wind-borne movement of adult Culicoides in a similar manner to the movement of BTV-4 from Morocco to Iberia during 2003 (Maan et al., 2009).

In 2007, the Algerian BTV-1 spread northwards to France and by late 2008, had arrived on the northern coast of the country becoming established in 2009, and

threatening the UK. These movements provided the first overlap between the northern European outbreak of BTV-8 and another BTV strain/serotype and are likely to provide opportunities for genome segment exchange, potentially leading to the generation of novel reassortant viruses (Maan et al., 2009).

Bluetongue is of significant veterinary concern to small ruminant producers, wildlife managers and veterinary diagnosticians because of the frequent occurrence of outbreaks among domestic and wild ruminants in geographical regions previously known to be BT-free (Saegerman et al., 2008). Recently Kardjadj et al. (2016) reported that the serological evidence of BTV exposure in Algeria was observed in 37 (17 cattle and 20 small ruminants herds) out of 225 herds accounting for 16.44% (95% CI 9.42-23.46) herd seroprevalence; 21.3% (95% CI 17.1-25.6) for cattle and 13.33% (95% CI 9.86-16.8) for small ruminants. At the individual level, our results reveal an individual seroprevalence of 6.96% (200/2871); 13.7% (62/450) for cattle and 5.70% (138/2421) for small ruminants.

MOLECULAR EPIDEMIOLOGY OF PPR IN ALGERIA

Similarly, following the PPR epizootiology in Morocco in 2008 where 257 outbreaks were recorded with severe economic losses (FAO, 2009), Sghaier et al. (2014) in Tunisia reported a PPRV strain belonging to lineage IV and genetically related to those isolated in Morocco. In 2011, PPR was reported in Sahrawi refugee camps in Tindouf district, at the south western border of Algeria with Western Sahara, Mauritania, and Morocco and the sequence analysis clustered the circulating virus under lineage IV of PPRV (De Nardi et al., 2012). A year later, Kardjadj et al. (2015a) described the first serological and molecular typing of the PPRV strain implicated in an outbreak in Ghardaïa district, in the center of Algeria. The strain was clustered with lineage IV of PPRV and shared between 97 to 99% similarity with the strain implicated in neighboring Morocco and Tunisia.

Regular PPRV epizootic activity across the tropical and sub-tropical areas of North Africa has resulted in the spread of the disease into uninfected areas within the continent. The recent results from the Food and Agriculture Organization (FAO)-funded project “Toward a harmonized strategy for the control of Peste des Petits Ruminants in North Africa FAO Project (TCP/RAB/3302) provide insights into the situation on PPR in the Northern African countries (Algeria, Egypt, Libya, Morocco, Mauritania and Tunisia) updated up to 2012 to 2013. The results of this project show a high herd seroprevalence in the region (40 to 70%), except in Morocco, which adopted four years of mass vaccination (the last was in 2011 in Eastern Morocco) (EFSA, 2015). The project TCP/ RAB/3302 was set up in 2010, following the emergence of PPR in Morocco in 2008. Indeed, Morocco was probably the last Northern African country to be infected by PPRV, which was first detected in Egypt during the 1980s (Ismail and House, 1990). An outbreak of PPR was later reported in the Nile delta in 2006 (Abd et al., 2010) and the phylogenetic analyses revealed that the circulating strain of PPRV belonged to lineage IV and was closely related to PPRV isolated in Morocco in 2008 (Kwiatek et al., 2011). Moreover, serological evidence of PPRV infection was observed in Tunisia in small ruminant samples collected in 2006 (Ayari-Fakhfakh et al., 2011). On the other hand, retrospective surveys on a Moroccan serological bank could not detect PPRV antibodies in small ruminant sera collected before 2008 (Ettair, 2012). The results of the molecular studies in each country show that the lineage IV of PPRV is circulating throughout the sub-region (Abd et al., 2010; De Nardi et al., 2012; Sghaier et al., 2014; Kardjadj et al., 2015a) except Mauritania, where El Arbi et al. (2014) reported the presence of the lineage II, therefore, highlighting the existence of a second lineage circulating in North Africa.

These findings stress the importance of an epidemiological survey at a national level to establish the status of the disease in Algeria and to recommend an adequate control strategy. Recently, Kardjadj et al. (2015b) described an overall PPR apparent flock seroprevalence of 42.66% (64/150) and showed a relatively uniform distribution of PPR seroprevalence among all Algerian regions, suggesting a widespread distribution and endemic establishment of PPR in Algerian small ruminant population. Subsequently, in September 2013, the Algerian Veterinary Authority proceeded for the first time with PPR vaccination in Ghardaïa and its neighboring districts (Laghouat, Adrar and El Bayadh) using the vaccine strain Nig.75/1 to avoid an endemic state of the disease in the area.

CONCLUSION

In recent years, molecular tools have been of tremendous advantage in allowing diagnosis and characterisation of transboundary animal diseases agents in Algeria with far greater accuracy than conventional diagnostic tools. The superior accuracy of the diagnostic tests invariably results in a higher degree of confidence for epidemiological statistics. Molecular tools have also contributed to the identification of origin that may influence the occurrence, the severity of disease and help in the choice of the adequate vaccine for control and possible eradication of TADs from the country.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Chlorophyll, nitrogen and antioxidant activities in Cumaru (*Dipteryx odorata* (Aubl.) Willd) (Fabaceae) in two water regimes

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The Cumaru (*Dipteryx odorata* (Aubl.) Willd.) is a species used by traditional populations and industries using timber and non-timber forest products. This study aimed to analyze the levels of chlorophyll A, B, total ammonia levels, nitrate, proline, electrolyte leakage and activity of oxidative enzymes in evaluation to tolerance of cumaru plants subjected to drought for 21 days of stress. The experiment was conducted in a greenhouse at the Federal Rural University of Amazonia (UFRA), Belém, Pará, in the period from March to July 2015. The results showed a significant decrease in the relative water content of 50.8 and 55% for chlorophyll b, 45% to total chlorophyll and an increase in proline to the plants under drought. There was no significant difference to chlorophyll a, ammonium and nitrate. Increases in electrolyte leak with 22.74% for roots and 39.55% for leaves were observed. The enzyme catalase (CAT) showed a significant increase from the 14th day of the experiment, while changes in superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities were observed from the 7th day of the experiment. Cumaru plants are not drought tolerant over 21 days; also, young plants of cumaru respond negatively to conditions of low water availability in the soil.

Key words: Drought, oxidative stress, chlorophyll, tolerance, *Dipteryx odorata*.

INTRODUCTION

Cumaru (*Dipteryx odorata* (Aubl.) Willd.) is a species used by traditional populations and industries using timber and non-timber forest products such as oils for medicinal and cosmetic properties, as well as with the

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reforestation of degraded areas, which in addition to the ecological benefits, increase the supply of wood from reforestation in the region, increasing the income on the farm and reducing the pressure on the remaining natural forest dependent of water resources (Shimizu, 1998).

As water resources become scarce, the commercial exploration of plants tolerant to drought becomes a priority for obtaining high yields (Matos et al., 2012). The impact of drought in the forestry and agricultural activities is an important socioeconomic consequence that affects millions of people around the world (Elliott et al., 2013). Among the various factors affecting the production plant, the water deficit occupies a prominent position, as well as affect the water balance in plants by altering their metabolism, is a phenomenon that occurs in large extensions of arable areas (Nogueira et al., 2001).

Among the many implications of drought on plant development, the restriction on the acquisition of nutrients and water is commonly recognized (Manivannan et al., 2008). Evidence suggests that drought causes oxidative stress in various plants, in which reactive oxygen species (ROS) such as superoxide radical (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2) and singlet oxigen ($^1O_2$), are produced (Jaleel et al., 2007).

To minimize the cytotoxic effects of ROS, the plants causes a complex antioxidant system where specific enzymes act by neutralizing the action of these radicals, starting with the superoxide dismutase (SOD), which inmute radical O_2 to H_2O_2. This, in turn, undergoes action of various enzymes such as catalase (CAT), responsible for the conversion of H_2O_2 to H_2O and O_2, and peroxidase, ascorbate peroxidase (APX) reducing the H_2O_2 to H_2O (Apel and Hirt, 2004). Besides, in water restriction, the plants should be able to handle ROSs particularly to prevent oxidative damage to lipids, proteins and nucleic acids; if there is an inability to adequately handle ROSs, oxidative damage may result in cell death (Demidchik, 2015).

Several methods are adopted by researchers to identify species tolerant to water stress, being more common selection through ecological descriptors associated with physiological and biochemical descriptors. According Pincelli (2010) water deficiency is one of the environmental stresses responsible for the reduction of pigments in the leaves, making the plant life cycle changes. Among these, related to the antioxidant system and osmotic adjustment are supported substantially in identifying promising species, and consequently, the progress of culture of works for improving drought resistance (Azevedo Neto et al., 2009).

The antioxidant enzyme activity is usually enhanced to promote better elimination of ROSs and promote increased cellular protection against oxidative damage (Jaleel et al., 2009). Considering then that collaboration between antioxidant enzymes should provide better protection against the deleterious effects of ROS, minimizing oxidative damage (Blokhina et al., 2003).

Given the above, the study aimed to analyze the content of chlorophyll A and B, ammonium, nitrate and proline as well as the activity of oxidative enzymes in evaluation tolerance of cumaru plants subjected to drought.

MATERIALS AND METHODS

Location and experimental conduction

The experiment was conducted in a greenhouse at the Federal Rural University of Amazonia (UFRA) belonging to the Institute of Agricultural Sciences (IAS), located in Belém, Pará, in the period from March to July 2015. The seedlings of cumaru (*Dipteryx odorata* (Aubl.) Willd.), from seeds were provided by AIMEX (Association of Industries Exporters of Wood in Pará) with four months old, they were placed in plastic pots with a capacity of 3.6 L. The substrate consisted of yellow dystrophic Latosol (EMBRAPA, 2013). Before the start of treatment, all plants were irrigated daily for three months, corresponding to the acclimation time. 5 ml of solution cocktail containing macro and micronutrients (Table 1) was added to all the samples at the start of acclimation, in the form of nutrient solution (Hoagland and Arnon, 1950), modified in Biodiversity Studies Laboratory in Higher Plants (EBPS), UFRA.

The plants were subjected to two water regimes: Irrigated (control) and water deficit, in which the imposition of water deficit was obtained by suspension of irrigation in 21 days, and the time 0 (zero days of drought), time 1 (7 days of drought), time 2 (14 days of drought) and 3 time (21 days of drought). During the period of analysis, control plants were irrigated daily to replace the water lost by evapotranspiration. There was also the weed control manually. It was not detected occurring nutritional deficiency symptoms, as well as the attack of pests and pathogens.

Experimental design and statistical analysis

The experimental design was completely randomized in split plot in time (four times evaluation and two water conditions: Control and drought), with 5 repetitions, totaling 40 experimental units, each experimental unit was composed of a plant/pot. Analysis of variance of the results was applied and when there was a significant difference, the means were compared by Tukey test at 5% significance level. Moreover, the standard deviations were calculated for each treatment, and statistical analyzes performed by Assistant Version 7.7 Beta program.

Relative water content (RWC)

The RWC was determined at 06:00h a.m in each collect. The method used was that described by Slavick (1979). Results were expressed as a percentage, according to the formula:

$$RWC = (FM1 - DM)/(FM2 - DM) \times 100\ (\%)$$

Where, FM1 = Fresh mass 1; FM2 = Fresh mass 2 with saturation; DM = Dry mass.

Determining the ammonium content

50 mg of previously lyophilised leaves and roots were weighed and put in a test tube containing 400 ml of total extract + 2.5 ml of solution A (5 g phenol + 0.025 g of sodium nitroprusside / 500 ml

Table 1. Solution cocktail containing macro and micronutrients.

Composition	Concentration (M)	ml /L
KNO_3	1	5
$Ca(NO_3)_2$	1	3
NH_4NO_3	1	2
KH_2PO_4	1	0,1
$MgSO_4$	1	1
Fe-EDTA		1
1. $FeSO_4$. $7H_2O$	0.1	
2. Na_2 (EDTA)	0.08	
Micronutrientes		1
1. H_3BO_3	0.04	
2. $MnCl_2$. 4 H_2O	0.009	
3. $CuSO_4$. $5H_2O$	0.0003	
4. $ZnSO_4$. 7 H_2O	0.0007	
5. Na_2MoO_4. 2 H_2O	0.0001	
$CoCl_2$. 6 H_2O	0.004	1
$Al_2(SO_4)_3$. 18 H_2O [(50 mM) pH= 4.0]	0.3	0,5
$Al_2(SO_4)_3$. 18 H_2O [(100 mM) pH= 4.0]	0.3	0,5
$Al_2(SO_4)_3$. 18 H_2O [(150 Mm) pH= 4.0]	0.3	0,5

distilled water) and homogenized by vortexing, adding 2.5 ml of Solution B (2.5 g NaOH + 12.6 ml of sodium hypochlorite / 500 ml distilled water), respectively. The free ammonium concentrations of the total extract were estimated from the standard curve constructed with $(NH_4)_2SO_4$ p.a. (Sigma) according to the method described by Weatherburn (1967).

Determination of nitrate

50 mg each of previously lyophilized leaves and roots was weighed and mixed with extract containing 100 mL + 200 of salicylic acid 5% solution (w / v) in concentrated sulfuric acid. After stirring vigorously in a vortex stirrer was slowly added 4700 uL of 2N NaOH. The concentration of nitrate was obtained from a standard curve with increasing concentrations of NO_3 (0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 µmol ml^{-1}) according the method described by Cataldo et al. (1975).

Determining the proline content

50 mg were weighed of previously lyophilised leaves and roots by adding in the test tubes the total extract, 1 ml of ninhydrin acid and 1 ml of glacial acetic acid 99.5%. It was determined through a standard calibration curve using proline and proline contents in samples were extrapolated from the curve and expressed in mmol g^{-1}. The dry matter (DM) was determined according to Bates et al. (1973).

Determination of photosynthetic pigments

The determination of photosynthetic pigments was realized according to Lichtenthaler (1987). The concentrations of chlorophyll A, B and total (mg. L^{-1}) were calculated using the formulas:

Chlorophyll A = $12.25 \times L_{(662)} - 2.79 \times L_{(644)}$ Chlorophyll B = $21.5 \times L_{(644)} - 5.1 \times L_{(662)}$
Total chlorophyll = $7.15 \times L_{(662)} + 18.71 \times L_{(644)}$

Membrane integrity (leak electrolytes)

The degree of membrane integrity was estimated by electrolyte leak according Blum and Ebercon (1981). The electrolyte leak was estimated by the following equation:

$EL\ (\%) = (C_1/C_2) \times 100$

Enzymatic activity

Superoxide dismutase (SOD)

The SOD activity was determined by inhibition of photoreduction of nitroblue tetrazolium chloride (NTC) according to Giannopolitis and Ries (1977).

Catalase (CAT)

CAT activity was determined by the method of Beers Jr. and Sizer (1952) with modifications.

Ascorbate peroxidase (APX)

The APX activity was determined by the method of Nakano and Asada (1981).

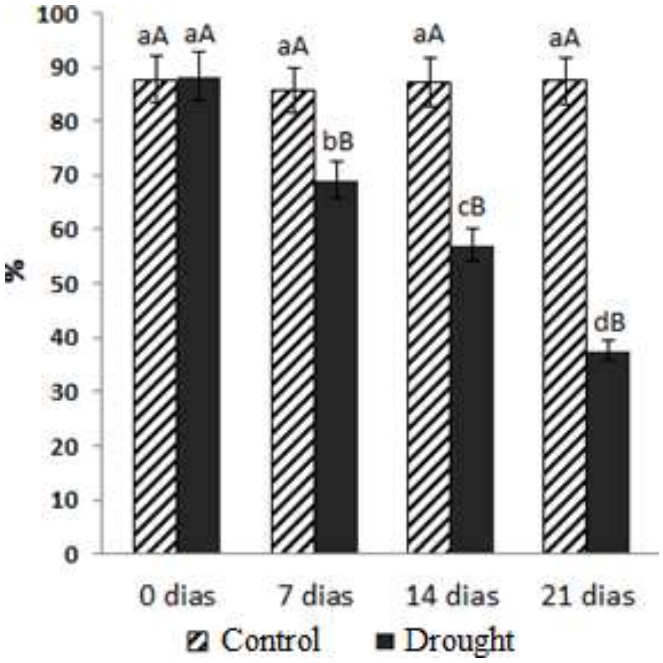

Figure 1. Relative water content in young plants Cumaru subjected to water deficit. Capital letters show statistical differences between water conditions and lower statistical differences between the collections time. Tukey test $p < 0.05$ probability was used for comparison.

RESULTS AND DISCUSION

Relative water content (RWC)

The relative water contents present in the leaves of cumaru under water stress decreased as the weeks went by, on average control plants showed water percentage between 87.7 and 85.5%, and plants under drought between 88.3 and 37.5%, representing a decrease of 50.8%.

The relative water content present in the leaves represent the water availability in the soil as well as the efficiency of the plant in pick up water in adverse conditions and maintaining water in the system reducing losses. Cumaru seedlings have the high water content in the leaf under normal conditions but had a sharp decrease due to lack of water. The decrease was significant from the 7th day of water suspension and decreasing over the 21 days of stress, as shown in Figure 1.

Photosynthetic pigments

The chlorophyll A contents do not vary significantly throughout the experiment (Tukey test at 5% significance level), while the chlorophyll **B** and total had a significant reduction in plants under drought compared to control plants. Mean values for chlorophyll A were 3.31 mmol.m^{-2}.s^{-1} for the control plants and 2.8 mmol.m^{-2}.s^{-1} for plants under drought. Chlorophyll **B** shows the mean values of 3.05 and 2.27 mmol.m^{-2}.s^{-1}, while total chlorophyll was 6.37and 5.08 mmol.m^{-2}.s^{-1} in control plants and plants under drought, respectively (Figure 2). Representing a decrease of 34% to chlorophyll A, 55% for chlorophyll B and 45% for total chlorophyll compared the two water conditions on the 21st day of the experiment. According to Morais et al. (2007), chlorophylls A and B are interconverted in the chlorophyll cycle and form complexes of chlorophyll-protein, that are important in the regulation and organization of the photosystem. Chlorophylls play an important role in photosynthesis, are responsible for capturing light energy, especially chlorophyll A as the main pigment of complex light collectors (LHC) for the photochemical reactions (Taiz and Zeiger, 2013).

Under reduced stomata conductance and consequently lower influx of CO_2 proceeds in reduction of net assimilation rate, which directly affects the biochemistry of photosynthesis and reduces the photochemical energy consumption (Carmo et al., 2014). In these situations there is constant production of reactive oxygen species and other chlorophyll degradation agents (Matos et al., 2012). Chlorophyll degradation occurs according to the level of stress in the plants are submitted and the implication is leaf senescence, occurrence found in this study (Carmo et al., 2014).

In this work the chlorophyll A showed no significant difference, a fact that may be in accordance with the statement of Dinakar et al. (2012), in which the chloroplasts are particularly susceptible to oxidative damage and when it comes to tolerance to drought periods as well as the production of antioxidants, chlorophyll content is maintained after the drying, to prevent the formation of reactive oxygen species (ROS's).

Ammonium, nitrate and proline content

The ammonium and nitrate concentrations had no significant change throughout the experiment in plants under drought and the control plants. Proline already had a significant increase from the 14th day in the leaves and 21th day in roots. The values for ammonium in the last day of collection were 11.2 and 11.5 mmol of NH_4^+.Kg^{-1} DM in roots and 7.2 and 6.4 mmol of NH_4^+.Kg^{-1} DM in leaves, control and drought, respectively (Figure 3A). Nitrate was of 0.07 and 0.08 mmol from NO_3^-.Kg^{-1} DM in roots and 0.06 and 0.06 mmol of NO_3^-.Kg^{-1} DM in leaves, control and drought, respectively (Figure 3B). Proline was of 3.8 and 20.8 mmol of Pro. g^{-1} DM in roots and 2.3 and 29.8 mmol of Pro.g^{-1} DM in leaves, control and drought, respectively (Figure 3C).

Most plants have a preference for nitrate ion as a nitrogen source, so it is common their levels were lower

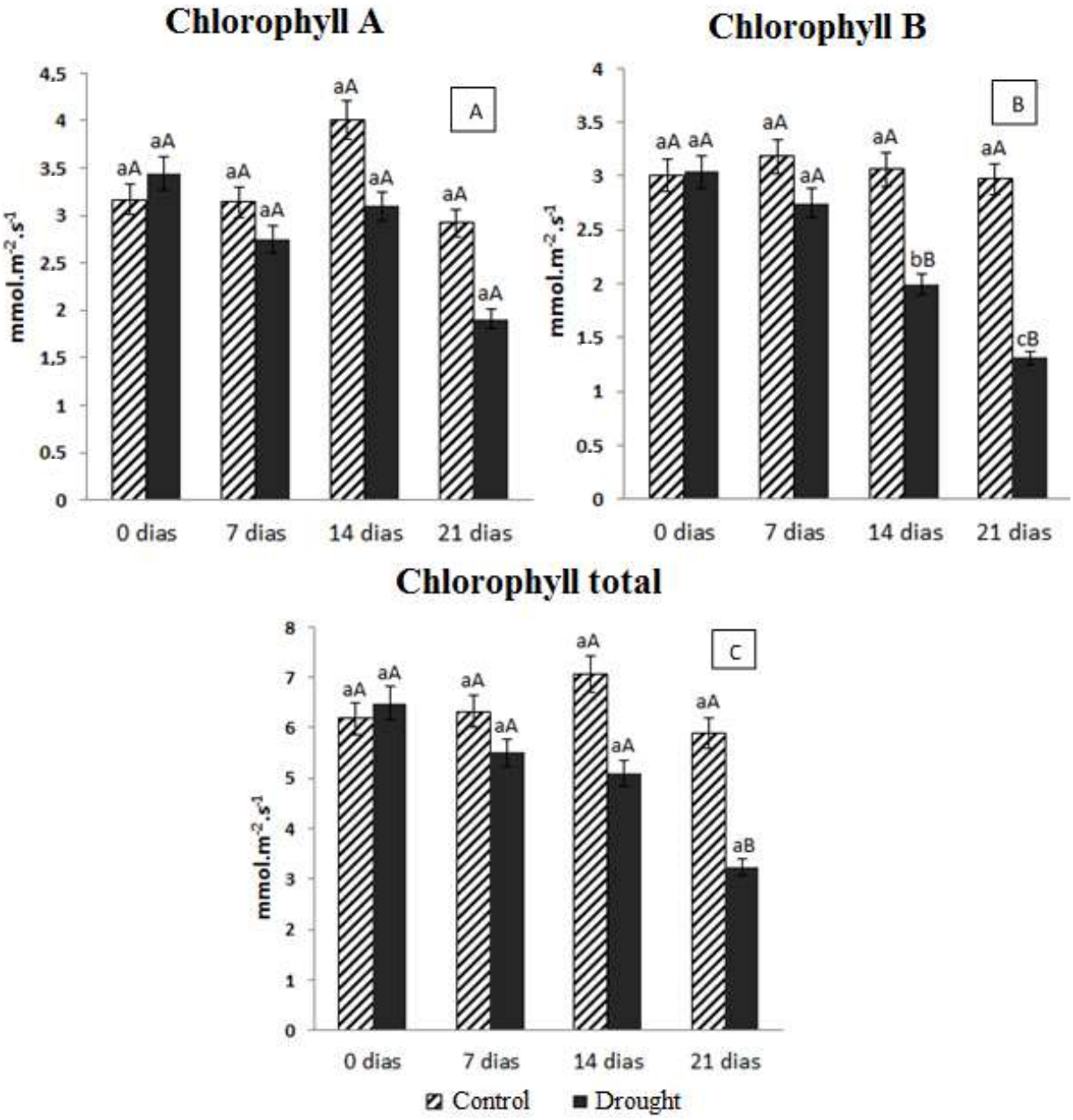

Figure 2. Chlorophyll content A (A), chlorophyll B (B) and chlorophyll total (C) in young plants Cumaru subjected to water deficit. Capital letters show statistical differences between water conditions and lower statistical differences between the collections time. Tukey test $p < 0.05$ probability was used for comparison.

than those found ammonium levels (Martinelli, 2003; Araújo et al., 2004), corroborating with these results.

The ammonium and the nitrate are the main forms of nitrogen available to plants, reduction processes and nitrogen assimilation can be absorbed both in the leaves and in the roots simultaneously or between these bodies becoming an essential process for the plant, since it is through it that It is controlled growth and development of the plant (Shan et al., 2012).

As a result, various forms of N available in the substrate can affect the morphological, physiological and biochemical plant, possibly in root growth, photosynthetic rates and catalytic activity of several enzymes (Li et al., 2013). In studies comparing the nutrition with nitrate (NO^{3-}) or ammonium (NH_4^+) show that these nitrogen sources can induce different metabolic responses (Patterson et al., 2010).

The accumulation of soluble solutes in plant cells provides a type of response to water deficit, called osmotic adjustment, which allows more negative water

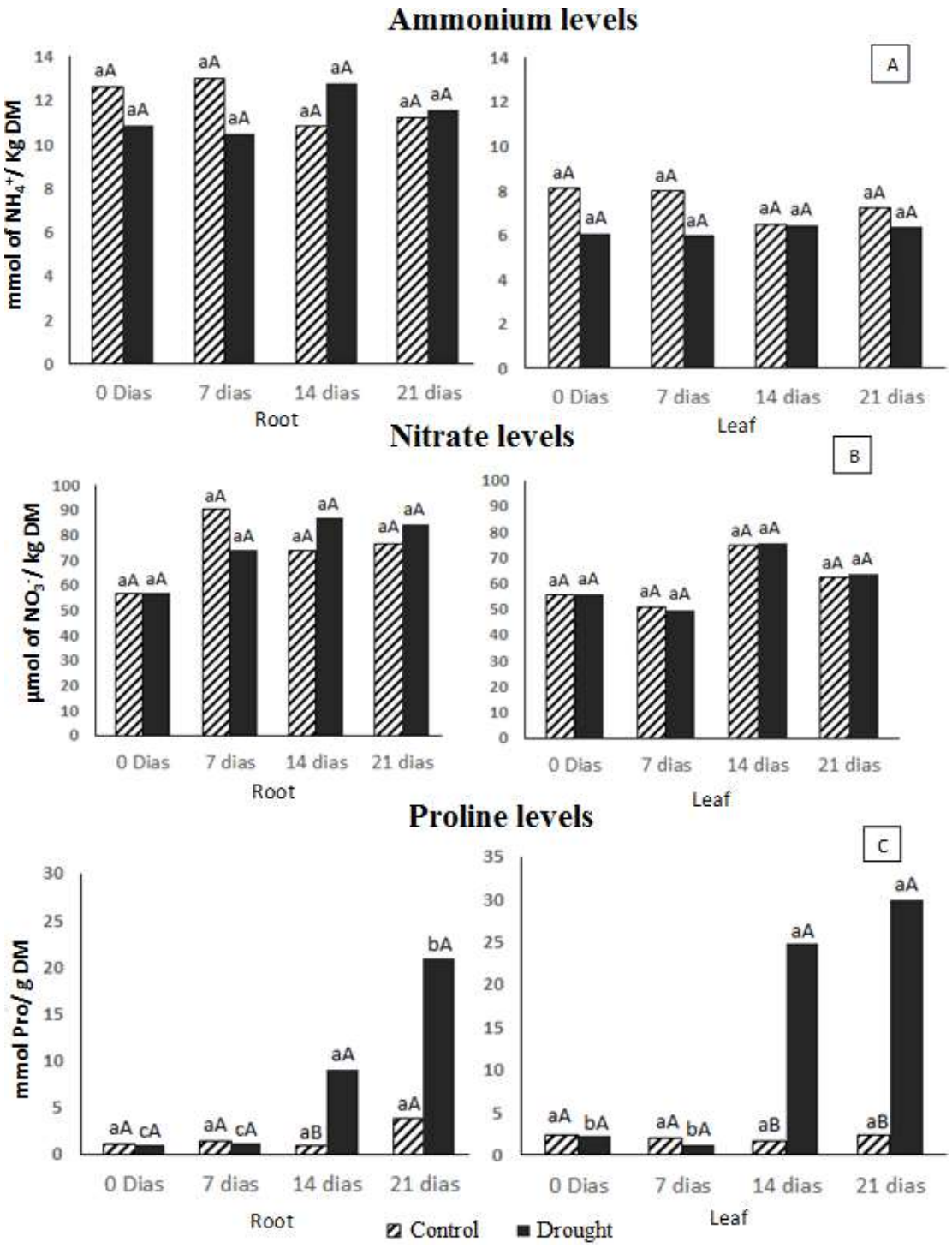

Figure 3. Ammonium levels (A), nitrate (B) and proline (C) in young plants Cumaru subjected to water deficit. Capital letters show statistical differences between water conditions and lower statistical differences between the collections time. Tukey test $p < 0.05$ probability was used for comparison.

potential in leaves, thus helping to keep the movement of water to the leaves (Silva et al., 2014). Proline has been highlighted as a compatible solute occurs in plants in response to environmental stresses that solute accumulates variety of plant species in response to stresses such as drought, heavy metals, extreme temperatures, salinity and ultraviolet radiation (Siripornadulsil et al., 2002).

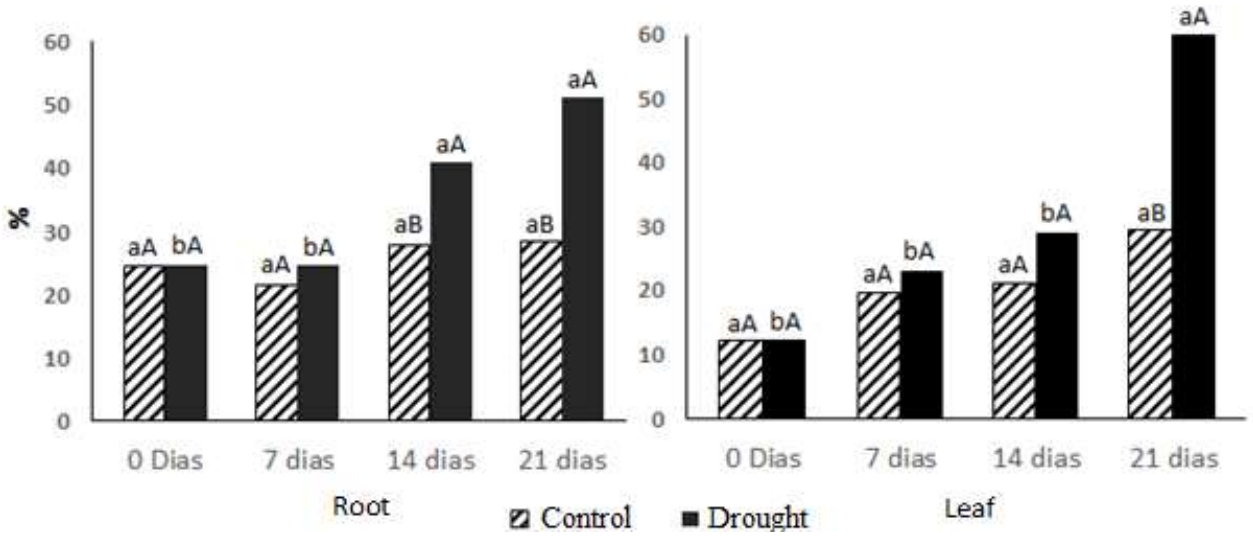

Figure 4. Electrolyte leak in young plants Cumaru subjected to water deficit. Capital letters show statistical differences between water conditions and lower statistical differences between the collections time. Tukey test $p < 0.05$ probability was used for comparison.

It is possible to note a proline increased much more significant in the leaves than the roots that this fact can be given by the need of the plant to have a more negative potential in the leaves so that the water can reach the highest parts of the plant. Proline contents only had increased from 14 days even if there is already a significant reduction in the RWC on the 7th day, this may be because the proline can be a compatible solute (osmoprotectors organic compound and osmoregulator) more secondary role in Cumaru species as it was the case in the study of Pinhão-manso (Sousa et al., 2012), which highlighted the glycine main osmoregulator and osmoprotectors.

Electrolyte Leak

Results show that there was a significant increase in both the leaves and the roots that were under water deficit with values of 12.2 to 29.58% and 12.2 to 60.73% for the leaves (plant control and drought, respectively). As well as 24.56 to 28.55% and 24.6 to 51.29% for the roots (plants control and drought), respectively (Figure 4) with a 39.55% increase in percentage for leaves and roots 22.74% to the 21 day of experiment. Lack of water causes a decrease in liquid photosynthesis and in this case the sharp reduction of water in the cumaru plants probably caused this decrease in liquid photosynthetic rate and to produce more O_2^- and H_2O_2 in chloroplasts (Blokhina et al., 2003; Reddy et al., 2004). The increased cellular leak in plants under drought is strongly related to the damage caused by free radicals O_2^- that attacks different parts of the plant as lipids and membrane proteins, nucleic acids and others causing cell death.

Enzymatic activity

Superoxide dismutase (SOD)

Plants subjected to drought showed a significant increase when compared to the control plants over the 21 days of experiment (Figure 5). The values for the roots were 49.86 to 50.85 mg^{-1}.protein, and of 49.79 to 58.59 mg^{-1}.protein (control plants and under drought), respectively. For the leaves the results were similar with values of 41.06 to 40.35 mg^{-1}.protein and of 40.91 to 49,2 mg^{-1}.protein (control plants and under drought), respectively. Plants have enzymatic systems of defense against reactive oxygen species, including SOD, CAT, APX. Activation of genes encoding these enzymes in response to oxidative stress was observed, for examples, tobacco (Bowler et al., 1991), soybean (Lee et al., 1999), and peanut (Sankar et al., 2007). Thus, increased activity of these enzymes is directly related to differential expression of the genes belonging to the antioxidant system, having as one of its functions to prevent H_2O_2 accumulation in cells (Eyidogan and Oz, 2007; Vaidyanathan et al., 2003).

Catalase (CAT)

The enzyme catalase showed significant difference from the 14th day of the experiment (Figure 6), with values for the roots of 0.042 mg^{-1}.protein (control plants) and of 0.042 to 0.054 mg^{-1}.protein (under drought), respectively. The leaves presents values of 0.043 to 0.042 mg^{-1}.protein and of 0.043 to 0.073 mg^{-1}.protein (control plants and under drought), respectively.

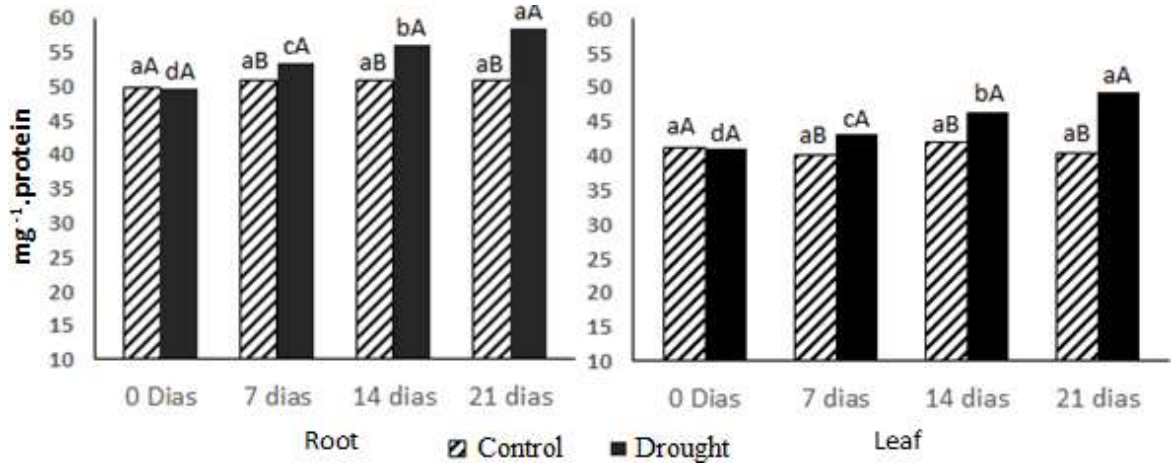

Figure 5. Superoxide dismutase enzyme activity in young plants Cumaru subjected to water deficit. Capital letters show statistical differences between water conditions and lower statistical differences between the collections time. Tukey test $p < 0.05$ probability was used for comparison.

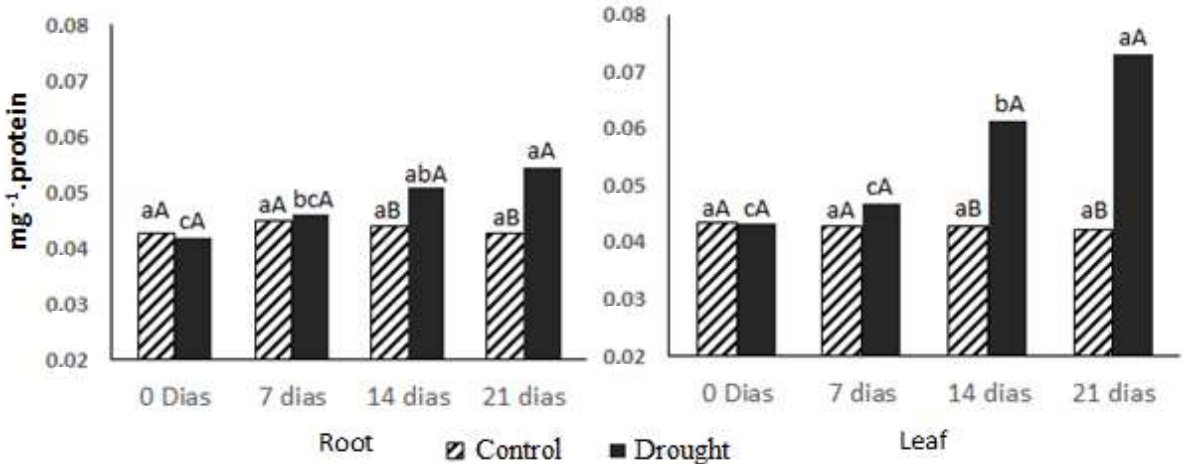

Figure 6. Enzyme catalase activity in young plants Cumaru subjected to water deficit. Capital letters show statistical differences between water conditions and lower statistical differences between the collections time. Tukey test $p < 0.05$ probability was used for comparison.

Akcay et al. (2010), who studied the CAT activity in creeping and erect peanut, found that the enzyme activity increased significantly when subjected to higher stress levels, confirming the results of this work. According to these authors, the CAT is one of the most effective defense enzymes in oxidative processes, since, in the resistant plants enables the integrity of the cell even when the stress is in a more rigorous stage. These results are reported in previous studies of water stress, salinity and other stresses, which reported that there is a reduced production of ROS in tolerant genotypes than in susceptible genotypes (Karabal et al., 2003; Chaitanya et al., 2002; Bhoomika et al., 2013). According to Sankar et al. (2007), as can be seen in his work, where an average increase of up to 230% of activity was obtained at the earliest material, when subjected to 10 days of water suppression.

Ascorbate peroxidase (APX)

The values of APX enzyme showed significant difference after 7 days of the experiment in plants were subjected to drought, when compared with control plants. The increase for the roots was from 0.0298 to 0.032 mmol.min^{-1} and of 0.0293 to 0.0376 mmol.min^{-1} in control plants and under drought, respectively. The leaves show values of 0.0315 to 0.0322 mmol.min^{-1} and of 0.0309 to 0.0405 mmol.min^{-1} in control plants and under drought, respectively (Figure 7). These results highlight that

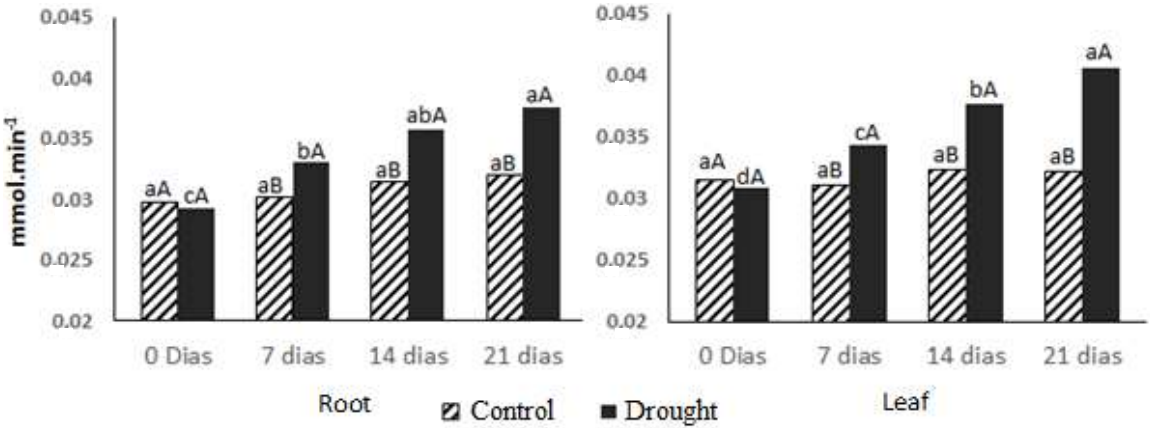

Figure 7. Enzyme peroxidase activity in young plants Cumaru subjected to water deficit. Capital letters show statistical differences between water conditions and lower statistical differences between the collections time. Tukey test $p < 0.05$ probability was used for comparison.

2013).

Thus, the potential oxidative damage due to drought was adequately mitigated by the constitutive activity APX (Cruz et al., 2013). Possibly, this gives rise to the triggering of multiple strategies antioxidants (Silva et al., 2015).

The species studied presents different mechanisms to overcome the drought periods, either by maintaining low RWC values and photosynthetic pigments, or by the increased activity of oxidative enzymes which are variables that can be used as water stress sensitivity indicator.

Young plants of cumaru are not tolerant to more than 21 days of water stress, and respond very negatively to the conditions of low water availability in the soil.

Conflict of Interests

The authors have not declared any conflict of interests.

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Enhancement of yield and nutritional value of soybean sprouts by persimmon fruit powder

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Large amount of soybean sprouts has been produced for human consumption in Korea for a long time. Various techniques and production methods have been implied to enhance the quality and yield of soybean sprouts. The objective of the present study was to investigate the effect of persimmon fruit powder treatment on yield and nutritional value of soybean sprouts. The sprout yield was increased by 16% with the addition of 5% (w/v) persimmon fruit powder during seed soaking. Vitamin C and polyphenol content were significantly (p<0.05) increased. Similarly, essential as well as total amino acid content were also enhanced. However, the moisture content and DPPH radical scavenging potential of soybean sprout were not significantly affected. On the other hand, the mineral elements especially potassium and calcium content were reduced with the application of persimmon fruit powder. The results of the present study suggest that addition of persimmon fruit powder could improve the yield, vitamin C and amino acid content, and antioxidant potential of soybean sprouts.

Key words: Persimmon fruit powder, nutritional value, soybean sprout, yield.

INTRODUCTION

Soybean is one of the important crops that has been included in Asian cuisine for centuries and is suggested to be included in Western diets because of their beneficial health effect (Sgarbieri, 1989). Germination has been proven to be one of the inexpensive and effective technologies to improve its nutritional potential (Paucar-Menacho et al., 2010) and reduce the effects of antinutritional factors such as lectins and enzyme inhibitors present in the seeds (Bau et al., 1997). Reports show that certain undesirable constituents, such as trypsin, chymotrypsin, lipoxygenase activity, phytic acid and oligosaccharides could be eliminated or reduced during germination in soybeans (Shi et al., 2010; Quinhone and Ida, 2015). In addition, the amounts of

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other constituents like vitamins, phytosterols, tocopherols and isoflavones increase (Shi et al., 2010).

Soybean sprouts have widely been consumed as an important vegetable in Korea for a long time. Huge amount of soybean sprouts is produced in Korea for human consumption (Hwang et al., 2004). Simple technology and considerably short time requirement are other advantages of soybean sprout production. Availability of functional foods in soybean sprouts is another reason for its high demand (Kim et al., 2004).

Germination process improves the nutritive value, texture and organoleptic characteristics of legumes (Frias et al., 2005; Granito et al., 2005); decreases undesirable anti-nutritional factors; and increases antioxidant potentials (Vidal-Valverde et al., 2002; Doblado et al., 2007). Different studies have been conducted investigating the effect of seed treatments and cultivation techniques that could enhance the yield and quality of soybean sprouts. Microbial safety was studied after exposing soybean seeds and sprouts to gamma radiation (Yun et al., 2013); biofortified soybean sprouts having enriched zinc content was produced with application of zinc sulphate solution (Zou et al., 2014); various light treatments were imposed to sprouts (Lee et al., 2007); influence of bacterial strains was investigated for their effect on bioactive contents and antioxidant activity of soybean sprouts (Algar et al., 2013); increased yield and inhibited rot of the soybean sprouts was found when watering the sprouts with grapefruit seed extract, chitosan and phosphate buffer (Choi et al., 2000) and *ginseng* extract improved the quality of soybean sprouts (Choi et al., 2003). Research on impact of persimmon fruit powder treatment on soybean sprouts has not been found so far although persimmon is rich in different nutrients and phytochemicals (Ebert and Gross, 1985; Gorinstein, 1999; Celik and Ercisli, 2007; Del Bubba et al., 2009). Since the persimmons fruits contain numerous nutraceutical properties, the objective of this work was to analyze the influence of persimmon fruit powder on yield and nutritional value of soybean sprouts.

MATERIALS AND METHODS

Soybean seed and persimmon powder

Soybean (*Glycine max* L.) seeds of cultivar Sowon with 12 g of 100-seed weight were purchased from a local market in Deagu, Korea. The soybean seeds were cleaned and kept into polyethylene containers and stored at 4°C until analysis. The freeze-dried persimmon fruits cv. Sangjudungsi were ground into powder (Speed Rotor Mill, Model KT-02A) and passed through a 200-mesh sieve.

Cultivation of soybean sprouts

One kilogram of intact seeds was thoroughly washed with tap water and excess water was drained out. The seeds were treated by soaking into water containing different amount of persimmon fruit powder. The treatments were named as control (seeds soaked in water without persimmon powder for 6 h), PP-1 (seeds soaked in water containing 0.5% (v/w) persimmon powder for 6 h), PP-2 (seeds soaked in water containing 1% (v/w) persimmon powder for 6 h), PP-3 (seeds soaked in water containing 2.5% (v/w) persimmon powder for 6 h), and PP-4 (seeds soaked in water containing 5% (v/w) persimmon powder for 6 h). The sprouts were watered with two hoses of 1 cm diameter for 2 min every 3 h. Soybean sprouts were grown at 20±1°C for 6 days. Samples for physicochemical studies were prepared by freeze-drying. The freeze-dried soybean sprouts were ground into powder (Speed Rotor Mill, Model KT-02A) and passed through a 100-mesh sieve. The strained samples were packed into airtight sample bottles and stored at -20°C until analysis.

Measurement of sprout yield

Total fresh weight of soybean sprouts in each batch was recorded at the end of the germination period of 6 days.

Determination of moisture and vitamin C content of soybean sprouts

Moisture and vitamin C content were determined in triplicate for each batch following the standard methods (AOAC, 1990).

Determination of mineral content of soybean sprouts

Sprout powder sample (0.5 g) was put into a cup and 15 ml of HNO_3 was added. A solution was diluted with equal volume of distilled water. Mineral concentrations were determined using inductively coupled plasma atomic emission spectrometer (ICP AES: Varian Vista, Varian Australia, Victoria, Australia) following Skujins (1998). The instrument was calibrated using known standards for each mineral. Average value of 2 replicate samples was reported.

Determination of total phenolic contents of soybean sprouts

The total phenols of the samples were estimated according to the Folin-Ciocalteau method (Singleton et al., 1999). A 50-µl sample was added to 250 µl of undiluted Folin-Ciocalteau-reagent. After 1 min, 750 µl of 20% (w/v) aqueous Na_2CO_3 were added, and the volume was made up to 5.0 ml with distilled water. The control contained all the reaction reagents except the extract. After 2 h of incubation at 25°C in dark, the absorbance was measured at 760 nm. Total phenols were determined as gallic acid equivalent (mg GAE/g extract), and the values were presented as average of triplicate analyses.

Determination of DPPH free radical scavenging potential of soybean sprouts

The 1,1-diphenyl-2-picrylhydrazol (DPPH) radical scavenging activity of sample was measured following Blois (1958) with some modifications. A 0.5 mM solution of DPPH in methanol and 0.05 M acetate buffer (pH 5.5) was prepared. An aliquot of 0.1 ml (at concentrations 0.5 to 1 mg/ml) of an antioxidant extract solution was added to 2 ml acetate buffer, 1.9 ml methanol and 1 ml DPPH solution.

Blanks contained 2 ml acetate buffer, 1.9 ml methanol and 0.1 ml cherry wine, while the control contained 2 ml acetate buffer, 1 ml DPPH and 2 ml methanol. The mixture was shaken immediately after adding DPPH and allowed to stand at room temperature in the dark, and the decrease in absorbance was measured at 517 nm

Table 1. Effect of different persimmon powders on yield and moisture and vitamin C contents of soybean sprouts cultivated for 6 days.

Sample	Total weight (g)	Moisture (%)	Vitamin C (mg/100 g)
Control	4624±62^{c} (100.0%)	86.21±0.07^{a}	16.00±0.37^{b}
PP-1	5036±69^{b} (108.9%)	86.28±0.19^{a}	16.52±0.44^{b}
PP-2	5068±32^{b} (109.6%)	87.12±1.21^{a}	16.32±0.52^{b}
PP-3	5378±55^{a} (116.3%)	85.99±3.12^{a}	17.21±0.14^{a}
PP-4	5410±48^{a} (112.3%)	84.32±3.00^{a}	17.28±0.22^{a}

Control, soybeans soaked in water for 6 h; PP-1, soybean soaked in water with 0.5% persimmon powder for 6 h; PP-2, soybeans soaked in water with 1% persimmon powder for 6 h; PP-3, soybeans soaked in water with 2.5% persimmon powder for 6 h; PP-4, soybeans soaked in water with 5% persimmon powder for 6 h. Percentage based on total weight of control. Values are expressed as mean ± standard deviation of 3 replicates. Values followed by different superscripts within a column indicate significant difference (p<0.05).

using a spectrophotometer (Shimadzu UV-1700UV, Shimadzu Scientific Instruments, Inc., Maryland, USA) after 30 min. All determinations were performed in duplicates. The inhibitory percentage of the DPPH radical by the samples was calculated as follows:

Scavenging effect (%) = $[A_0 - (A - A_b)/A_0] \times 100$

Where, A_0 is the absorbance of DPPH without sample (control), A is the absorbance of sample and DPPH, and A_b is the absorbance of sample without DPPH (blank).

Determination of free amino acid content of soybean sprouts

Amino acid contents were analyzed following the procedure of Je et al. (2005) with some modifications. Soybean sprout sample (1 g) was hydrolyzed with 6 N HCl (10 ml) in a sealed-vacuum ampoule at 110°C for 24 h for amino acid composition analysis. The HCl was removed from the hydrolyzed sample on a rotary evaporator, brought to a known volume (5 ml) with 0.2 M sodium citrate buffer (pH 2.2). The sample was passed through a C-18 Sep Pak (Waters Co. Milford, USA) cartridge and filtered through a 0.22 µm membrane filter (Millipore, USA). Amino acids were determined using an automatic amino acid analyzer (Biochrom- 20, Pharacia Biotech Co., Swiss).

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and differences between means at p<0.05 were analyzed using the Tukey test. The statistics package version 4.0 (Analytical Software, Tucson, AZ, USA) was used for statistical analysis.

RESULTS AND DISCUSSION

Yield and moisture and vitamin C contents of soybean sprouts

Treatment of soybean seeds with different concentration of persimmon fruit powder significantly (p<0.05) increased the soybean sprout yield (Table 1) after 6 days of germination. The highest sprout yield was found in the treatments PP-3 (5378 g) and PP-4 (5410 g) which were 16.3 and 12.3%higher than that of the control (4624 g). The higher yield of soybean sprouts with persimmon powder treatment might be due to some plant growth promoting substances present in the persimmon fruits. The results of this experiment show that yield of soybean sprouts could be improved using persimmon juice. Treatment of soybean seeds with persimmon powder did not affect the moisture content of soybean sprouts significantly (Table 1).

Vitamin C content of soybean sprout with persimmon fruit powder treatment PP-3 (17.21 mg/100 g) and PP-4 (17.28 mg/100 g) was significantly high as compared to untreated control (16 mg/100 g) and PP-1 and PP-2 samples (Table 1). The reason for higher vitamin C content in the persimmon fruit treated soybean sprouts was not well understood.

DPPH radical scavenging activities and total phenolic contents of soybean sprouts

DPPH radical scavenging capacity of soybean sprouts with persimmon fruit powder treatment was not significantly different; however, total polyphenol was significantly (p<0.05) high as compared to that of the untreated control (Table 2). The amount of polyphenol content of soybean sprout was found to be increased with the increased amount of persimmon powder, with the lowest value for PP-1 (338 µg GAE/g) and the highest for PP-4 (359.72 µg GAE/g). The higher polyphenol content of persimmon fruit powder treated soybean sprouts might be due to the polyphenols present in the persimmon fruits (Jang et al., 2010, 2011) as the application of zinc sulphate solution enriched the soybean sprouts with zinc content (Zou et al., 2014). Phenolic compounds are considered to possess antioxidant properties of foods, crops, vegetables and natural plants (Rice-Evans et al.,

Table 2. Effect of different persimmon powders on DPPH radical scavenging activities and total phenol contents of soybean sprouts cultivated for 6 days.

Sample	DPPH(% Inhibition)	Total phenol content (µg GAE/g of sample)
Control	96.66±1.31[a]	329.19±3.17[d]
PP-1	96.63±1.01[a]	338.36±4.21[c]
PP-2	96.65±0.98[a]	347.47±4.92[bc]
PP-3	96.00±1.21[a]	354.42±6.31[ab]
PP-4	96.10±1.80[a]	359.72±5.00[a]

Control, soybeans soaked in water for 6 h; PP-1, soybean soaked in water with 0.5% persimmon powder for 6 h; PP-2, soybeans soaked in water with 1% persimmon powder for 6 h; PP-3, soybeans soaked in water with 2.5% persimmon powder for 6 h; PP-4, soybeans soaked in water with 5% persimmon powder for 6 h. GAE, Gallic acid equivalents. Values are expressed as mean ± standard deviation of 3 replicates. Values followed by different superscripts within a column indicate significant difference (p<0.05).

1995; Maksimovic et al., 2005). Therefore, the total polyphenol content may contribute significantly to the antioxidant potential in the soybean sprouts.

Amino acid content of soybean sprouts

There were 38 amino acids analyzed in the sprout samples out of which 8 were essential and 9 others were not detected in either sample (Table 3). The sum of essential amino acids (72.16-89.2 mg/g) as well as total amino acid contents (311.5-344.08 mg/g) in persimmon fruit powder treated soybean sprouts was higher than those in the untreated control (70.56 and 305.94 mg/g of essential and total amino acids, respectively). Among the persimmon fruit powder treated sprouts, PP-4 showed the best results in terms of amount of essential as well as total amino acids content in the sprouts. Glutamic acid was the most abundantly found amino acid in the sprout samples. The content of γ-amino-n-butyric acid (GABA) was the highest for PP-4 (19.84 mg/g) followed by PP-3 (17.06 mg/g). Amino acids GABA and glycine are associated with learning and memory, stroke and neurodegenerative diseases; mediate signals between neurons that inhibit neutral amino acids and thus relieve anxiety, sedation, anticonvulsant and muscle relaxation functions (Krogsgaard-Larsen, 1989; Mody et al., 1994; Oh and Oh, 2004). GABA rich foods are also known as brain food which has bioactive capabilities to enhance blood cholesterol and triglyceride blood pressure suppression, improved cerebral blood flow, antioxidant, diuretic, insomnia, depression and anxiety stabilizingeffect on nerves and pain (Dhakal et al., 2011). The amount of amino acids is one of the key factors in determining the nutritional qualities of fruits, vegetables and foods (Basarova and Janousek, 2000). The results of this study reveal that total amino acid content in soybean sprouts could be increased by soaking the soybean seeds in water containing persimmon juice.

Mineral contents of soybean sprouts

Potassium was the most, whereas the amount of Cu was the least abundant minerals found in the soybean sprouts. The amount of Ca, Fe, and K was found reduced in the soybean sprouts which were treated with persimmon fruit powder (Table 4). Application of 5% persimmon fruit powder slightly increased the amount of Cu, Mg, Mn, Na and Zn. Overall performance of persimmon application to increase the mineral content was not found effective. Elements, As, Pb, Cd and Hg were not detected in sprout samples. Total amount of mineral content in persimmon fruit powder treated soybean sprouts were lower than that of the untreated control. The reason for the reduced mineral content in the treated samples was not well understood.

Conclusions

This study shows that yield and nutritional values of soybean sprouts could be enhanced by soaking the seeds in water containing persimmon fruit powder. The sprout yield was increased by 16% with the addition of 5% (w/v) persimmon fruit powder during seed soaking. Vitamin C content was also significantly increased with the treatment. Similarly, total polyphenol and essential as well as total amino acid content of soybean sprouts were also increased as a result of the seed treatment. However, the mineral elements especially potassium and calcium content were significantly reduced with the application of persimmon fruit powder. This study shows that soybean seed soaking in water containing persimmon

Table 3. Free amino acid composition (mg/g of dry weight) of soybean sprouts cultivated by different persimmon powders for 6 days.

Amino acid	Sample				
	Control	PP-1	PP-2	PP-3	PP-4
Essential amino acid					
L-Threonine	13.91± 2.12^{b}	14.61± 3.10^{ab}	14.91± 1.02^{b}	17.31± 0.37^{a}	16.92± 2.20^{ab}
L-Valine	19.58± 1.02^{b}	21.01± 1.92^{b}	21.06± 1.80^{b}	25.41± 1.99^{a}	24.88± 0.31^{a}
L-Methionine	1.64± 0.02^{a}	1.60± 0.01^{a}	1.49± 0.03^{c}	1.58± 0.10^{ab}	1.56± 0.04^{bc}
L-Isoleucine	10.48± 0.10^{c}	11.10± 0.12^{b}	10.73± 0.91^{b}	13.58± 1.31^{a}	13.85± 2.00^{a}
L-Leucine	5.92± 0.02^{d}	6.03± 0.01^{c}	5.41± 0.87^{d}	7.16± 0.50^{b}	8.17± 0.03^{a}
L-Phenylalanine	17.05± 0.03^{c}	17.60± 0.07^{c}	18.08± 0.06^{b}	22.10± 1.00^{a}	21.60± 0.98^{a}
L-Lysine	0.56± 0.02^{b}	0.58± 0.01^{a}	0.48± 0.01^{c}	0.54± 0.01^{b}	0.58± 0.02^{a}
L-Histidine	1.42± 0.01^{c}	1.50± 0.01^{b}	ND	ND	1.64± 0.02^{a}
Total essential amino acid	70.56	74.03	72.16	87.68	89.2
Non-essential amino acid					
O-Phospho-L-serine	ND	ND	ND	ND	ND
Taurine	ND	ND	ND	ND	ND
O-Phospho ethanol amine	0.53± 0.01^{d}	0.66± 0.02^{c}	0.53± 0.01^{d}	0.73± 0.02^{b}	0.82± 0.01^{a}
Urea	12.94± 0.03^{b}	13.85± 0.51^{a}	12.50± 0.31^{b}	14.58± 0.71^{a}	13.45± 0.61^{ab}
L-Aspartic acid	9.70± 0.07^{b}	10.16± 1.01^{a}	8.68± 0.98^{b}	9.39± 1.31^{ab}	10.61± 1.69^{a}
L-Serine	28.45± 1.12^{b}	29.48± 2.02^{b}	29.90± 1.38^{b}	33.76± 1.30^{a}	33.19± 2.00^{a}
L-Glutamic acid	79.78± 1.87^{b}	84.06± 2.00^{a}	86.67± 1.02^{a}	84.32± 1.81^{a}	79.68± 1.90^{b}
L-Sarcosine	ND	0.11± 0.01	0.11± 0.02	0.13± 0.03	0.08± 0.05
L-α-Aminoadipic acid	3.50± 0.02^{a}	3.34± 0.05^{b}	3.18± 0.07^{c}	3.40± 0.08^{b}	3.59± 0.10^{a}
Glycine	2.22± 0.31^{a}	2.27± 0.31^{a}	2.32± 0.51^{a}	2.51± 0.50^{a}	2.45± 0.59^{a}
L-Alanine	23.31± 2.10^{a}	24.47± 3.12^{a}	24.08± 0.91^{a}	26.66± 2.31^{a}	25.38± 1.61^{a}
L-Citrulline	ND	ND	ND	ND	ND
L-α-Amino-n-butylric acid	0.98± 0.02^{c}	1.10± 0.03^{b}	1.03± 0.05^{b}	1.27± 0.01^{a}	1.25± 0.04^{a}
L-Cystine	ND	ND	ND	ND	ND
Cystathionine	0.13± 0.01	0.15± 0.01	0.14± 0.01	0.17± 0.02	ND
L-Tyrosine	1.53± 0.01^{b}	1.52± 0.02^{b}	1.19± 0.01^{d}	1.33± 0.03^{c}	1.61± 0.02^{a}
β-Alanine	1.88± 0.04^{d}	2.19± 0.02^{ab}	2.13± 0.04^{b}	2.24± 0.03^{a}	2.00± 0.02^{c}
D,L-β-Aminoisobutyric acid	0.53± 0.01^{b}	0.53± 0.31^{b}	0.46± 0.02^{c}	0.53± 0.01^{b}	0.66± 0.02^{a}
γ-Amino-n-butyric acid	14.96± 0.09^{c}	14.42± 0.08^{c}	11.86± 0.02^{d}	17.06± 0.04^{b}	19.84± 0.03^{a}
Ethanolamin	2.99± 0.02^{d}	3.27± 0.01^{b}	3.10± 0.01^{c}	3.41± 0.02^{a}	3.27± 0.02^{b}
Ammonia	2.03± 0.02^{d}	2.32± 0.02^{c}	2.05± 0.11^{d}	2.87± 0.03^{b}	3.09± 0.04^{a}
Hydroxylysine	ND	ND	ND	ND	ND
L-Ornithine	ND	ND	ND	ND	ND
1-Methyl-L-histidine	15.00± 0.07^{c}	13.44± 0.03^{e}	13.85± 0.07^{d}	15.16± 0.01^{b}	15.59± 0.03^{a}
3-Methly-L-histidine	31.87± 0.04^{c}	32.38± 0.07^{b}	32.39± 0.01^{b}	36.36± 0.06^{a}	35.27± 0.06^{a}
L-Anserine	ND	ND	ND	ND	ND
L-Carnosine	ND	ND	ND	ND	ND
L-Arginine	ND	ND	ND	ND	ND

Table 3. Contd.

Hydroxy proline	0.56± 0.06^{a}	0.50± 0.03^{a}	0.53± 0.06^{a}	0.42± 0.01^{b}	0.33± 0.03^{c}
Proline	2.49± 0.01^{e}	2.55± 0.02^{d}	2.64± 0.01^{c}	2.84± 0.02^{a}	2.72± 0.02^{b}
Total non-essential amino acid	235.38	242.77	239.34	259.14	254.88
Total	305.94	316.8	311.5	346.82	344.08

Control, soybeans soaked in water for 6 h; PP-1, soybean soaked in water with 0.5% persimmon powder for 6 h; PP-2, soybeans soaked in water with 1% persimmon powder for 6 h; PP-3, soybeans soaked in water with 2.5% persimmon powder for 6 h; PP-4, soybeans soaked in water with 5% persimmon powder for 6 h. ND: Not detected. Values are expressed as Mean ± standard deviation of 3 replicates. Values followed by different superscripts within a row indicate significant difference ($p<0.05$).

Table 4. Mineral contents (mg/kg of dry weight) of soybean sprouts cultivated by different persimmon powders for 6 days.

Element	Sample				
	Control	**PP-1**	**PP-2**	**PP-3**	**PP-4**
Ca	3,089.92± 12.12^{a}	2,747.85± 20.02^{d}	2,116.91± 10.20^{e}	2,921.61± 6.44^{c}	3,066.19± 8.99^{b}
Cu	8.96± 0.04^{d}	11.11± 0.03a	10.00± 0.09^{c}	10.41± 0.10^{b}	9.99± 0.02^{c}
Fe	64.96± 5.21^{a}	58.81± 6.31^{ab}	62.24± 3.33^{a}	54.69± 1.21^{b}	56.42± 3.33^{ab}
K	13,577.38± 20.31^{a}	12,527.06± 18.22^{d}	12,863.96± 10.33^{e}	12,937.24± 11.22^{b}	12,614.16± 15.00^{c}
Mg	1,472.97± 9.32^{c}	1,443.21± 8.20^{d}	1,521.29± 9.00^{a}	1,477.87± 8.77^{c}	1,510.16± 5.31^{b}
Mn	30.21± 0.99^{a}	29.68± 2.10^{a}	31.58± 0.39^{a}	29.82± 1.31^{a}	31.71± 1.00^{a}
Na	213.19± 2.31^{c}	210.82± 1.98^{c}	214.13± 5.21^{bc}	223.90± 1.21^{a}	218.20± 1.98^{b}
Zn	52.08± 1.33^{b}	48.72± 2.00^{c}	56.49± 1.88^{b}	60.63± 2.44^{a}	62.51± 1.90^{a}
As	ND	ND	ND	ND	ND
Cd	ND	ND	ND	ND	ND
Hg	ND	ND	ND	ND	ND
Pb	ND	ND	ND	ND	ND
Total	18,509.67	17,077.26	16,876.60	17,716.17	17,569.34

Control, soybeans soaked in water for 6 h; PP-1, soybean soaked in water with 0.5% persimmon powder for 6 h; PP-2, soybeans soaked in water with 1% persimmon powder for 6 h; PP-3, soybeans soaked in water with 2.5% persimmon powder for 6 h; PP-4, soybeans soaked in water with 5% persimmon powder for 6 h. ND: Not detected. Values are expressed as Mean ± standard deviation of 3 replicates. Values followed by different superscripts within a row indicate significant difference ($p<0.05$).

fruit powder could enhance the yield, vitamin C content and antioxidative potential of soybean sprouts.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

In vitro direct organogenesis in response to floral reversion in lily

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Our previous study indicated that the tiger lily (*Lilium lancifolium* var. Flore Pleno) has a great ability to produce inflorescence bulbils in nature as a form of natural phenomenon of floral reversion in plants. This present research was carried out to investigate the artificial floral reversion in *in vitro* culture of two lilies (Asiatic hybrid cv."Black out"), and (*Lilium longiflorum* cv "White heaven") based on the type and developmental stage of explants plus the different concentrations of naphthalene acetic acid (NAA) and benzyl aminopurine (BA). Developmental changes were observed in both lilies in response to floral reversion which was enhanced by growth regulators under *in vitro* condition. The regeneration of vegetative organs was associated with certain degeneration of floral organs. Large bulblets and multiple shoots were formed only in specific regions in floral organs, precisely in two attached points: the boundary region between the receptacle with other floral organs and the branching point where the peduncle joins the pedicel. This direct organogenesis was highly dependent on type of lily, type and developmental stage of explants in addition to the concentration of BA and NAA in *in vitro* culture. However, 1 mg/L BA combined with 0.1 mg/L NAA was the optimum for regenerating shoots and bulblets in *in vitro* culture of both lilies after six weeks.

Key words: Floral reversion, organogenesis, lilies, active points, growth regulators, *in vitro*, bulblets.

INTRODUCTION

In nature, there are more than 250,000 species of flowering plants, and they represent the most wide spread groups of plants. Flowers are important sexual reproductive organs of flowering plants and source of fruit and seed for completing plants' life cycle.

However, in some species, the phenomenon of floral reversion occurs rarely in nature in response to adverse environment and it is affected by photoperiod and hormones. Furthermore, this phenomenon can be efficiently induced in some species *in vitro* under optimal concentrations of auxins and cytokinins (Tooke et al., 2005; lashman and Kamenetsky, 2006; Supriyo et al., 2013). It can also be an excellent method for propagating some ornamental geophytes. It has been shown that it is a great alternative to explants of underground storage organ for overcoming the problem of heavy contamination which usually occurs in these organs (Ziv and Lilien- Kipnis, 2000; Poluboyarova et al., 2011)

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Various floral organs such as pedicel and filament were taken from some geophytes as explants to regenerate shoots and bulblets in *in vitro* culture (Kumar et al., 2006; Nhut et al., 2001). The study of Liu and Burger (1986) observed that the explants which were taken very close from the receptacle and the most distilled section of the pedicel produced the greatest number of buds in *in vitro* culture (*Lilium longiflorum*).

In recent years, the histological study of *Allium altissimum* (Poluboyarova et al., 2014) showed that the morphogenic tissue in the fused area of stamens and sepals had the potency to regenerate shoot in *in vitro* culture. Moreover, several molecular genetic studies confirmed that the pedicel parts are different in their developmental processes in the main model plant (*Arabidopsis thaliana*) (Douglas and Riggs, 2005) and are genetically regulated by several genes (Cho and Cosgrove, 2000; Kirik et al., 1998; Song and Clark, 2005; Ragni et al., 2008).

The objective of the current study was to investigate the artificial floral reversion of two lilies based on the type and developmental stage of explants in addition to the concentrations of naphthalene acetic acid (NAA) and benzyl aminopurine (BA) in *in vitro* culture.

MATERIALS AND METHODS

This study was designed to investigate the organogenetic response of two lilies, Asiatic hybrid cv."Black out" and *L. longiflorum* cv "White heaven" to floral reversion process in *in vitro* culture. Three cultural experiments were done *in vitro*. The inflorescence segments were collected from plants grown in computerized greenhouses at the school of biological sciences, University of Plymouth during the year 2014. The explants were carefully washed and sterilized with 10% v:v sodium hypochlorite for 15 min. They were washed three to four times with sterilized distilled water before culturing. The explants were then cultured on Murashige and Skoog (MS) basal medium containing (30 g L^{-1}) sucrose (8 g L^{-1}) agar, pH 5.7, supplemented with different concentrations of NAA and BA. All cultures were incubated in a Gallenkamp growth cabinet under 16 h photoperiod, provided by cool-white fluorescent lamps with an irradiance of 100 µmol m^{-2} s^{-1} at a constant temperature of (25°C).

Three types of explants were taken for this study: explants of receptacle, explants of the branching point where the peduncle joins the pedicel and explants of whole flower bud. Four concentrations of BA and NAA: (1 mg/L BA+1 mg NAA), (1 mg/L BA+0.5 mg NAA), (1 mg/L BA+0.1 mg NAA) and (0 mg/L BA+0 mg NAA) as control were used. Two different developmental stages of explants were chosen: young explants were taken when the size of floral bud was 4 to 6 cm and mature explants were taken at fully mature stage. The receptacle explants were prepared by cutting the portion of receptacle into two identical pieces and the half piece was cultured horizontally on agar ; otherwise, all other explants were placed vertically on the agar.

Each experimental treatment was carried out with at least 15 explants per treatment. The experiment was arranged in a completely randomized block design. The number and weight (g) of bulblets and roots per explant, the number, weight (g) and length (cm) of shoots and the weight of ovary per explant were recorded after six weeks of culture *in vitro.*

The statistical analysis SAS system (SAS, 2012) was used to show the effect of different factors on the study parameters. Significant difference (LSD) test was used in this study to compare between means at the 0.05 level of significance.

RESULTS

Plate 1 (for Asiatic hybrid lily cv. Black out) and Plate 2 (for *L. longiflorum* cv. White heaven) shows that the processes of floral reversion after six weeks in *in vitro* culture, converting floral to vegetative organogenesis resulted in the formation of a wide range of vegetative organs using young and mature receptacle explants with different concentrations of naphthalene acetic acid (NAA) and benzyl aminopurine (BA) (Plates 1 and 2).

Plate 3 records the *in vitro* culture of Asiatic hybrid lily after six weeks using whole floral buds explants with different concentrations of (NAA) and (BA). The bulblets and shoots appeared on specific points in floral organs: receptacle boundary and in branching points of pedicel-peduncle of explants. Plate 4 shows the *in vitro* culture of Asiatic hybrid lily using young and mature branch explants of pedicel- peduncle with constant concentration of 1 mg/L BA combined with 0.1 mg/l NAA. Plate 5 shows the *in vitro* culture of longiflorum lily after six weeks using mature whole floral bud explants and mature receptacle explants with constant concentration of 1 mg/L BA combined with 0.1 mg/L NAA. Figure 1 records the results of the *in vitro* cultures of Asiatic hybrid lily for six weeks using different type and developmental stage of explants plus different concentrations of NAA and BA.

The explants of inflorescence stalk branch have higher ability to regenerate bulblets and shoots than receptacle ones (Figure 1A). The young explants produced more shoots but less bulblets compared to the mature ones (Figure 1B). 1 mg/L BA combined with 0.1 mg/L NAA was the optimum for regeneration of shoots and bulblets compared to others (Figure 1C). The interaction effect of all these experimental factors is indicated in Figure 1D and the results confirm that the regeneration ability of explants is dependent on all these factors. Figure 2 shows the results of *in vitro* cultures of both lilies for six weeks using different concentrations of NAA and BA with young receptacle explants. Longiflorum lily has higher ability to regenerate bulblets and shoots than Asiatic hybrid lily (Figure 2A). The interaction effect between the type of lily and concentrations of NAA and BA is recorded in Figure 2B, and the results confirm that the regeneration ability of explants is clearly dependent on these experimental factors.

Figure 3 records the results of the experiment of *in vitro* cultures of both lilies for six weeks using different type and developmental stage of explants with constant concentration of 1 mg/L BA combined with 0.1 mg/L NAA.

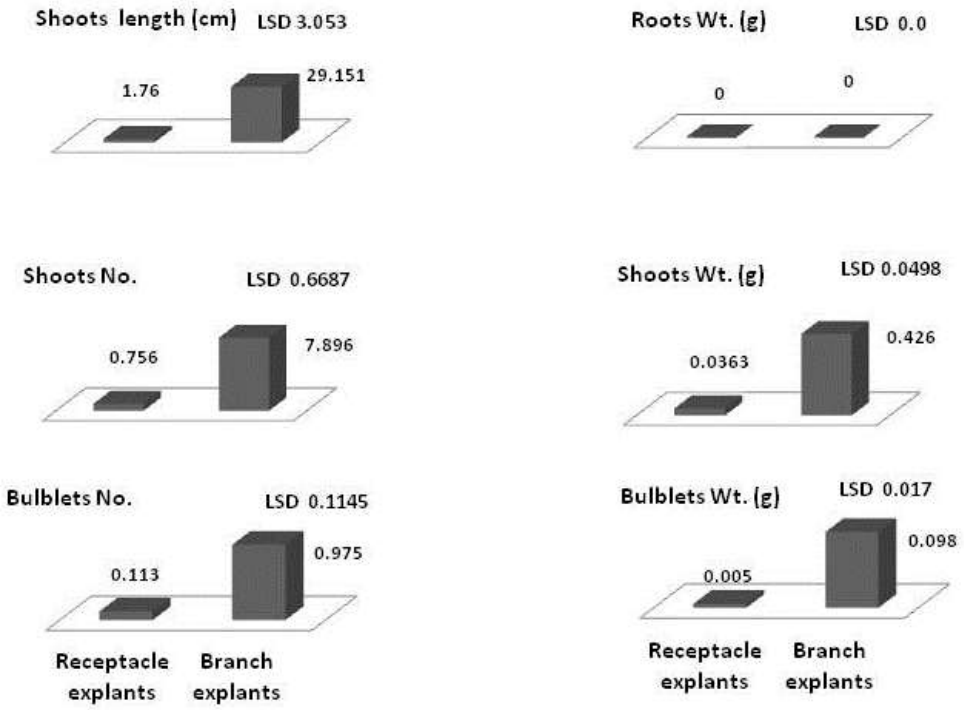

Figure 1A. Regeneration ability of explants of Asiatic hybrid lily after six weeks of culture *in vitro* as influenced by the type of explants. Using LSD test (p≤0.05).

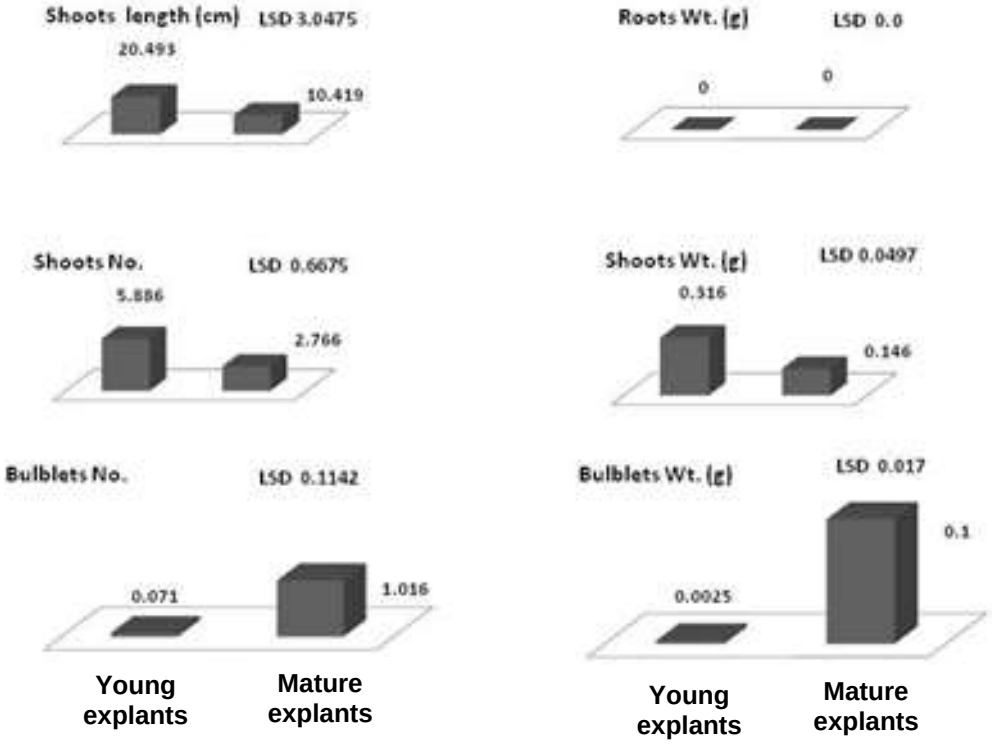

Figure 1B. Regeneration ability of explants of Asiatic hybrid lily after six weeks of culture *in vitro* as influenced by the developmental stage of explants. Using LSD test (p≤0.05).

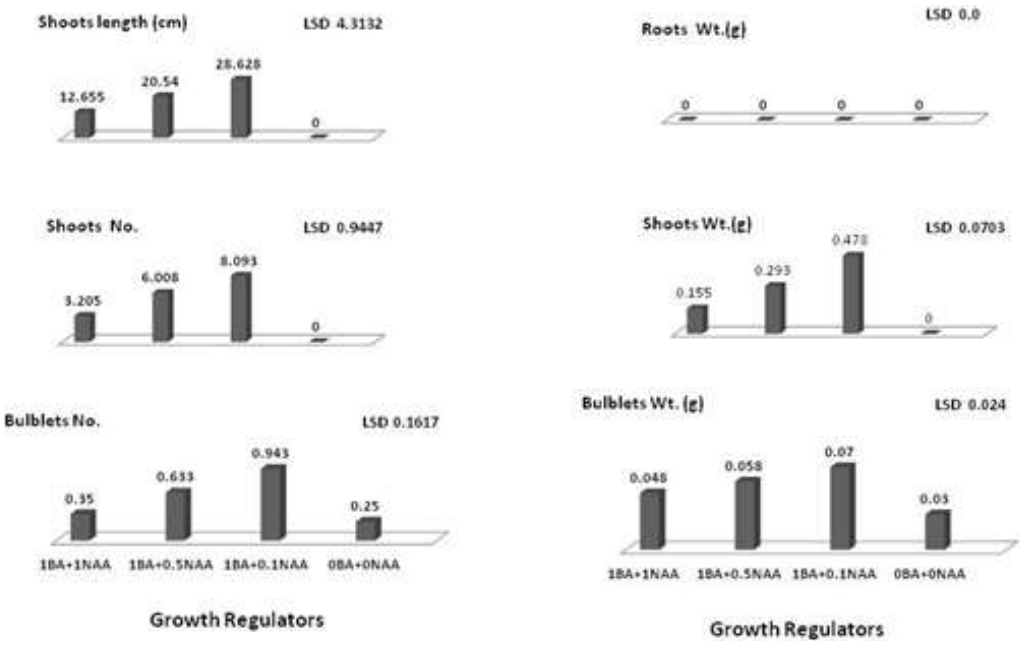

Figure 1C. Regeneration ability of explants of Asiatic hybrid lily after six weeks of culture *in vitro* as influenced by the concentration of NAA and BA. Using LSD test (p≤0.05).

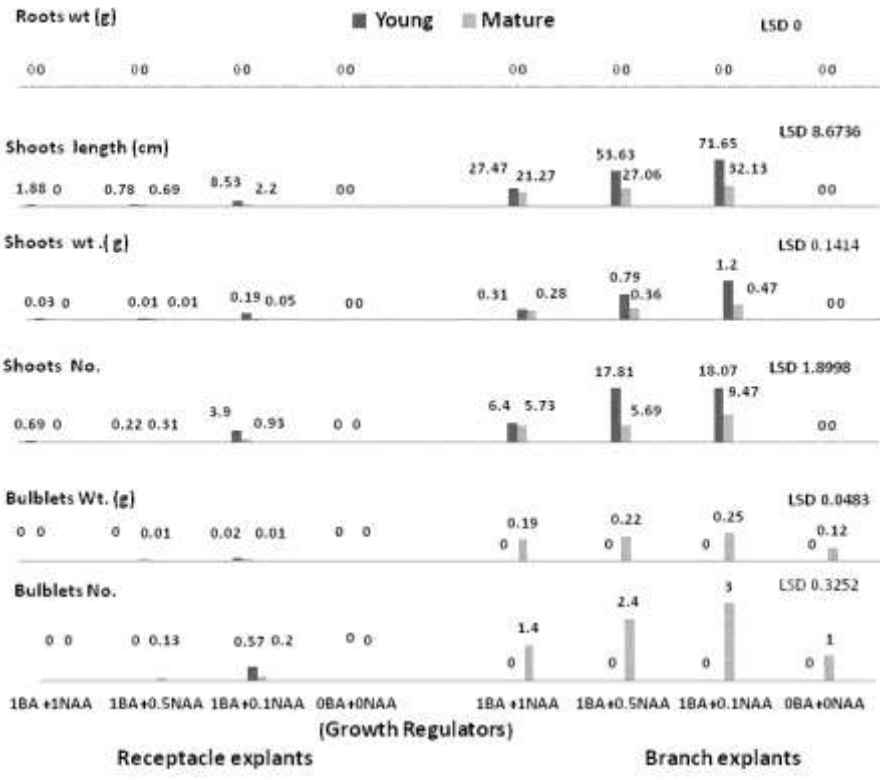

Figure 1D. Interaction effect of different concentration of plant growth regulators, type of explants and development stage of explants on the bulblets, shoots and roots regeneration of Asiatic lily in vitro. Using LDS test (p≤0.05).

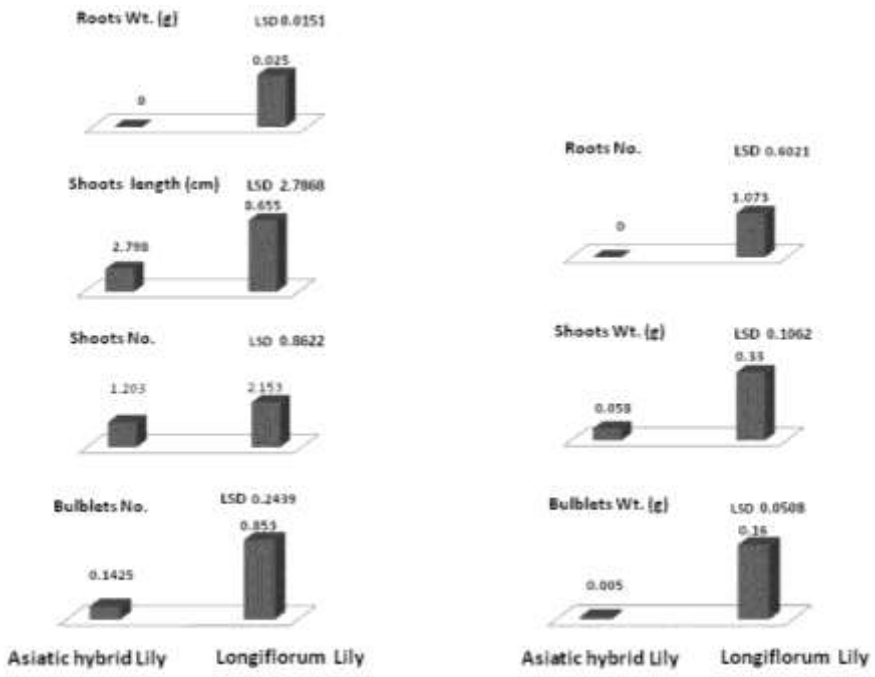

Figure 2A. Effect type of lily on the regeneration ability of explants after 6 weeks of culture *in vitro* using young receptacle explants using LSD test (p≤0.05).

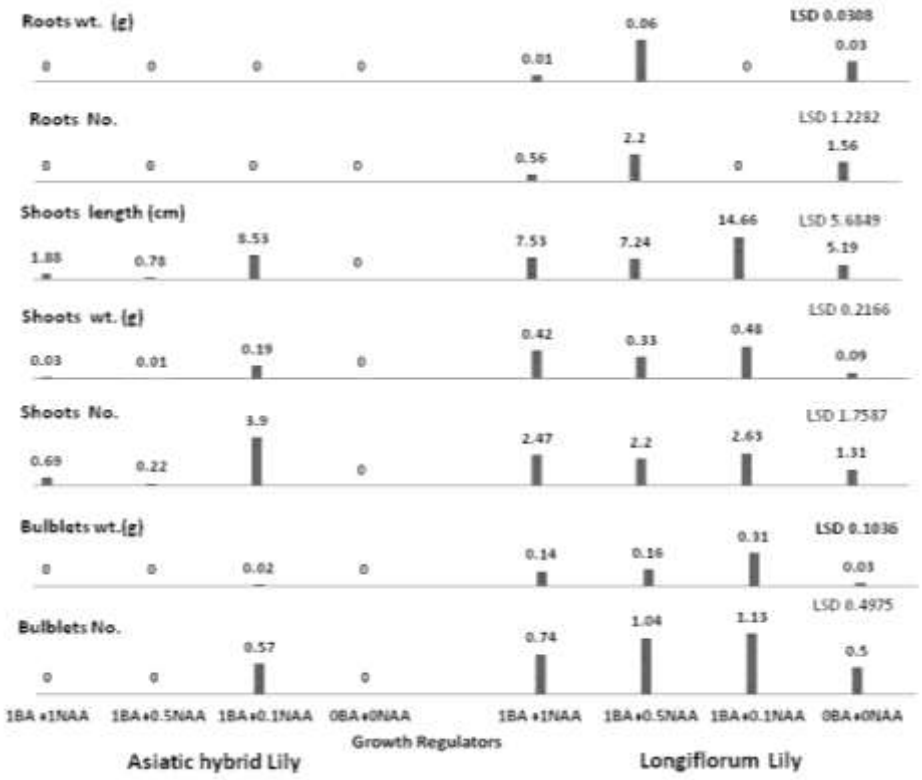

Figure 2B. Interaction effect of type of lily and different concentration of plant growth regulators on the bulblets, shoots and roots regeneration.

Plate 1. Floral reversion of Aslatic hybrid cv. Black out converting floral to vegetative organogenesis resulting in formation of a wide range of vegetative organs in young and mature receptacle explants after six weeks of *in vitro* culture using different concentration of naphthalene acetic acid (NAA) and benzyl aminopurine (BA). **A)** Great appearance of large bulblets on the boundary of receptacle-floral organs in young receptacle explants. **B)** Regeneration of multiple shoots on the boundary of receptacle-floral organs in young explants. **C)** Observation of buds and roots on the boundary of receptacle-flora organs in young explants. **D)** Formation of roots on young explants. **E)** Formation multiple roots in mature receptacle explants. **F)** The enlargements of ovary and shoot regeneration in mature receptacle explants.

Plate 2. Floral reversion of *Lilium longiflorum* cv. White heaven converting floral to vegetative organogenesis resulting information of a wide range of vegetative organs in young and mature receptacle explants after 6 weeks *in vitro* culture using different concentration of NAA and BA. **A)** Formation of large buds and shoots in young receptacle explants. **B)** Regeneration of shoots bulblets and roots in young explants. **C)** Observation of roots in the distal part of young explants. **D)** Formation of buds on the distal part of young receptacle explants. **E)** Appearance of abnormal shoots on the distal part of young explants. **F)** Development of shoots on the boundary of receptacle-floral organs of mature receptacle explants G-ovary enlargement and seeds formation were observed in mature receptacle explants.

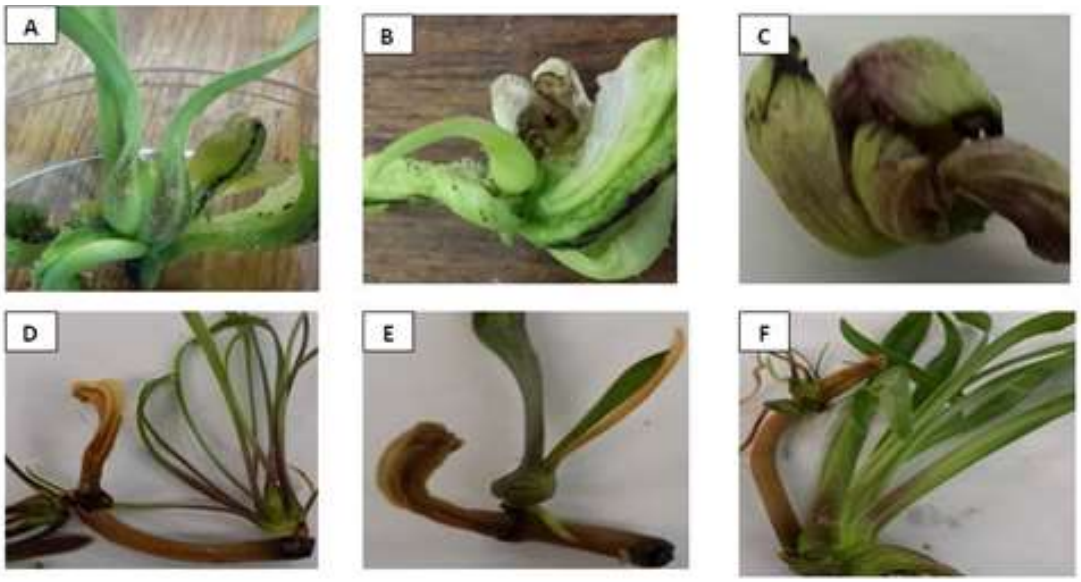

Plate 3. *In vitro* culture of Aslatic hybrid cv. Black out the bulblets and shoots rising from specific points. Flora organs-receptacle boundary and branching points of pedicle- peduncle in whole flora buds explants using different concentration of NAA and BA. **A)** Large bulblets appearance on the floral organs-receptacle boundary of young floral bud explants. **B)** Bulblet and shoots regeneration is associated with the floral organs degeneration. **C)** Floral reverse degeneration processes in young explants. **D)** Appearance of enlargement of whole ovary and the regeneration of bulblets and shoots on the both active points of mature explants. **E)** Large bulblets formation on the receptacle point of mature explants. **F)** Regeneration bulbelts, shoots and roots on the both active points of mature explants.

Plate 4. *In vitro* culture of Asiatic hybrid cv. Black out, after six weeks of culture in presence BA and NAA using young and mature branch explants of pedicel-peduncle, the result showed that the shoot and bulblets appeared only in branching points where pedicel join peduncle. **A)** Efficient regeneration of large multiple shoots on the young pedicle-peduncle branch explants. **B)** Development of shoots on the branching points in young explants. **C)** High performance of shoots regeneration of young explants. **D)** Bulblets and shoots regeneration on the branching points of mature pedicel-peduncle explants. **E)** Appearance of large bulblets on the point where the leave join the stem in mature explants. **F)** Development of large bulblet and shoots on the mature explants.

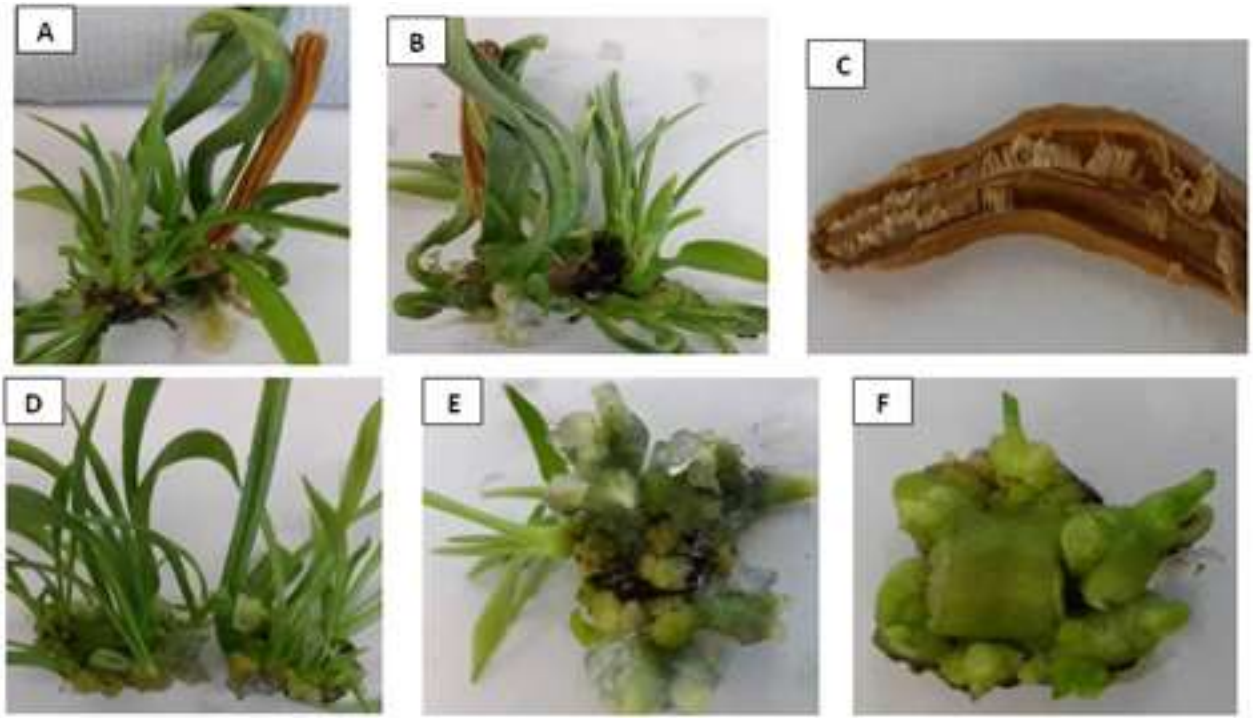

Plate 5. *In vitro* culture of *Lilium longiflorum* cv. White heaven, after 6weeks of culture using the mature whole floral buds explants and the mature receptacle explants with constant concentration of 1 mg/l BA combined with 0.1 mg/l NAA. A). Regeneration of shoots, bulblets and enlargements of ovary in mature whole floral buds explants. **B)** The vegetative organs regeneration in the boundary of receptacle-floral organs and distal part of receptacle in the mature whole flora bud explants. **C)** Seed formation inside ovary in mature whole flora buds explants. **D)** Shoots and bulblets regeneration in mature receptacle explants. E) Buds regeneration underneath adixal side which attached agar surface in mature receptacle explants. **F)** The mature receptacle explants were completely surrounded by the extensive numbers of buds and shoots on around cut surfaces.

The results of Figure 3A confirm that the whole floral bud explants had higher ability to regenerate bulblets, shoots and roots compared to receptacle explants. Figure 3B indicates that the regeneration ability of explants is highly dependent on all these experimental factors. However, the ovary enlargement was clearly observed in treatment of the mature receptacle of longiflorum lily.

DISCUSSION

Our previous study was concerned with the natural phenomenon of floral reversion in tiger lily *L. lancifolium* var. Flore Pleno, with high ability to produce inflorescence bulbils in nature (Asker, 2105). This present study investigates the artificial floral reversion processes in both lilies: Asiatic hybrid cv.”Black out" and *L. longiflorum* cv "White heaven”. They were enhanced by growth regulators under *in vitro* condition. Thus, many developmental changes were observed in response to this reversion when the regeneration of vegetative organs was associated with the degeneration of floral organs. Large bulblets and multiple shoots appeared in specific regions in floral organs, precisely in two attached points: the boundary region between the receptacle with other floral organs and the branching point where the peduncle joins the pedicel. This is in line with an histological study on *A. altissimum* (Poluboyarova et al., 2014) which reported that the morphogenic tissue in the area of fusion between stamens and sepals had the highest potency for direct shoot regeneration. This also agreed with the study of Ziv and Lilien- Kipnis (1997), which indicated that the pedicel - peduncle junction had high shoot regeneration in some geophytes.

That generated vegetative organs which appear only in specific points may be because the pedicel parts are different in their development process. Douglas and Riggs (2005) reported that the proximal portion and bulged distal region are different in their development in the model plant *(Arabidopsis thaliana*). Moreover, it was found that pedicle development process is under genetically control by several genes (Cho and Cosgrove, 2000; Kirik et al., 1998; Song and Clark, 2005; Ragni et al., 2008). The current results also showed that the direct

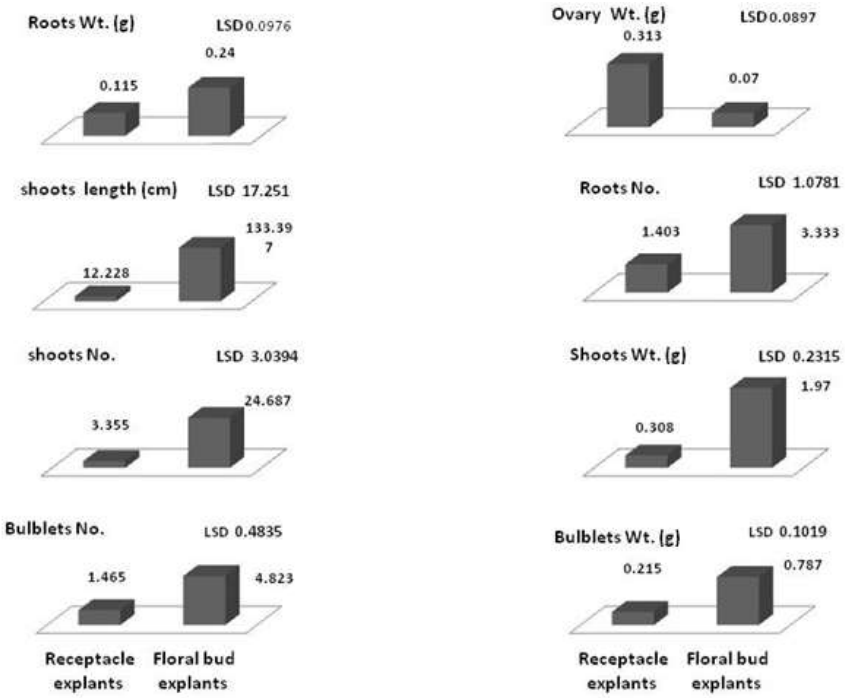

Figure 3A. Effect type of explants on the regeneration ability of explants after 6 weeks of of culture in vitro of both lilies using the concentration of 1mg/ 1BA combined with 0.1mg/1 NAA. Using LDS test (p≤0.05).

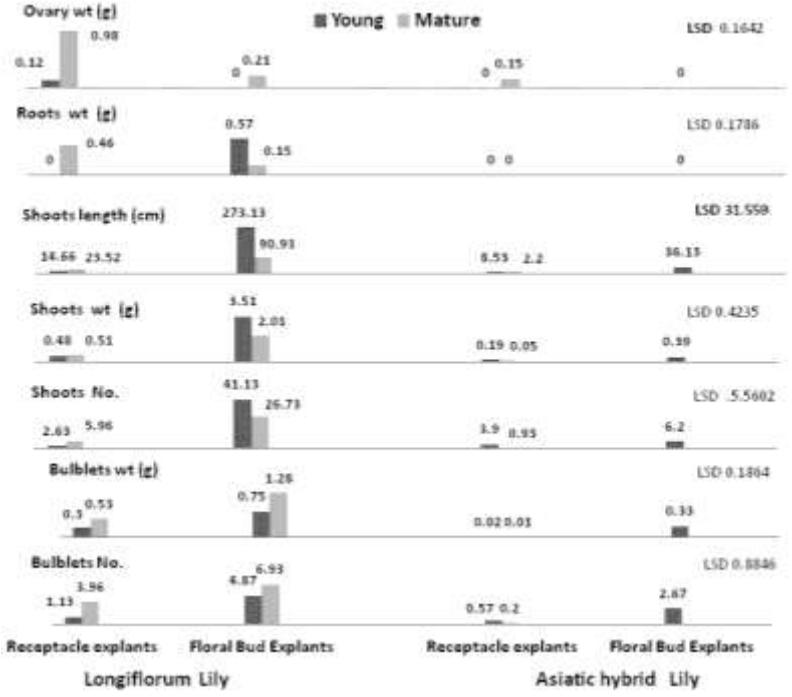

Figure 3B. Interaction effect of type of lily and the type and developmental stage of explants on the bulblets, shoot and roots regeneration after 6 weeks of culture in vitro using the concentration of 1mg/ 1BA combined with 0.1mg/1 NAA. Using LDS test (p≤0.05).

organogenesis which occurred in both lilies in response to floral reversion was highly dependent on type of lily, type of explants, developmental stage of explants in addition to the concentration of naphthalene acetic acid (NAA) and benzyl aminopurine (BA) *in vitro* culture.

L. longiflorum cv "White heaven" showed higher performance in regenerating bulblets and shoots and greater ability to enlarge ovary in response to floral reversion process compared to Asiatic hybrid cv."Black out". The inflorescence stalk branch explants and the whole floral bud explants produced more bulblets and shoots compared to the receptacle explants. Cytokinin (BA) combined with auxin (NAA) had great effect on the floral reversion process and subsequently on the performance of floral organs to regenerate bulblets, shoots and roots in *in vitro* culture. This result agrees with studies of Ziv and Lilien Kipnis (2000), Kumar et al. (2006), Werner and Schmulling (2009), Sakakibara (2006) and Bartrina et al. (2011) who demonstrated that these plant growth regulators play an essential role in plant morphogenesis *in vitro.*

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Genetic diversity and population structure of maize landraces from Côte d'Ivoire

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Maize (*Zea mays* L.) occupies an important place among food crops in Côte d'Ivoire. However, no study on the genetic diversity of the species has been performed to date. This study aims at analyzing the diversity and genetic structure of 35 maize accessions using 10 microsatellite markers. These accessions are from different agro-ecological zones representative of the Ivorian territory. The results showed that for all accessions studied, 47 alleles were detected, with an average of 6.71 ± 2.21 alleles per locus. The percentage of polymorphic loci and total genetic diversity, were 91.34% and 0.524 ± 0.159. A slight excess of heterozygotes was observed in the accessions (4.6%). The molecular analysis of variance showed that the total genetic diversity is mainly due to the intra-accession diversity (85.17%). The inter-accession diversity rate was estimated at 14.87%, which is in favor of moderate differentiation between the accessions. The Bayesian analysis grouped the accessions into two populations, regardless of geographical origin. These results provide basic information potentially useful in selection. The cross between two individuals from different groups might help exploit the phenomenon of heterosis.

Key words: *Ex situ* conservation, Côte d'Ivoire, genetic diversity, maize, microsatellite marker.

INTRODUCTION

Maize (*Zea mays* L.) is an annual herbaceous tropical crop. It was domesticated about 9000 years ago from teosinte (*Zea mays parviglumis* subspecies) in a medium-altitude region located in southern Mexico City (Van Heerwaarden et al., 2010). In West Africa, maize and rice (*Oryza* spp.) make up the main source of food for largely rural populations. In Côte d'Ivoire, the area planted with maize is 349 470 ha with an annual yield of 680 000 tons (FAOSTAT, 2015). It is grown in all the agro-ecological zones of the country and has various uses. Its grains are highly consumed, but they also serve as raw material for feed mills, breweries, oil-mills, manufacture of flour and starch. Despite such food and economic importance, the average maize yield remains low. It is estimated at

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1.95 t/ha (FAOSTAT, 2015) against more than 6 tons in Argentina and more than 10 in Chile and French Guiana (FAOSTAT, 2015). This remains a real problem and a serious limitation to the fight against food insecurity. Serious actions should be taken so as to improve maize yields in Côte d'Ivoire.

Local maize varieties are of major importance. They contain a range of genetic diversity necessary to increase and maintain the yield potential of maize in Côte d'Ivoire. In addition, they provide new sources of resistance to various biotic and abiotic stresses (Hammer and Teklu, 2008). The genetic diversity of local varieties is unanimously accepted as a prerequisite for enhancing agricultural productivity and food security. The optimal use of this diversity presupposes that it is characterized in all its aspects. The study of the morphological diversity of Ivorian maize has been discussed in some previous works. To our knowledge, the diversity based on molecular description has never been studied so far. This work is the first approach to the genetic variability of local varieties of Ivorian maize.

Microsatellites are considered to be good molecular markers. They are co-dominant, multiallelic and neutral vis-à-vis the selection process (Mondini et al., 2009). Microsatellites are relatively small and, therefore, are easily amplified using the PCR ("Polymerase Chain Reaction") technique. The procedure is relatively simple and fast, followed by a migration of the amplified fragments on an acrylamide gel or on a sequencing gel. The availability of automated DNA sequencers enables a high-speed analysis of a large number of samples. Several loci can be studied simultaneously when the primers used are labeled with different colored fluorophores (multiplex PCR). The technique requires a very low quantity of DNA. These technical and genetic features justify now the choice of microsatellites in estimating the genetic diversity of several cultivated species such as maize (Oppong et al., 2014), rice (Kumbhar et al., 2015), sorghum (Adugna, 2014), millet (Danjuma et al., 2014), wheat (Arora et al., 2014), barley (Chen et al., 2012) and quinoa (Bazile et al., 2014).

This study aims at assessing the variability and genetic structure of local varieties of Ivorian maize using microsatellite markers. The results of the assessment can contribute to the *ex situ* conservation and the development of selection strategies in order to improve maize yield in Côte d'Ivoire.

MATERIAL AND METHODS

Plant Material

This study concerned 35 maize accessions. Previously, a set of 116 accessions collected in different agro-ecological zones of Côte d'Ivoire was analyzed using 22 morphological and agronomic descriptors. Multivariate analyses helped to structure these 116 maize accessions into five groups. These 35 accessions were selected from five phenotypic groups identified by multivariate analyses, so as to represent the genetic diversity of the species. They were also selected based on their geographical origin. The accessions and their collection area are listed in Table 1.

Extraction of genomic DNA

Fifteen seedlings per accession were analyzed. The choice of this number was based on the studies of Warburton et al. (2010) and Wasala and Prasanna (2013). The total number of individuals to be analyzed was 525. Genomic DNA was extracted from 100 mg of fresh plant material taken from 10 to 15 days old seedlings. The extractions were performed according to the extraction kit protocol, *Thermo Scientific GeneJET Plant Genomic DNA Purification Mini Kit* (available on the website: www.thermoscientific.com/onebio) at the Central Laboratory of Biotechnology of the CNRA. The purity and concentration of the extracted DNA were verified respectively by electrophoresis on 0.8% agarose gel and by assay using the Nanodrop spectrophotometer (Thermo Scientific NanoDrop 2000). The extracted DNA was placed at -20°C.

Microsatellite markers used were 16 microsatellite loci markers (Supplied by InqabaBiotec) covering the entire maize genome which was analyzed for this study. These microsatellites were selected from the maize database (Maize GDB, 2016). The choice of markers was mainly based on the pattern, size and amplification quality. They are known to be polymorphic and used successfully in the study of the genetic diversity of maize by several authors (Matsuoka et al., 2002; N'guyen et al., 2012; and Pineda-Hidalgo et al., 2013). The sequence of primers, the location of such sequences in the genome and the repeated patterns are available on the website: http://www.maizegdb.org.

Amplification of microsatellite markers using PCR

PCR amplification was performed in 96-microwell plate. The final volume for PCR reaction was 10 µl and contained *DreamTaq* ™ *Green PCR Master Mix* 2X (Thermo Scientific, Inqaba Biotec), 0.20 µM 5'-tailed M13 forward and reverse primers (Inqaba Biotec) and 5ng DNA. The conditions of the PCR performed in a *GeneAmp PCR System 9700*-type thermal cycler (Applied Biosystems) were as follows (Warburton et al., 2002): a first denaturation at 94°C for 2 min, followed by 30 cycles, each comprising a denaturation at 94°C (30 s), a hybridization at the determined optimum temperature X°C (1 min) and an elongation at 72°C (1 min). A final elongation step at 72°C (5 min) was scheduled. X°C refers to hybridization temperatures of each primer, determined by the following equation: Ta = [2°C (A + T) + 4°C (G + C) - 5°C] (Newton and Graham, 1997). Each amplification of maize DNA extracts was assayed by electrophoresis on 2% agarose gel before genotyping. The amplification products were genotyped using a LI-COR® sequencer (LI-COR 4300 DNA Analyser, USA). The interpretation of genotypes was then carried out through the SAGA 2.0 software.

Data analysis

Genotyping data are represented as a matrix with markers in columns, and individuals in lines. Each marker is represented by two allelic forms. The matrix is cleaned by removal of individuals who showed a high number of missing data (over 20%). From this matrix, the standard parameters measuring genetic diversity were calculated for each locus, each accession and for all accessions. Prior to analyses, the hypotheses necessary for the implementation

Table 1. Maize accessions studied, and their origin.

N°	Code[a]	Collection site	Latitude	Longitude	Altitude (*masl*)[b]	No.ind.
1	Acc113	North-center	10°23′28″	6°26′13″	351 m	15
2	Acc-97	North-center	10°37′29″	6°15′8″	321 m	15
3	Acc-85	North-center	9°39′34″	7°37′28″	435 m	15
4	Acc-147	North-center	9°29′20″	5°21′43″	325 m	15
5	Acc-157	North-center	9°30′08″	5°31′24″	332 m	15
6	Acc-451	North-center	7°40′59″	5°01′59″	313 m	15
7	Acc-356	North-east	8°01′59″	2°47′59″	344 m	15
8	Acc-83	North-west	9°30′03″	7°42′32″	456m	15
9	Acc-176	Center-north	8°23′90″	4°30′47″	300 m	15
10	Acc-159	Center-north	8°17′40″	5°9′51″	318 m	15
11	Acc-163	Center-north	8°10′13″	5°6′38″	318 m	15
12	Acc-584	Center	7° 41′ 07″	5° 01′ 50″	353 m	15
13	Acc-578	Center	7° 41′ 07″	5° 01′ 50″	353 m	15
14	Acc-798	Center-West	6°20’52’	5°23’62’	153 m	15
15	Acc-800	Center-West	6°20’52’	5°23’62’	153 m	15
16	Acc-771	Center-West	6°01’28’	6°13’39’	214 m	15
17	Acc-569	Center-West	6°24.52’	5°22.07’	148 m	15
18	Acc-760	Center-West	6°05’1’’	6°4’49"	214 m	15
19	Acc-848	Center-West	-	-	-	15
20	Acc-782	Center-West	5°48’11’	5°20’36’	-	15
21	Acc-701	South	6°11.40’	3°48.12’	154 m	15
22	Acc-706	South	6°19.00’	3°57.15’	121 m	15
23	Acc-712	South	6°23.13’	3°53.01’	125 m	15
24	Acc-644	South	5°52 85’	4° 48 08’	40 m	15
25	Acc-750	South	5°28.78’	3°51.09’	26 m	15
26	Acc-597	South	5°29 81’	4°31 73’	66 m	15
27	Acc-628	South	5°38 86’	4°44 92’	42 m	15
28	Acc-645	South	5°55 62’	4°57 63’	88 m	15
29	Acc-608	South	5°52 85’	4° 48 08’	40 m	15
30	Acc-692	South	5°55.20’	4°20.66’	79 m	15
31	Acc-725	South	5°16.33’	2°58.77’	39 m	15
32	Acc-633	South	5°52 85’	4° 48 08’	40 m	15
33	Acc-621	South	5°29 81’	4°31 73’	66 m	15
34	Acc-814	South-West	5°54’21’	5°11’99’	-	15
35	Acc-788	South-West	6°06’45’	5°41’26’	222 m	15
Total						**525**

aAcc (Accession) bmasl (meters above sea level).

of population genetic models were verified (accordance with Hardy-Weinberg equilibrium and absence of linkage disequilibrium) as well as the potential presence of null alleles. The parameters measured were the percentage of polymorphic loci (*P* = number of polymorphic loci/total number of loci) at 95% threshold, the average number of alleles per locus, expressing allelic richness of loci (*Na* = number of alleles/number of loci), the number of efficient alleles (*Ne* = $1/\Sigma\ pi^2$), the number of private alleles (Nap: alleles exclusively present in a given accession), the heterozygosity observed (Ho) and the unbiased estimate of expected heterozygosity (*He* = $1-\Sigma pi^2$) under the Hardy-Weinberg hypothesis and the total genetic diversity (Ht), defined by Nei (1975). All these indices were calculated using the PopGene Version 1.32 software (Yeh et al., 1999) except for the number of private alleles calculated with GenAlex version 6.5 (Peakall and Smouse, 2006). Since the input format differs from one software to another, the software PGDSpider_2.0.9.1 (Lischer and Excoffier, 2012) was used to convert the files. The Micro-Checker software (Oosterhout et al., 2004) was used to verify the potential presence of null alleles.

The structure and level of differentiation of accessions were analyzed at different levels in order to highlight variability in the most optimal way. Genetic differentiation of populations was addressed in part by the standard F-statistics (FST, FIS and FIT) initially described by Wright (1978), corrected by Weir and

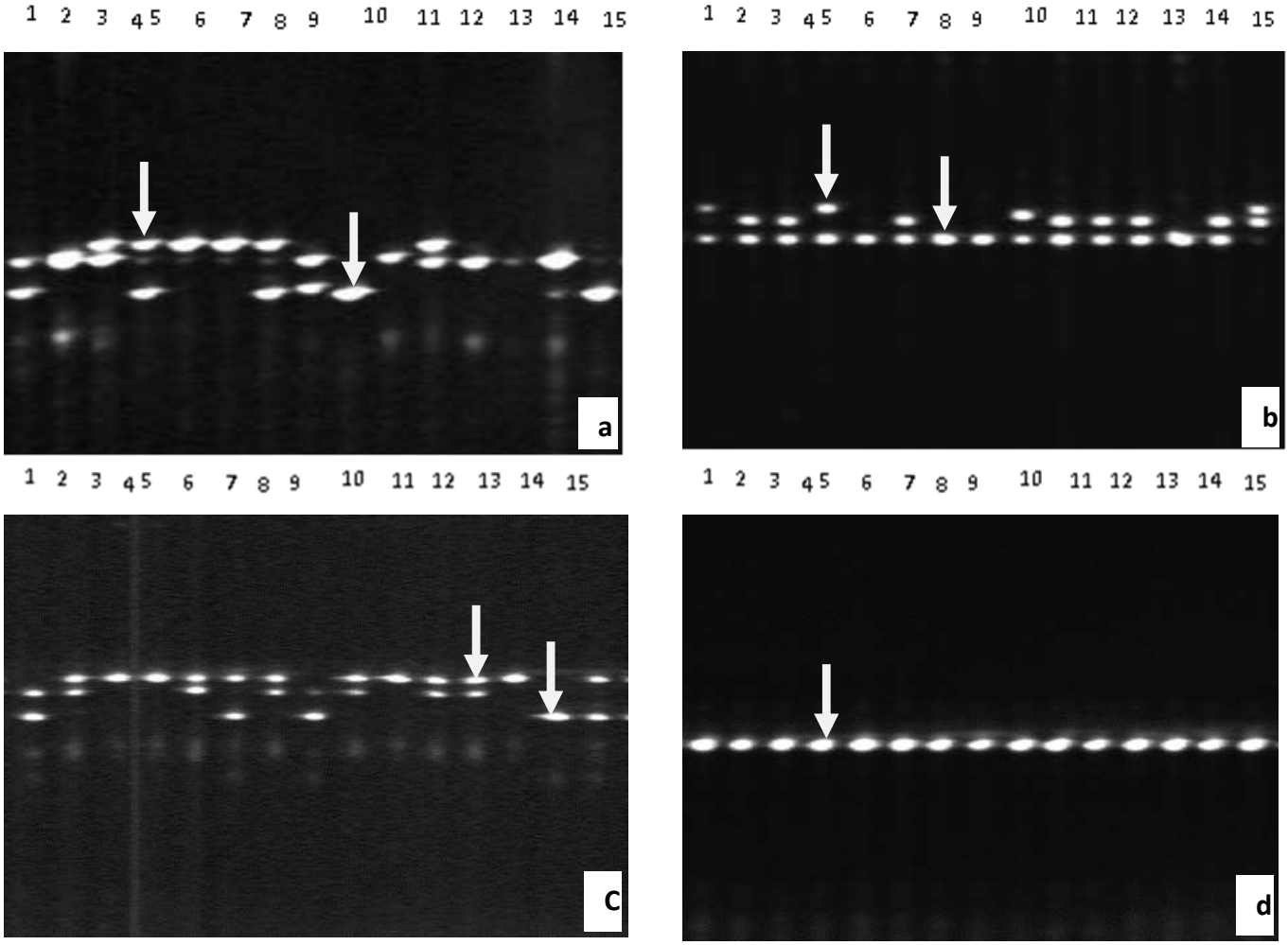

Figure 1. An example of a microsatellite profile in 33 corn accessions collected from Côte d'Ivoire, using the microsatellite primers phi085 (a), phi056 (b), phi083 (c), phi041 (d). The number of sampled individuals for each accession is shown in table 1.

Cockerham (1984) so as to derive unbiased estimators therefrom. These parameters were estimated using the FSTAT software for Windows, Version 2.9.3.2 (Goudet, 2002). On the other hand, a molecular analysis of variance (AMOVA) was performed using the Arlequin 3.5 software (Excoffier et al., 2005) to check whether the genetic variation was greater within accessions or between the accessions. A Bayesian analysis was performed using the STRUCTURE 2.3.4 software and method of Pritchard et al. (2000), with a view to detect a structuring of accessions in genetically different groups. This approach defines "K" subpopulations (classes), assigning individuals to groups under Hardy-Weinberg equilibrium, without a priori information on their membership in a population. Mixture models "admixture model" and correlated allele frequencies were used (Falush et al., 2003), with *Ks* varying from 1 to 10. Each model was set up using 10 000 iterations per100 000 burn-in, repeated 10 times for each value of K. The results of iterations were visualized using the STRUCTURE HARVESTER program (Earl and VonHoldt, 2012), available at: http://taylor0.biology.ucla.edu/structureHarvester/.The optimal number of "K"classes was determined according to the Evanno et al. (2005) method. Accessions having more than 60% of their genome from a group were assigned to such group. Below 60%, individuals were classified as intermediate (Yang et al., 2011).

RESULTS

Polymorphism of microsatellite markers

Of the 16 primers tested, six were eliminated as they showed no bands. Of the ten loci for which profiles were observed, three (phi041, phi102228, phi233376) proved monomorphic in all samples (Figure 1). Those markers were excluded from the analysis. Seven primers having polymorphic bands were used for analyses (Figure 1). Similarly, individuals having more than half of their markers to no avail were excluded, as too many missing data might unbalance and bias the analysis. The number of individuals selected for the analysis was 491.

Table 2. Diversity indices of the SSR loci used in the study.

Locus	Na	Ne	Nap	Ho*	He*	Nei (Ht)**
phi056	7	2.588	0	0.600	0.614	0.614
phi064	11	3.316	2 (5-11)	0.668	0.699	0.698
Phi083	6	2.185	1 (6)	0.593	0.543	0.542
Phi072	7	2.196	1 (7)	0.377	0.545	0.545
Phi085	7	2.788	1 (7)	0.488	0.642	0.641
Phi389203	4	1.624	0	0.318	0.385	0.384
Phi112	5	1.317	1 (1)	0.231	0.240	0.240
Total	47	16.014	6	-	-	-
Mean	6.71	2.288	-	0.468	0.524	0.524
SD	2.21	0.683	-	0.163	0.160	0.159

* Expected homozygosty and heterozygosity were computed using Levene (1949) ** Nei's (1975) expected heterozygosity.

The total number of alleles detected was 47 alleles, of which 6 were specific to a single accession (12.76%). The number of alleles per locus ranged from 4 (phi389203) to 11 (phi064) with an average of 6.71 ± 2.21 different alleles. The number of efficient alleles (Ne) ranged between 1.317 (phi112) and 3.316 (phi064), with an overall average of 2.288 ± 0.683. The heterozygosity observed switched from 0.231 for locus phi112 to 0.668 for locus phi064. The expected heterozygosity switched from 0.240 for phi112 to 0.699 for phi064. The average values of Ho and He were 0.468 ± 0.163 and 0.524 ± 0.160 respectively. The genetic diversity of Nei per locus switched from 0.240 for phi112 to 0.698 for phi064. Its multilocus value was high with an average of 0.524 ± 0.159 (Table 2).

Genetic diversity within accessions

The average values of genetic diversity indices per accession are presented in Table 3. The average number of alleles observed for seven loci within an accession ranged from 1.714 (acc356 and acc584) to 3.857 (acc760). Six accessions (acc97, acc569, acc608, acc628, acc645, acc706 and acc725) had a private allele each, of which 2 could be considered as rare alleles (Freq < 0.05). The percentage of polymorphic loci obtained for all accessions was 91.34% and ranged from 57.14% (acc159 and acc356) to 100% (acc97; acc85; acc147; acc83; acc176; acc798; acc800; acc771; acc569; acc760; acc848; acc750; acc597; acc628; acc645; acc608; acc725; acc633; acc788). The average values of Ho ranged from 0.260 ± 0.341 to 0.642 ± 0.176, respectively for accessions acc356 and acc848. The average values of He were in general lower than those observed. The lowest heterozygosity rate expected was recorded in accession acc356 (0.233 ± 0.277), while the highest rate was that of accession acc85 (0.630 ± 0.094). The highest genetic diversity of Nei was observed in accession acc85 (0.609 ± 0.09), while the lowest value concerned accession acc356 (0.225 ± 0.267).

Structure of accessions

F-statistics was calculated for each locus, and all loci are shown in Table 4. Fixation indices (Fis) were different from 0 and negative for some loci and for all of the analyzed loci (Fis = -0.046 ± 0.046). Fit values switched from -0.087 ± 0.082 for locus phi083 to 0.028 ± 0.065 for locus phi056. A moderate genetic differentiation was observed between the accessions. The Fst ranged from 0.100 ± 0.022 (phi064) to 0.233 ± 0.037 (phi072). The average change in all loci was 0.150 ± 0.022.

The molecular analysis of variance applied to all the accessions (Table 5) showed that the greatest portion of variance (85.17%) was due to the variation between individuals within accessions. The variation due to accessions within regions was low (14.87%), while the one due to regions was very low and even negative (-0.04%). Of the three sources of variation, the difference between regions was not significant ($p = 0.49071 > 0.05$).

The Bayesian analysis showed a structuring of accessions in homogeneous genetic groups. The values of Delta *K* Evanno ($\Delta K = 43.475$) showed that the most relevant partition was the one in two groups ($K = 2$). Figure 2 shows the assignment profiles of all accessions for *K* equal to two. Each color bar, red, green, or mixed, corresponds to an individual. Cluster 1 "red", consisted of 13 accessions of which half were represented by accessions from the north (7) and the other half by accessions from the center (3) and the South (3). Cluster 2 "green", gathered 12 accessions of which four were

Table 3. Summary of population diversity indices averaged over the 7 loci.

Accessions	P (0.99)	Na	Ne	Nap (Freq.)	Ho	He	Nei (Ht)
Acc113	85.71	2.714±1.113	1.733±0.744	-	0.390±0.302	0.358±0.233	0.346±0.225
Acc-97	100	3.000±0.817	2.332±0.788	1 (0.167)	0.573±0.221	0.554±0.130	0.536±0.126
Acc-85	100	3.429±0.976	2.690±0.694	-	0.601±0.249	0.630±0.094	0.609±0.091
Acc-147	100	3.429±1.134	2.528±0.870	-	0.468±0.240	0.560±0.240	0.539±0.231
Acc-157	85.71	2.429±0.787	1.745±0.575	-	0.378±0.273	0.372±0.252	0.359±0.243
Acc-451	85.71	2.571±1.134	1.901±0.655	-	0.395±0.296	0.415±0.267	0.400±0.257
Acc-356	57.14	1.714±0.756	1.468±0.614	-	0.260±0.341	0.233±0.277	0.225±0.267
Acc-83	100	2.714±0.951	2.127±0.576	-	0.519±0.230	0.522±0.115	0.504±0.112
Acc-176	100	3.429±1.272	2.513±0.822	-	0.451±0.304	0.568±0.210	0.549±0.203
Acc-159	57.14	1.857±0.899	1.592±0.559	-	0.331±0.337	0.300±0.281	0.290±0.272
Acc-163	85.71	3.000±1.528	2.030±0.700	-	0.438±0.285	0.461±0.233	0.446±0.225
Acc-584	71.43	1.714±0.488	1.402±0.441	-	0.295±0.322	0.235±0.229	0.228±0.221
Acc-798	100	2.857±0.690	2.057±0.590	-	0.517±0.333	0.495±0.168	0.475±0.161
Acc-800	100	3.000±1.000	2.269±0.656	-	0.533±0.144	0.544±0.144	0.525±0.139
Acc-771	100	2.143±0.378	1.626±0.287	-	0.503±0.238	0.381±0.116	0.368±0.112
Acc-569	100	3.429±1.397	1.848±0.559	1 (0.300)	0.421±0.189	0.432±0.175	0.417±0.169
Acc-760	100	3.857±1.676	2.581±1.052	-	0.598± 0.205	0.566±0.208	0.547±0.201
Acc-848	100	3.000±1.155	2.226±0.702	-	0.642±0.176	0.532±0.142	0.514±0.137
Acc-782	85.71	2.571±0.976	2.071±0.697	-	0.511±0.335	0.476±0.223	0.459±0.220
Acc-701	85.71	2.429±0.976	1.580±0.496	-	0.409±0.364	0.318±0.232	0.308±0.225
Acc-706	85.71	2.571±1.272	1.913±0.924	1 (0.269)	0.497±0.301	0.405±0.241	0.391±0.232
Acc-644	85.71	2.714±1.113	1.862±0.997	-	0.362±0.315	0.351±0.291	0.340±0.281
Acc-750	100	3.143±1.069	2.022±0.660	-	0.560±0.171	0.481±0.155	0.465±0.150
Acc-597	100	3.143±1.345	2.487±1.170	-	0.396±0.252	0.529±0.246	0.511±0.238
Acc-628	100	3.143±1.865	1.861±0.954	1 (0.033)	0.352±0.260	0.378±0.248	0.366±0.239
Acc-645	100	2.571±0.535	1.973±0.504	1 (0.033)	0.554±0.305	0.474±0.173	0.458±0.167
Acc-608	100	2.429±0.787	1.630±0.329	-	0.413±0.209	0.377±0.137	0.364±0.132
Acc-692	85.71	2.429±0.976	1.587±0.298	-	0.412±0.223	0.357±0.166	0.344±0.161
Acc-725	100	3.143±1.215	2.279±0.468	1 (0.067)	0.599±0.289	0.567±0.082	0.548±0.079
Acc-633	100	3.429±1.512	2.671±0.854	-	0.613±0.143	0.618±0.111	0.597±0.107
Acc-621	85.71	3.000±1.414	2.320±0.780	-	0.579±0.336	0.522±0.247	0.505±0.239
Acc-814	71.43	2.857±1.773	1.812±0.890	-	0.405±0.377	0.342±0.303	0.330±0.292
Acc-788	100	2.857±0.690	1.914±0.429	-	0.486±0.137	0.468±0.136	0.453±0.132
Mean	91.34	2.810±0.507	2.020±0.361	6	0.469±0.099	0.449±0.106	0.434±0.102

from the north, three from the center and three five from the south. The other eight accessions made up the intermediate group.

DISCUSSION

The local genetic resources constitute a basis for the improvement of production capacities of a species in its environment and in other areas. In the characterization of genetic resources, the first works concern most of the time agro-morphological studies. However, morphological studies alone do not provide sufficient information to help understand neither the genetic diversity of species nor their resemblance to other species. The molecular analysis by microsatellite provides additional information on genetic diversity. Previous studies have shown that maize contains an abundant number of microsatellites (Senior et al., 1998), highly polymorphic even between samples of small size (Chin et al., 1996). To our knowledge, it is the first time the study of the genetic diversity of maize has been performed using microsatellite markers in Côte d'Ivoire.

This study revealed a relatively high allelic richness in the different maize accessions analyzed. The average number of alleles per primer is almost similar to the one

Table 4. Wright's F statistics for 7 polymorphic loci for x corn accessions.

Locus	f=Fis*	F=Fit*	θ = Fst*	*Nm*
phi056	-0.104±0.053	0.028±0.065	0.119±0.033	1.517
phi064	-0.057±0.061	0.049±0.064	0.100±0.022	1.760
phi072	0.106±0.065	0.314±0.062	0.233±0.037	0.729
phi112	-0.118±0.062	0.042±0.055	0.143±0.018	1.274
phi389203	0.053±0.068	0.176±0.061	0.131±0.020	1.334
phi083	-0.236±0.074	-0.087±0.082	0.121±0.034	1.565
phi085	0.050±0.071	0.244±0.068	0.204±0.036	0.855
Averages per loci	-0.046±0.046	0.111±0.057	0.150±0.022	1.279±0.351

* Fis = the fixation index of individuals within populations; Fit = the fixation index with respect to the total population; Fst = the proportion of genetic differentiation; Nm = gene flow.

Table 5. Analysis of molecular variance (AMOVA) between the maize regions of collection, between the accessions within geographical regions, and within maize landrace populations.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P**	Fixation Indices
Between groups	2	18.415	-0.00065 va	-0.04	0.49071	F_{CT} = -0.00036
Between populations within groups	30	282.739	0.26557 vb	14.87	0.00000	F_{SC} = 0.14861
Within populations	949	1443.841	1.52143 vc	85.17	0.00000	F_{ST} = 0.14830
Total	981	1744.995	1.78636	-	-	-

*Probability of having statistical and variance valuesΦhigher than the values observed, due to chance only. Significance tests (1023 permutations).

reported by Barcaccia et al. (2003). But it is high, compared to the value 3.85 observed by Legesse et al. (2007) and 5.34 found by Yu et al. (2007). The genetic diversity observed in this study is also higher than the one reported by Rupp et al. (2009) and Park et al. (2008) on maize. These results could be explained in part by the sampling technique applied which took into account not only geographic differences but also morphological differences. Bogyo et al. (1980) showed that the sampling based on geographic origin and morphological variation is the most efficient way, to capture the maximum genetic diversity.

These results might also be due to the genetic nature of plant material. Indeed, these authors in their studies have focused on hybrid varieties or lines. But these varieties have less diversity than population varieties because of the selection pressure. However, the genetic diversity estimates obtained in this work seem less important than those obtained for microsatellite loci in other works on maize. Kostova et al. (2007) reported an average number of alleles by 9.1 and genetic diversity by 0.713 for Bulgarian maize, using 18 SSRs. Qi-Lun et al. (2008) reported that the Na and He values for local varieties in China were 7.93 and 0.70 respectively. Wasala and Prasanna (2013) analyzed the genetic diversity of local populations of India maize using 42 microsatellites. They deduced therefrom Na by 13.1 and He by 0.63. Similarly, Oppong et al. (2014) found by means of 20 microsatellite a Na value equal to 7.3 for local varieties of Ghanaian maize. The number of alleles reported in a diversity study is usually proportional to the sample size (Foulley et al., 2006). Some differences observed here could be attributed to the sampling difference. However, another factor affecting the number of alleles is the use of di-nucleotide microsatellite sequence. According to Liu et al. (2003) the di-nucleotide microsatellite sequences produce a higher number of alleles and show greater genetic diversity. The significant allelic richness observed in these previous studies might be partly linked to the high number of di-nucleotide used.

The genetic structure analysis suggests a slight excess of heterozygote relatively having panmictic proportions. This result is in accordance with the observations made by Eloi et al. (2012). In the literature, a deficit of heterozygotes was often encountered in maize (Yao et al., 2007; Pineda-Hidalgo et al., 2013.). Brown (1979) explained that a widespread deficit of heterozygotes is found among preferentially allogamous species. The results obtained do not confirm the observations of those authors. The excess of heterozygosity observed might

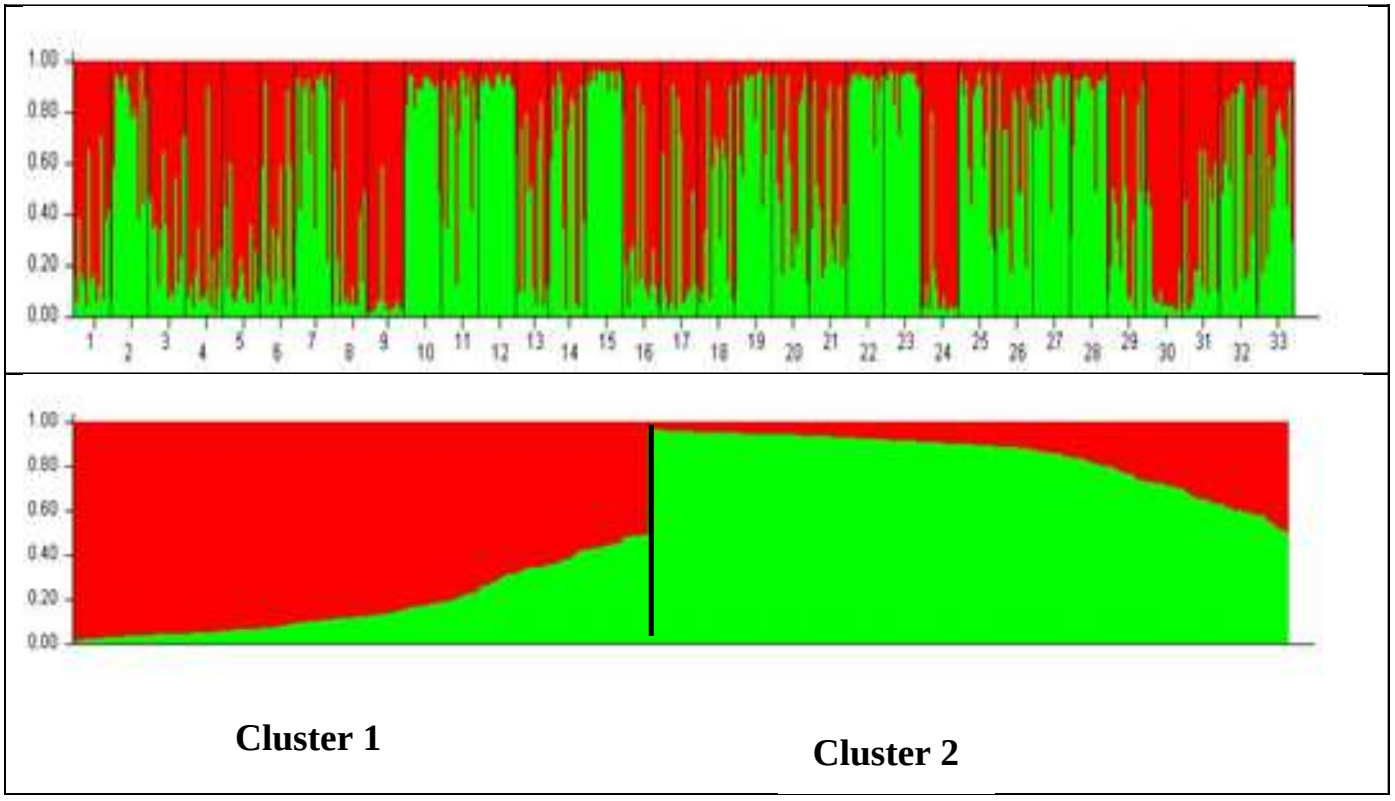

Figure 2. Bayesian individual clustering results with Structure for k=2. Colored bars represent proportions of membership of each individual to each cluster. The accession numbers of corn are shown in Table 1.

come from a strong selection in favor of heterozygotes. In maize, inbreeding depression negatively affects the size and shape of the ears, while heterosis produces longer ears, more and often larger grains (Jain and Bharadwaj, 2014). By choosing as seed to renew their crops the biggest and most beautiful ears, farmers unwittingly select heterozygous individuals.

The coefficient of genetic differentiation (Fst = 0.150 ± 0.022) reveals that there is a moderate differentiation between accessions (Wright, 1978). This result is in accordance with the results of Qi-Lun et al. (2008) and those of Pineda-Hidalgo et al. (2013). This could be explained by the fact that there are moderate gene flows, due to the proximity of neighboring fields or seed exchange between different groups living in different regions. For a naturally allogamous species like maize, these biological events are more probable, as exchanges between populations are favored by cross-pollination. This value is still higher than the one obtained by Pressoir and Berthaud (2004) for local populations of maize in the region of Oaxaca, Mexico (Fst = 0.011). The low genetic differentiation observed by these authors might be due to the geographical distance between populations. These authors worked on populations from a single region. But according to Affre et al. (2003), genetic differentiation increases with the geographic distance of agricultural plots. Fst values are very low on short distances because gene flows are more frequent and increase, generally, when agricultural plots are increasingly remote.

The AMOVA analysis showed that genetic diversity is greater within accessions than between them, which confirms the results of Fst previously calculated. The significant genetic variability within each accession could be favored by the mode of reproduction preferentially allogamous found in maize. This result is consistent with previous studies carried out on this species (Hoxha et al., 2004) and more generally on allogamous species (Wanjala et al., 2013). According to Affre et al. (2003) and Nybom (2004) allogamous species usually maintain a strong genetic variation within populations and a low genetic differentiation between populations, conversely, in autogamous species. These trends are similar to those reported by Hamrick and God (1997), in a benchmark study in which these authors showed that intra accession genetic diversity ranges from 0.103 to 0.266 in allogamous crops. Exchange or mixture of seeds by farmers is also another factor justifying the high variability within accessions. Generally, varieties grown by farmers are mixtures of populations.

The Bayesian clustering analysis by structure described two genetic groups, regardless of geographical origin. These results are similar to those obtained by Jia et al. (2013) on green foxtail. This structuring can be explained by the existence of a common genetic basis between the different accessions despite the geographical and phenotypic divergence. Indeed, the detailed examination of the genetic profile of each accession unveils a genetic introgression, reflecting the gene flows occurring between the different accessions.

CONCLUSION AND IMPLICATIONS

This study has helped in identifying seven polymorphic microsatellite markers that could be used more widely in the characterization of maize in Côte d'Ivoire. These markers showed a quite significant genetic variability in the accessions analyzed. Of all the 491 genotyped individuals, a total of 47 different alleles were identified. Some accessions showed alleles that are specific to them, that is, a percentage of 12.76% of total alleles. A slight excess of heterozygotes was observed in accessions (4.6%), which is the result of a peasant selection in favor of heterozygotes. The accessions studied show high levels of intra-accession genetic diversity and moderate differentiation. The conservation of small number of accessions *ex situ*, could be enough to represent the diversity found in cultivars, as each accession is genetically rich. Such conservation must involve first the accessions which showed a high level of polymorphism and private alleles. In the case of this study, priority accessions could be: acc97, acc569, acc628, acc633, acc645 and acc706. The fact that the rare alleles have more chance of disappearing by genetic drift is another factor to consider in setting conservation priorities.

A structuring into two populations has been highlighted. The genetic diversity observed is promising for the development of new cultivars. Both groups obtained can be used for the formation and improvement of heterotic pools. The cross between two individuals of different pools might help to exploit the phenomenon of heterosis. However, this work remains a preliminary study of genetic diversity of corn in Côte d'Ivoire. It should be further strengthened by increasing the number of microsatellite as well as the number of samples studied. Moreover, the use of much more variable markers such as SNPs, will enable to better structure and describe genetic diversity, with a view to its management and exploitation in plant breeding programs.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Molecular genetic diversity in a core of cocoa (*Theobroma cacao* L.) clones with potential for selection of disease resistance, plant height and fruit production

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This study aimed to assess the genetic variability in groups of 11 clones of *Theobroma cacao* L., from different geographical regions, based on microsatellite markers, with the interest to characterize germplasm for breeding. The products of the amplification of these materials with 15 simple sequence repeat (SSR) markers were separated into ABI377 sequencer. The genotype encoded data were analyzed by means of Roger's genetic distances, which was employed in the main coordinate's analysis. The high heterozygosity observed in this group of clones (Ho = 0.7276) and genetic distances between pairs of clones (average 0.75) show that there is a high diversity among these clones. In cluster analysis, the cluster of Trinitario clones and hybrids was separated from the others according to their genealogy, while Forasteros were classified into different groups. The variability among these clones makes them important materials for parent selection in order to obtain hybrid progenies.

Key words: *Theobroma cacao,* genetic diversity, simple sequence repeat (SSR), germplasm, breeding.

INTRODUCTION

Theobroma cacao L. is relatively new in domestication (Dias, 2001) and its morphological features vary. The genus, *Theobroma* is typical of neotropical regions, with a natural distribution that covers the tropical rainforest in the western hemisphere, between latitudes 18° N and 15° S, spreading from southern Mexico to the Amazon Forest (Cuatrecasas, 1964). But this distribution may have been greatly influenced by pre-Columbian civilizations, which were responsible for the domestication of cacao and its distribution throughout Latin America over 2000 years of

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cultivation. According to Venturieri (1993), there are 22 species of the genus, *Theobroma* which are restricted to tropical America. Nine of them are found in the Brazilian Amazon and are fit for cooking, and only five fit the preference for chocolate production. Among them, the species *T. cacao* and *Theobroma grandiflorum* S. stand out in the chocolate industry. The probable center of genetic diversity of cocoa, according to Cheesman (1944), covers the region of Napo, Putumayo and Caqueta, in the upper Amazon.

According to the characteristics of fruits and seeds and the geographic distribution, cocoa can be classified into three major racial groups: (i) the Forasteros, with flat, purple seeds, high hardiness and high yield potential (the Forasteros of the upper Amazon are vigorous, early maturing and resistant to certain pathogens; while the species found at the lower Amazon is known for the uniformity of its fruits, called Amelonados), (ii) the Criollo, which were originally cultivated by indigenous peoples of Mesoamerica (Mexico, Guatemala, Belize, Honduras and El Salvador) and have large seeds, rounded cotyledons and white or light violet when they are wet; (iii) and the Trinitario, natural hybrids between Forasteros and Criollo; although they are included in the group of Forasteros, they form a group that share intermediate characteristics (Dias, 2001; Bartley, 2005; Almeida and Valle, 2007).

Several studies on the genetic diversity of cacao sought to characterize and define genetic groups (Santos et al., 2005; Sounigo et al., 2005; Faleiro et al., 2004a, b; Dias, 2001; Risterucci et al., 2000; Lanaud et al., 1999; Lerceteau, 1997a, b; Russel et al., 1993). Some studies made the assumption that it is possible to differentiate the Criollo of Forasteros by isoenzymes (Ronning and Schnell, 1994) or by means of DNA markers (Laurent et al., 1993a, b). Attempts to categorize the genotypes of cocoa in the three racial groups were frustrated when using morphological descriptors (Engels, 1983) and isoenzymes (Lanaud et al., 1987) which prevented the separation into races of vegetables as initially proposed. The use of molecular markers could prove that the upper Amazon Forasteros, lower Amazon Forastero, Criollo and Trinitario have genetic differences. Studies of genetic diversity in *T. cacao* have been previously used mainly for demarcation of geographical regions (Figueira et al., 1994), discrimination of subgroups, determination of the ancestry of Criollo and modern Criollo, within the group Criollo (Motamayor et al., 2002) and delimitation of the breakdown of material Trinitario, Criollo and lower Amazon Forastero (Motamayor et al., 2003).

The international collections of germplasm are being characterized by their genetic diversity based on microsatellite markers. By means of the use of SSR markers to analyze hundreds of genotypes from commercial plantations and compare them with 246 genotypes from five collections of cocoa (germplasm banks and groups of parents of African cocoa clones), it was shown that there is great genetic differentiation among populations, except among the genebank of the West Africa and materials cultivated in countries of that region (Aikpokpodion et al., 2009). Among the 612 accessions from six groups of old cocoa genotypes, only 316 were kept for diversity analysis, since these markers allowed excluding the remaining accessions and identified as duplicates or misclassified. Such diversity analysis revealed strong structure of genetic diversity, consistent with the hydrographic division of the main rivers of the Peruvian Amazon (Zhang et al., 2009). Therefore, microsatellites have been instrumental in the management of germplasm banks and in understanding the structure of cocoa populations.

The planting of large commercial cocoa growing regions have been characterized by genetic diversity and possible involvement of different genetic groups in their composition. In Madagascar, SSR were used in diversity analysis of 27 cocoa clones and nine Trinitarians Criollo. This investigation revealed the distribution of Trinitario along a continuum between the Criollo and foreigners of the lower Amazon, except that the clones had high introgressions of alleles from Amazon. This shows that the Trinitario have as likely parent, the Amazonian individuals whose genetic diversity is low (Motamayor et al., 2002). Additionally, the hybrids with at least one parent of the high Amazon showed marked diversity. The low genetic diversity found in the family relationship between Trinitario and low Amelonados Amazon is the result of a few low Amazonian genotypes involved in the genealogy of Trinitario (Motamayor et al., 2003). Cocoa clones resistant to witches' broom grown in southern Bahia, Brazil, have high inter-genetic relationship due to the predominance of crossings of Scavina 6 with about a dozen other clones (Faleiro et al., 2001). In the analysis of 30 clones grown in this region, and four international clones of different geographic origins, it was observed that the genetic distances computed on the basis of microsatellite markers range from 0.13 to 0.71, revealing high genetic diversity among these materials (Faleiro et al., 2004a). In the analysis of 36 clones currently grown in southern Bahia, it was found that clones with potential tolerance to drought stress have genetic diversity comparable to international groups of clones such as TSH 1188 and CCN 51 (Bertolde et al., 2010). By examining 332 genotypes from farms in West Africa based on 12 SSR loci, the genetic variability and the presence of alleles associated with amelonado materials (potentially associated with high organoleptic qualities of cocoa) was shown. But this materials has been rarely used in these crop production in West Africa, highlighting the need to include local selections in germplasm banks, for conservation purposes (Aikpokpodion et al., 2009). The large-scale studies of genetic diversity of cacao has indicated more comprehensive ways to define the cacao genetic groups and eliminate access of misidentifications in the germplasm bank (Motamayor et al., 2008). In several other studies where cocoa is grown, also there

Table 1. Identification, geographical origin and type (racial groups), and height and disease resistance traits of 11 clones of *T. cacao*.

Clone	Name of clone	Origin	Historic	[a]Type	[b]Height	[c]Resist.	[d]Ref.
AMAZ 15	Amazon River	Peru-Ecuador	Wild	UA	M	VB	1
IMC 47	Iquitos Maraño (or Mixed) Calabacilo	Peru-Amazon	Wild	UA	M-H	Ce	2
SCA 24	Scavina	Peru	Wild	UA	L	VB	3
LCT EEN 37A	London Cocoa Trade Experimental Station of Napo	Ecuador	Wild	UA	M	VB	4
LCT EEN 162/1010	London Cocoa Trade Experimental Station of Napo	Ecuador	Wild	UA	M	VB	5
CCN 51	Collección Castro Naranjal	Ecuador	Cultivated	CH	M	VB	6
ICS 95	Imperial College Selection	Trinidad	Cultivated	T	L-M	Mo	7
TSH 1188	Trinidad Selection Hybrid	Trinidad	Cultivated	UH	H	VB, PP	8
COCA 3370/5	Coca River	Ecuador	Wild	UA	M-H	VB	9
GU 261	French Guyanese	French Guiana	Cultivated	LA	M-H	VB	10
H 56	Huallaga	Peru	Wild	UA	M-H	Mo	11

[a]Type: UA = Upper Amazon Forastero; CH = complex hybrid; T = Trinitario; UH = Upper Amazon Forastero Hybrids; LA = Lower Amazon Forastero. [b]Height: M = median; L = low; H = high. [c]Disease Resistance: VB = vassoura-de-bruxa; PP = podridão parda; mo monilíase; Ce = Ceratocystis. [d]References: 1 – Sounigo et al., 2005; Bekele and Bekele, 1994; ICGD, 2007; Dias, 2001; Pires, 2003. 2 – Dias, 2001; Crouzillat et al., 2000; ICGD, 2007; Pires, 2003. 3 – Bartley, 1994; Dias, 2001; Sounigo et al., 2005; ICGD, 2007; Pires, 2003. 4 – Sounigo et al., 2005; Risterucci et al., 2000; ICGD, 2007; Dias, 2001; Pires, 2003. 5 – Sounigo et al., 2005; Risterucci et al., 2000; ICGD, 2007; Dias, 2001; Pires, 2003. 6 – Neto et al., 2005; ICGD, 2007; Dias, 2001. 7 – Dias, 2001; Sounigo et al., 2005; Bartley, 1994; ICGD, 2007. 8 – Bartley, 1994; Dias, 2001; ICGD, 2007. 9 – Faleiro et al., 2004a; Bartley, 1994; ICGD, 2007; Dias, 2001. 10 – Lecerteau, 1997a; ICGD, 2007; Dias, 2001. 11 – ICGD, 2007; Dias, 2001; Pires, 2003.

are studies on the genetic diversity of local materials. These studies make it possible to better understand the genetic basis and potential use of these materials in improvement (Lima et al., 2013, Santos et al., 2015; Thondaiman et al., 2013). All these studies demonstrate that microsatellites are useful to characterize the genetic materials included in cocoa breeding programs around the word.

In the cocoa region of Bahia, Brazil, several local institutions develop research with cocoa tree, and groups of clones that best match the focus of improving their breeding programs. The germplasm characterization involves local and international clones for use in breeding programs. In this sense, this work was carried out to verify the genetic variability and clusters obtained with molecular data of 11 clones from different geographical regions, aimed to help the breeding programs of cocoa.

MATERIALS AND METHODS

The 11 clones of cocoa used in this study are from different geographical regions and are part of Brazilian breeding program (Table 1). These clones were *a priori* selected by the MCCS based on agronomic traits in order to hybridize and subsequently identify the progenies with combining ability for plant size, quality of products derived and to characterize routinely required as yield and disease resistance. Among clones considered promising, are some from the upper Amazon region that were used as parents of commercial hybrids in crosses with the lower Amazon Forastero (Amelonado), aiming at selection for combining ability for resistance to black pod (Tahi et al., 2006) and production components, such as weight per plant and seeds per fruit (Dias, 2001).

DNA samples were extracted from 300 mg of fresh leaves and healthy plants collected from each clone at intermediate age of maturity, using previously modified method (Doyle and Doyle, 1990; Bertolde et al., 2010). After extraction, all samples of DNA were purified with Prep-A-Kit Gene Mapris BIO-RAD according to the manufacturer's recommendations. Amplifications were performed in a total volume of 13 µL of reaction solution with the concentrations previously described (Bertolde et al., 2010), in a thermal cycler programmed for 35 cycles according to the following program: 94°C for 2 min for DNA denaturation, 46°C or 51°C (temperature specific to primer) for 1 min for annealing of primer, 72°C for 1 min extension of the DNA molecule by Taq DNA polymerase. After these 35 cycles of amplification, a final step at 72°C for 7 min was performed.

The 15 microsatellite primers used for amplification are specific for *T. cacao* and were previously developed by Lanaud et al. (1999) and Risterucci et al. (2000). These primers are available at NCBI (2009) and are encoded as: AJ271827 (mTcCIR 35), AJ271942 (mTcCIR 37), AJ271945 (mTcCIR 43), AJ271946 (mTcCIR 44), AJ271953 (mTcCIR 54), AJ271956 (mTcCIR 57), Y16977 (mTcCIR 3), Y16978 (mTcCIR 2), Y16980 (mTcCIR 6), Y16982 (mTcCIR 8), Y16983 (mTcCIR 9), Y16984 (mTcCIR 10), Y16985 (mTcCIR 11), Y16986 (mTcCIR 12) and Y16987 (MTcCIR 13). Among these 15 primers, 10 were also used by other authors to characterize germplasm international collections of cocoa (Motilal et al., 2009).

Table 2. Description of microsatellite loci, observed heterozygosity (Ho) and length of the amplicons (pb) in 11 clones of *T. cacao*.

Loci	Patern*	A**	H_o	Length of amplicons (pb)
AJ271827	235	3	0.5413	222, 232, 238
AJ271942	210	5	0.7500	198, 203, 204, 205, 206
AJ271945	206	4	0.7107	248, 256, 258, 260
AJ271946	178	4	0.6016	149, 175, 183, 185
AJ271953	165	7	0.8395	229, 233, 235, 241, 243, 251, 255
AJ271956	253	7	0.8223	209, 211, 215, 217, 219, 227, 229
Y16977	249	9	0.8750	141, 147, 153, 155, 157, 159, 161, 163, 165
Y16978	254	4	0.6563	240, 250, 254, 256
Y16980	231	4	0.5826	248, 252, 254, 256
Y16982	301	5	0.6777	283, 291, 293, 295, 307
Y16983	143	7	0.8140	273, 275, 277,285, 287, 289, 295
Y16984	208	7	0.7603	198, 200, 202, 204, 206, 208, 210
Y16985	298	5	0.7521	292, 304, 306, 308, 318
Y16986	188	7	0.8182	188, 202, 204, 206, 212, 214, 210
Y16987	258	5	0.6983	142, 148, 156, 160, 164
Average	-	5.53	0.7267	-

*Number of base pairs (bp) in the fragment amplified with the primers AJ in clone Catongo (Lanaud et al., 1999) and with primers Y on IFC 5 clone (Risterucci et al., 2000). **A = number of alleles per primer generated by amplifying DNA from 11 different clones of *Theobroma cacao*.

The products of amplification were separated on ABI Prism 377 (Applied Biosystems) automated sequencer and genetically analyzed according to Bertolde et al. (2010). The genotype data generated by the programs Genescan and Genotype (Applied Biosystems) were coded into numerical matrices. The level of heterozygosity was calculated based on the ratio between the number of heterozygosis loci and the total number of loci analyzed. The matrix of modified Rogers' genetic distances (Goodman and Stuber, 1983) was used in the graphic dispersion analysis of data by principal coordinates (Gower, 1996), the program NTSYS-pcversion 2.0 (Rohlf, 1997). For each dendrogram, the value of cophenetic correlation was calculated between the genetic distance matrix and the matrix of cophenetic values.

RESULTS AND DISCUSSION

The 15 pairs of primers generated a total of 83 alleles and the group of analyzed clones showed an observed heterozygosity (Ho) of 0.7276 (Table 2). The number of alleles varied according to the primer: the most informative primer Y16977 (Ho = 0.87500) generated nine alleles and the less informative primer AJ271827 (Ho =0.5413) generated three alleles. The value of Ho in this group of clones was higher than that observed in studies with upper Amazon genotypes and clones grown in Africa, for which were reported Ho = 0.5790 (Aikpokpodion et al., 2009). The average number of alleles per primer (Na = 5.53) detected in these genotypes is lower than Na = 13.8 obtained in 35 commercial clones used in plantations in southern Bahia and resulting from crosses or from different places (Bertolde et al., 2010) and Na = 8.8 alleles detected in 60 clones sampled in different international collections of cocoa (Motilal et al., 2009). However, in the subgroup of 13 upper Amazon Forasteros, Motilal et al. (2009) found Na = 7.5, a value similar to that found in the present study. In the present study, eight out 11 clones analyzed was formed by upper Amazon clones. This lower number of alleles per locus is slightly higher to that found in other studies with natural populations (Na = 4.45) of hits from the Brazilian Amazon (Sereno et al., 2006).

Based on 15 microsatellite markers, high genetic variation among clones was detected (Table 3). The genetic distances ranged from 0.55900 (between genotypes COCA 3370 / 5 and AMAZ 15) to 0.89190 (between genotypes ICS 95 and SCA 24), averaging 0.75 for all combinations. The pairs of genotypes that had the greatest distances, around 0.8, were IMC 47 x LCTEEN 162/1010; SCA 24 x LCTEEN 162/1010; H 56 x LCTEEN 162/1010; IMC 47 x AMAZ 15; H 56 x AMAZ 15; GU 261 x AMAZ 15; SCA 24 x LCTEEN 37 A; GU 261 x LCTEEN 37 A; CCN 51 x SCA 24; H 56 x SCA 24; GU 261 x SCA 24; ICS 95 x SCA 24; H 56 x IMC 47; COCA 3370/5 x IMC 47; COCA 3370/5 X H 56; ICS 95 x H56. The other distances in the crosses had values ranging from 0.60 to 0.79. One of the smallest distances was observed between clone TSH 1188 and ICS 95 and average distances with IMC 67 24 and SCA 24. This result partially agrees with the genealogy of the clone, because TSH 1188 is the result of crosses between SCA 6 and ICS 1 IMC 67. A smaller distance between SCA 24 and IMC 47 shows tendency already expected for these two clones, since they were collected in the Amazon Forest of Peru (Pound, 1938) and are also classified as Upper Amazon or Wild.

Table 3. Genetic distances of 11 *Theobroma cacao* clones, estimated from 15 microsatellite alleles based on modified Rogers' coefficient.

Clone no.	Clone name	1	2	3	4	5	6	7	8	9	10	11
1	LCT 162/1010	0.0000										
2	AMAZ 15	0.6055	0.0000									
3	LCT 37 A	0.7853	0.6325	0.0000								
4	SCA 24	0.8466	0.7528	0.8062	0.0000							
5	IMC 47	0.8756	0.8266	0.7303	0.6583	0.0000						
6	H 56	0.8165	0.8165	0.7853	0.8466	0.8266	0.0000					
7	GU 261	0.8660	0.8292	0.8036	0.8036	0.7773	0.7906	0.0000				
8	COCA3370/5	0.6292	0.5590	0.6614	0.7500	0.8165	0.8416	0.7538	0.0000			
9	CCN 51	0.7416	0.7416	0.7071	0.8756	0.7638	0.7638	0.7638	0.6922	0.0000		
10	TSH 1188	0.6831	0.5916	0.6325	0.7416	0.6831	0.7188	0.7638	0.6922	0.6055	0.0000	
11	ICS 95	0.7538	0.7230	0.7687	0.8919	0.7385	0.8118	0.7977	0.6892	0.6571	0.6030	0.0000

Underlined numbers indicate the genetic distances between Amazon clones (1-8) and the hybrids (9-10) or Trinitario (11) clones.

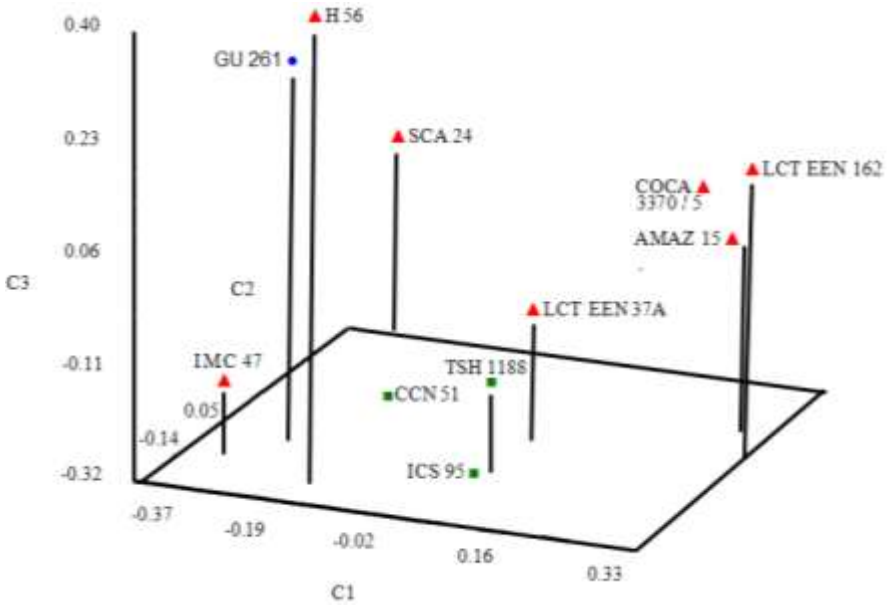

Figure 1. Principal coordinates' analysis of 11 clones of *Theobroma cacao* based on genetic distances from the data of 15 microsatellite primer pairs. (▲) Upper Amazon Forastero; (■) Hybrids and Trinitario; (●) Lower Amazon Forastero.

Distances between all Trinitario clones ranged from 0.60 to 0.66 and the distances between all Forasteros ranged from 0.56 to 0.88 (Table 3). Thus, the increased variability found between Forastero clones as compared to Trinitario is expected because the sample is formed by more Forastero than Trinitario. Additionally, the genealogy of Trinitario sampled here contributes to these differences. The following average genetic distance was observed among clones: 0.62 among clones Trinitario; 0.75 between Trinitario and Forastero; 0.74 among Forastero. Greater variability within the Forasteros as compared to Trinitarios was also observed in other genotypes based on molecular markers (Lecerteau et al., 1997ab). It was found that the largest genetic distances are found both between clones belonging to the same racial group and between the two racial groups. Thus, the parent selection both between and within racial groups may be considered aiming for the selection of economically important traits present in this group of germplasm.

In cluster analysis, hybrids clones (TSH 1188 and CCN 51) and a Trinitario clone (ICS 95) stayed together, while the remaining clones were distributed into different plans (Figure 1). This pattern of group pattern shows that different materials do not form homogeneous groups according to the molecular diversity of racial groups,

since the apparent homogeneity of the three genotypes of Trinitario and hybrids of Trinitario can be explained by genealogy and not by racial group. The Forastero (LCTEEN 37 A) remained alone, but in an intermediate position between hybrids and a group formed by tree high Amazon clones (AMAZ 15, LCT EEN 162/1010 and COCA 3370/5). Indeed, the molecular markers alone are not a single security classification of racial groups as observed before by Lecerteau et al. (1997a) and Figueira et al. (1994). But, it is useful to show the genetic diversity of the germoplasm included in breeding.

In this study, when considering the classification of wild and domesticated clones, the formation of groups is also not clearly seen. Wild clones (AMAZ 15, LCT EEN 162/1010, LCT EEN37 A, SCA 24, IMC 47, COCA 3370/5, H 56) did not differ from the cultivated ones (TSH 1188, ICS 95, CCN 51 and GU 261). According to Figueira et al. (1994), the wild group is composed of upper Amazon Forastero, while all others are domesticated, since they suffer a lot of anthropogenic manipulation.

In this work, different clones from Ecuador were grouped with two clones from Trinidad (ICS 95 and TSH 1188) and one Peruvian clone (H 56). This result can be attributed to the structure of the populations grown in Ecuador which are offspring of parents known as “refractario”, which in turn are derived from hybridization events between genotypes of three different possible regions and then selected for resistance to witches' broom (Bartley, 2001). Thus, the genetic structure of the Ecuadorian population can be attributed to (i) crosses between genotypes of Trinidad, Venezuela and Colombia, which were introduced in Ecuador in the late XIX century, (ii) National Cocoa and (iii) genotypes of Forastero from part of the Ecuadorian Amazon or Peru, at the middle Napo River (Bartley, 2001). Based on the grouping shown in this study, AMAZ 15 was allocated to the other Ecuadorians clones. Two clones from Peru (SCA 24 and IMC 47), lower Amazon clone of French Guiana (GU 261) are in the second group. According to Risterucci et al. (2000) and Sounigo et al. (2005), genotypes were classified by the acronym AMAZ which can also be classified as material of Peru or Ecuador. Sounigo et al. (2005) classified them as belonging to the Peruvian genotype AMAZ series, because the other genotypes were grouped in the same region of collection (SCA and IMC).

In conclusion, there is divergence between the materials that enable the selection of parents for hybridizations of crosses involving these genotypes. Also, there is no agreement among the clusters obtained at molecular level, geographic regions, level of domestication and racial groups for the germplasm in this work.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Agronomic evaluation and quality characteristics of three *Cucurbitaceae* varieties acclimated in Benin

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The present study aimed to evaluate some agronomical, physical and nutritional characteristics of seeds of three species of Cucurbitaceous, namely *Citrulus lanatus, Lagenaria siceraria* and *Cucumeropsis edulus.* Agronomic experiments were investigated over a two-year period during which, some agronomic performances of the tested varieties were evaluated. The physical characteristics of the collected fruits were also determined. The nutritional composition of the seeds, in particular their moisture, oils and proteins contents were then evaluated. Finally, the physicochemical characteristics, such as acidity, peroxide index, saponification index and the composition of fatty acids present in the oils extracted from seeds were performed. Results show that the species of *C. lanatus* and *L. siceraria* germinated respectively five and seven days after sowing. The flowering was observed 36 and 42 days, respectively, after sowing while the fructification appeared 44 and 58 days after sowing. According to results obtained during the two years of experimentation, the three species of *Cucurbitaceous* investigated in the present study could be ranged in short-cycle species (*C. lanatus* and *L. siceraria*) and long cycle species (*C. edulus).* Moreover, *C. lanatus* could be classified as the most productive as compared to others species of Cucurbitaceous plants investigated in the present study. *C. edulus* seeds are the longest (1.76 and 1.83 cm) in opposition to the seeds from *C. lanatus* (1.33 and 1.49 cm). Results of physicochemical analyses revealed the presence of fat (49.5 - 51.9%), with a high protein contents (18.46 - 31.41%). The composition of the fatty acids detected in oils extracted from seeds showed a strong concentration of linoleic acid (66.65%) and the presence of oleic acid (13.76%). The high unsaturated fatty acids proportion detected in oils from *L. siceraria* seeds was 80.40%. The saturated fatty acids detected were C16:0 and C18:0; and were more concentrated in the species of *C. lanatus*.

Key words: *Cucurbitaceous*, *Citrullus lanatus*, *Lagenaria siceraria*, *Cucumeropsis edulis,* oil, seeds, Benin.

INTRODUCTION

In developing countries, the exploitation of local plant resources is certainly a way to achieve food security, especially in countries with a high demographic growth (Sabo, 2014). However, it requires the preservation and availability of a high level of genetic diversity of these resources. Among these vegetal resources, Cucurbitaceous have an important place in the various uses of plant resources. *Citrullus lanatus*, *Lagenaria*

siceraria and *Cucumeropsis edulis* were cultivated for their high content in oils and proteins in West Africa (Levi et al., 2001; Zoro Bi et al., 2003; Schippers, 2004). In Benin, these three species are also widely grown by farmers (Vodouhe et al., 2001). However, little research was focused on the characterization of Cucurbitaceous for their valorization. Previously, studies reported a high content of trace elements with at least 45% of oil content in Cucurbitaceous plants (Vodounhe et al., 2008). They are also rich in polyunsaturated fatty acids which are known to be essential for human health (Schippers, 2004).

All parts of the plant are used in food production or in folk medicine. Indeed, in rural areas, more than 80% of the populations depend on food mainly based on cereals. The animal protein consumption is exceptional because the best proteins are those of animal origin (egg, milk, meat and fish). However, these animal products are expensive and very often exceed the financial capacities of the populations. Therefore, for nutritional safety of the populations, the introduction of vegetal proteins into the food chain appeared suitable. Based on this, it is very important to inquire vegetal with good sources of proteins which can improve the nutritional value of food accessible by the low income populations. In order to achieve this objective, the present study was interested in seeds of some Cucurbitaceous such as *C. lanatus*, *L. siceraria* and *C. edulis*. Although, several studies were carried out on their pharmacological potential (Prajapati et al., 2010) and the various rough levels of nutriments (Nmila et al., 2002; Sabo et al., 2005a, b; Sadou et al., 2007; Ullah et al., 2012), very few works were focused on the biochemical composition of these seeds (Abiodun and Adeleke, 2010). In Benin, previous researches were focused on the agronomic evaluation of some species of Cucurbitaceous used in the foodstuff in Benin (Achigan Dako et al., 2006) in other to develop a model of prediction. Then, the present study aimed to improve scientific knowledge on some species of Cucurbitaceous (*C. lanatus*, *L. siceraria* and *C. edulis)* through the identification of some agronomic aspects during two years of experimentation and evaluated the physical properties of seeds and physicochemical characteristics of oils extracted from these seeds for their industrial valorization.

MATERIALS AND METHODS

Collection of seeds for farming practices

Seeds of *Cucurbitace*, namely, *C. edulus*, *L. siceraria, C. lanatus* used for farming practices were previously collected at Abomey-calavi (south Benin) and were identified at the national herbarium, where voucher specimens are deposited. These species were chosen, because they were the three important species of *Cucurbitaceae* mostly cultivated in Benin (Vodouhe et al., 2008).

Sites and experimentation

The sites used for agricultural experimentation were located at Polytechnic School of Abomey-Calavi's University (Benin). Growing experimentations were conducted during the first year from June 2013 to January 2014 and in the second year from April to December 2014.
During growing, some agronomical parameters, such as durations of germination, flowering and fructification, as well as the number of fruits per seedling were evaluated. The seeds obtained were then collected and kept in dry place for analysis.

Determination of the durations of germination flowering and fructification

The duration of germination is evaluated starting from the date of sowing to the period of appearance of the cotyledons above the ground. The duration of flowering is calculated, starting from the date of sowing to the period of appearance of the first floral button. The duration of fructification corresponded to the time which separated the sowing date and the appearance of the first fruits.

Determination of the shape of seeds

The length and the breath of 10 seeds were measured per species, using a slide caliper. Then, the shape index calculated as the ratio breath/length was determined for each seed in order to appreciate their forms (Cowan and Smith, 1993). This ratio expresses the degree of circularity of seed. It is equal to the unit for seeds round and close to zero for lengthened seeds (Cowan and Smith, 1993). In addition, three forms according to the value obtained from the relationship between width (l) and the length (L) were observed (Cowan and Smith, 1993): l/L = 1 (seeds are round); l/L > 1 (seeds are broad); l/L <1 (seeds are lengthened). At the complete maturation of the fruits, the mass of the fruits, the average number of seeds per fruit, the mass of seeds, the ratio masses/average of seeds, the mass of 100 peeled seeds and unpeeled seeds, and the ratio almonds report/seeds were determined.

Chemical and biochemical analyses

Moisture content of samples was determined by desiccation using the method of De Knegt and Brink (1998). Protein was analyzed by the Microkjedhal nitrogen method, using a conversion factor of 6.25 and fat content was obtained by Soxhlet extraction. Ash was determined according to the standard methods described by the Association of Official Analytical Chemists (AOAC, 1990). Acidity of extracted oils was determined by titration with 0.01 mol/L of sodium hydroxide solution, using phenolphthalein as indicator

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Table 1. Agronomic characteristic of three species of *Cucurbitaceae* cultivated.

Species	Germination duration (days)		Flowering date (days)		Fructification date (days)		Number of fruits per plant	
	1st Year	2nd Year	1st Year	2nd Year	1st Year	2nd Year	1st Year	2nd Year
Citrulus lanatus	5.01±1.38[a]	5.09±1.17[a]	36.32±0.24[a]	35.74±0.64[a]	45.26±6.18[a]	44.31±5.58[a]	08.02±0.04[a]	08.23±0.01[a]
Lagenaria siceraria	7.14±0.88[a]	7.11±0.18[a]	42.23±0.54[a]	42.18±0.23[a]	58.04±9.21[b]	58.10±8.81[b]	06.08±0.15[a]	06.06±0.10[a]
Cucumeropsis edulus	10.76±0.44[b]	10.54±0.34[b]	81.23±0.54[b]	46.23±0.54[a]	-	68.22±4.17[c]	-	02±0.07[b]

Values are means. The means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests.

(AOAC, 1990). Peroxide and saponification indexes of extracted oils, were performed as described respectively by NF T 60-220 (1993) and NF ISO 3657 (1993). The composition in fatty acids of the oil extracted from seeds of three investigated Cucurbitaceous species are performed as follows: the methyl esters of the oil samples were prepared by transesterification with sodium methylate of sodium according to NF standard T60-233 (1995), and then analyzed using an Agilent series 6890 chromatograph equipped with a capillary column Supelcowax 10. The temperatures of the detector and the injector were respectively 250 and 270°C and the oven was programmed from 150 to 225°C with a gradient of 5°C/min. The carrier gas is helium with a flow rate of 1 ml/minute. The identification of methyl esters was done by comparison of retention time with the methyl esters of standard previously analyzed.

Statistical analysis

Experiments were performed in triplicate, and data analyzed were mean subjected to one-way ANOVA. Means were separated by the Turkey's multiple range tests when ANOVA was significant ($p < 0.05$) (SPSS 10.0; Chicago, IL, USA).

RESULTS AND DISCUSSION

Agronomic characteristic of cultivated species

To provide samples for agronomic and physicochemical characteristics, a two-year seeds production of three Cucurbitaceae species was experimented. As listed in Table 1, results related to the determination of germination, flowering, fructification duration, as well as those relating to the number of fruits per plant was reported. Statistical analysis indicated that, the duration of germination as well as the time of flowering of the species such as *C. lanatus* and *L. siceraria*, were not significantly different ($p < 0.05$) during the first and the second year of the experimentation. Indeed, the germination of seeds was observed, respectively at the fifth and the seventh days after sowing, and their flowering periods respectively took place thirty-six and forty-two days after sowing. In addition, there are significant differences between results obtained with *C. edulus* which has a germination and flowering periods of eleven (11) and eighty-one (81) days respectively, as compared to those of *C. lanatus* and *L. siceraria* during the first year. However, during the second year of the experimentation, the difference was not statistically significantly ($p < 0.05$). Based on the fructification duration, the obtained results indicated a significant difference between investigated species, with an average period of forty four (44), fifty eight (58) and sixty eight (68) days after sowing, respectively for *C. lanatus*, *L.siceraria* and *C. edulus.* The number of fruits is also statistically different with an average of two (02), six (06) and eight (08) fruits per plant, respectively for *C. edulus, L. siceraria* and *C. lanatus.* According to the results listed above during the two years of experimentation, the three species of Cucurbitaceous investigated in the present study, could range in short-cycle species (*C. lanatus*, *L. siceraria*) and long cycle species (*C. edulus).* Moreover, *C. lanatus* could be classified as the most productive as compared to other species of Cucurbitaceous plants investigated in the present study. In the work of Sabo (2014), which described Cucurbitaceous plant from Niger, *L. siceraria* was reported as lower productive species and an average of 3-10 berries was found in the first year.

Physical characteristic and results of proximate analysis

Cucurbitaceae are among the most important plant families supplying humans with edible products. The study of physicochemical characteristics of the seeds of these plants is of great interest. As observed, the tested species have diversity of fruit shape (Table 2). According to these results, seeds length of *C. edulus* ranged from1.76 to 1.83 cm. It then represents the species with longest seeds length, as compared to other species investigated in the study. The shortest one was collected from

Table 2. Characteristics of the three tested species of *Cucurbitaceae* seeds.

Species	Length of seeds (cm)		Width of seeds (cm)		Width/Length	
	1st Year	2nd Year	1st Year	2nd Year	1st Year	2nd Year
Citrulus lanatus	1.33±0.06[a]	1.49±0.03[a]	0.76±0.04[a]	0.81±0.04[a]	0.57±0.07	0.54±0.05
Lagenaria siceraria	1.63±0.09[b]	1.56±0.09[a]	0.64±0.17[b]	0.62±0.07[b]	0.49±0.06	0.39±0.01
Cucumeropsis edulus	1.76±0.99[b]	1.83±0.11[b]	0.53±0.06[c]	0.57±0.02[b]	0.30±0.08	0.31±0.05

Values are means. The means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests.

Table 3. Physical composition of the produced fruits of three species of *Cucurbitaceae* tested.

Species	Weight of the fruits (kg)		Numbers of seeds by fruit		Weight of seeds by fruit (g)		Ratio of weight of seeds to weight of the fruits	
	1st Year	2nd Year	1st Year	2nd Year	1st Year	2nd Year	1st Year	2nd Year
Citrulus lanatus	0.80±0.39[a]	0.80±0.91[a]	160.79±27.80[a]	189.21±15.72[a]	32.42±1.69[a]	36.78±1.64[a]	0.04	0.04
Lagenaria siceraria	0.99±0.17[a]	1.08±0.27[b]	186.46±23.82[b]	196.35±15.32[a]	36.58±1.18[a]	39.77±3.11[b]	0.03	0.03
Cucumeropsis edulus	-	1.55±0.09[c]	-	420.91±17.24[b]	-	60.43±1.27[c]	-	0.03

Values are means. The means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests.

C.lanatus (1.33 - 1.49 cm respectively in first and the second year). The shape index (ratio width/length) obtained, indicated that the whole of produced seeds were lengthened forms (ratio lower than 1). These results are similar to those of Sabo et al. (2014) on species of *C. colocynthis* and *L. siceraria* used for food in Niger. The physical characteristics of the produced fruits are given in Table 3. A significant difference can be observed during the second year between the three cultivated species. However, statistical difference ($p < 0.05$) was observed during the first year of production. In addition, there was proportionality between the numbers of seeds by fruit and the masses of the fruits. Statistical analyses revealed a significant difference between the investigated species when compared the mass of 100 of unpeeled fruits (Table 4).

However, when the fruits were peeled a difference between the various species was observed. The ratio of masses of almond to the mass of seeds varied from 65 to 77% during the first year while it however varied from 66 to 78% during the second year. Heaviest seeds were those of *C. edulus* while the least heavy are those of the species *C. lanatus*. On the other hand, results of proximate analyses of seeds are presented in Table 5. The moisture content varied from 6.31 to 9.24% and the ash content ranged from 4.46 and 4.65%. Statistical analysis indicated that there are no difference in moisture and fat contents of seeds from *C. lanatus* and *C. edulus.* However, there are significant differences in protein contents in seeds from Cucurbitaceous species ($p=0.05$). The highest protein content is observed in the seeds of *C. edulus*. This protein content is similar to those reported for important vegetal which contain, in general, 7.8 to 22.8 g/100 g (Bullock et al., 1989) but is higher than those reported in locust bean pulp (Dahouenon-Ahoussi et al., 2012). This high protein content could be lead to classified *C. edulus* in the group of vegetal in which valorization could be contributed to overcome the nutritional problems. Indeed, according to Umaru et al. (2007), edible wild indigenous plants are an alternative source of food with high potential of proteins and other interesting elements, particularly during seasonal

Table 4. Weight of 100 seeds (g) of three tested species of *Cucurbitaceae*.

Species	Weight of 100 not peeled seeds		Weight of 100 peeled seeds		Weight of 100 hulls		Ratio of Almonds/seeds (%)	
	1st Year	2nd Year	1st Year	2nd Year	1st Year	2nd Year	1st Year	2nd Year
Citrulus lanatus	14.65±0.70[a]	15.10±0.40[a]	9.58±0.61[a]	9.99±0.34[a]	5.07±0.02[a]	5.11±0.03[a]	65±0.01[a]	66±0.00[a]
Lagenaria *siceraria*	16.15±0.04[b]	16.50±0.07[b]	11.65±0.06[a]	11.86±0.23[b]	4.50 ±0.01[a]	4.64 ±0.05[a]	72±0.00[b]	71±0.08[b]
Cucumeropsis edulus	16.63±0.13[b]	16.68±0.04[b]	12.81±0.12[b]	13.03±0.03[c]	3.65±0.05[b]	77±0.00[b]	77±0.00[b]	78±0.01[c]

Values are means. The means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests.

Table 5. Complete proximate analysis of seeds of three species of *Cucurbitaceae* tested.

Variety	Water (%)		Ash (%)		Oil (%)		Protein (%)		Total sugar (%)	
	1st Year	2nd Year	1st Year	2nd Year	1st Year	2nd Year	1st Year	2nd Year	1st Year	2nd Year
Citrulus lanatus	9.14±0.22[a]	9.04±0.19[a]	4.57±0.11[a]	4.65±0.22[a]	49.78±0.81[a]	49.87±0.69[a]	18.46±1.98[a]	19.64±0.4[a]	4.42±0.06[a]	4.41±0.04[a]
Lagenaria siceraria	6.32±0.30[b]	6.31±0.76[b]	4.46±0.08[a]	4.48±0.07[a]	52.15±0.71[b]	51.90±0.91[b]	27.42±0.96[b]	27.79±0.82[b]	7.66±0.04[b]	7.68±0.90[b]
Cucumeropsisedulus	9.23±0.23[a]	9.24±0.17[a]	4.72±0.26[a]	4.68±0.29[a]	49.53±1.93[a]	49.51±1.86[a]	31.41±0.69[c]	31.27±0.54[c]	4.12±0.66[a]	4.09±0.57[a]

Values are means. The means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests.

Table 6. Composition of fatty acids of the oil of three species of *Cucurbitaceae* tested.

Variety	C16 :0		C18 :0		C18 :1		C18 :2	
	1st Year	2nd Year	1st Year	2nd Year	1st Year	2nd Year	1st Year	2nd Year
Citrulus lanatus	15.1	14.97	13.80	13.81	17.1	16.99	54.1	54.23
Lagenaria siceraria	13.2	13.21	6.4	6.4	13.76	13.74	66.64	66.65
Cucumeropsis edulus	11.2	11.**21**	10.4	10.39	19.89	19.89	58.1	58.11

food shortage.

Characteristics of extracted oils

Besides protein content of *Cucurbitacea* plants, some species showed potential uses as edible oils. In the present work, fatty acid composition of oils extracted from harvested seeds was investigated. As listed in Table 6, palmitic, oleic, stearic and linoleic acids were the fatty acids detected in all the recovered oils. There are more unsaturated fatty acids than saturated acids in the analyzed oils. Moreover, linolenic acid was in high concentration in all the oils. The high unsaturated oils described above from fatty acid composition were confirmed by the high iodine values presented in Table 7. Besides fatty acid composition of the oil seeds, biological active part of oils such as unsaponifiable matter was also extracted and determined (Table 7). Highest amount of unsaponifiable fraction was found in oil extracted from *C. lanatus* seeds. The characteristics of analyzed oils were similar to common edible oils and those reported by Kapseu (1993).

Table 7. Quality parameters of the oil of various varieties of *Cucurbitaceous.*

Variety	Iodine value (g I_2/100g)		Unsaponifiable (%)	
	1st Year	2nd Year	1st Year	2nd Year
Citrulus lanatus	242.39±4.58[a]	2 38.47±5.24[a]	1.61±0.13[a]	1.58±0.23[a]
Lagenaria siceraria	183.76±6.23[b]	188.63±7.03[b]	0.59±0.18[b]	0.62±0.06[b]
Cucumeropsis edulus	202.79±4.6[c]	201.97±5.15[c]	0.96±0.21[c]	0.95±0.61[c]

Values are means. The means followed by same letter in the same column are not significantly different according to ANOVA and Tukey's multiple comparison tests.

Conclusion

The present work contributes to knowledge on seeds of three varieties of *Cucurbitaceae* for agronomic and technological improvements. *C. lanatus* and *L. siceraria* show good agronomic performances. All cucurbitaceous seeds investigated were also rich in oil and protein. These potentials of cucurbitaceous seeds investigated, could be taken into account in the traditional plants development programs

Conflict of Interests

The authors have not declared any conflict of interests.

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