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African Journal of Biotechnology

Review

Azospirillum spp. potential for maize growth and yield

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The importance of biotechnology involved in the availability of nutrients to plants in different production systems is well known. In the search for agricultural sustainability, biological nitrogen fixation process stands out, especially in tropical regions where soil organic matter can be rapidly mineralized. In this aspect, researches have pointed out the potentialities of the use of diazotrophic bacteria, as well as other growth-promoting bacteria in *Poaceae*. Maize crop, especially, stands out in the international scenario, requiring a deepening of the research aiming to raise the contribution potential of microorganisms including *Azospirillum* spp. in reducing the consumption of fertilizers from non-renewable sources while promoting an increase in agricultural productivity and mitigating environmental impacts.

Key words: *Zea mays*, diazotrophic bacteria, nitrogen, biological fixation, growth promoting.

INTRODUCTION

Maize (*Zea mays* L.), one of the most important cereals grown throughout the world, is used as a source of food for humans, animal feed and as raw material for industries. The United States of America is the world's largest producer of this crop, followed by China and Brazil, respectively (USDA, 2018). The maize crop is generally influenced by environmental stress problems, among which are those related to low soil fertility, mainly in terms of nitrogen (N) availability (Novakowiski et al., 2011). In this way, nitrogen fertilizers are commonly used in maize crops in order to supply the deficiency of this nutrient in the soil, meet the physiological needs of the plant and provide high yields. However, due to the economic and environmental cost of industrial manufacturing processes for the increasing food demand, it is necessary to develop technological innovations and incorporate them into the agricultural activity aiming to rationalize the use of nitrogen fertilizers. Among the viable alternatives, the use of beneficials generated by the prokaryotic microorganisms is able to fix atmospheric nitrogen and make them available to the plants through association with the plant roots. The microorganisms with these features are representatives of several bacterial phylogenetic groups, called diazotrophic, capable of colonizing both the interior of the roots and the rhizosphere of plants. This type of symbiosis generates a number of benefits including the stimulus to root growth, making it more voluminous and so absorb larger

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quantities of water and nutrients (Martínez-Morales et al., 2003). Probably because of the higher root volume and better plant nutrition, there are also several reports of higher tolerance to plant pathogens (Correa et al., 2008).

Usually, inoculation with *Azospirillum brasilense* provides an increase of dry mass, N accumulation in plants and grain yield, especially if there is correlation between bacteria and unimproved genotypes under conditions of low N availability (Okon and Vanderleyden, 1997). In addition, plant nutritional status, exudate quality, competing microorganisms and strain choice are also factors that may influence the interaction between the maize plant and the bacterium as well as the efficiency of biological nitrogen fixation (BNF) (Quadros, 2009). Due to the incompatibility of *A. brasilense* with the chemical products used in seed treatment (Croes et al., 1993), there is a need to study alternative methods of seed inoculation. Thus, a method that has been developed is the inoculation of bacteria in the sowing furrow. According to Basi et al. (2011), inoculation with *A. brasilense* (Abv5/Abv6 strains) in the seeds or on the sowing furrow increased the yield of the maize crop independently of the N rate applied in topdressing (Pereira et al., 2015).

In addition to the increase in maize productivity, there are reports in the literature about nitrogen fertilizer economics when the crop is submitted to inoculation with *A. brasilense* (Cheng et al., 2011). The researches related to the efficiency of the use of inoculants based on *A. brasilense* were neglected for many years due to the inconsistency of the results, however, recently they have been more focused on the necessity for development of more sustainable agriculture. Gramineae have some advantages when compared to Leguminosae. They have a fasciculated root system, having advantages over the pivotal system of legumes to extract water and nutrients from the soil, which, together with other physiological factors, promotes greater photosynthetic activity. Therefore, the interest in the biological fixation in Gramineae is great. Not all of the nitrogen required in maize is provided by the bacteria, making the technique a form of N supplementation for the crop. However, this alternative may lead to a reduction in the use of nitrogenous fertilizers and this cost may be equal to or higher than legumes that may be self-sufficient for nitrogen (Döbereiner, 1992). In this way, several investigations involving the association of maize genotypes with *A. brasilense* found positive results, so that Hungria (2011) obtained yield increases of 30% in relation to the uninoculated control and without nitrogen topdressing fertilization when maize genotypes were inoculated with *A. brasilense* whereas Braccini et al. (2012) observed a 20% increase in grain yield under the same conditions. Müller et al. (2016) obtained an increase of up to 28% of productivity in maize, when plants were inoculated with *A. brasilense* at different doses of N. Portugal et al. (2016) verified higher yields in the four N doses tested (0, 30, 60 and 90 kg ha^{-1}) by spraying *A. brasilense* on maize leaves.

However, the adoption of this technology in the agricultural systems is still incipient, because the results vary according to the cultivar, edaphoclimatic conditions, inoculation types and methodology of conduction of the research. Genotypes may interfere with nitrogen uptake, so the identification, selection, and use of more efficient maize genotypes in nitrogen uptake and assimilation is an important strategy, as they contribute to the development of the crop, as well as reduce the contamination of the environment by nitrogen residues (Reis Júnior et al., 2000). Thus, to find responsive maize genotypes to the inoculation with bacteria of the genus *Azospirillum*, associating them with genetic and breeding programs aiming to increase grain production are really important for the development of new cultivars.

MAIZE CROP

Maize (*Zea mays* L.) which belongs to the family of Gramineae (Poaceae), is a monoic, allogamous, annual, robust, erect and diploid (2n = 2x = 20) crop (Paterniani and Campos, 1999). It originates from the teosinte, a Mexican subspecies (*Zea mays* ssp. Mexicana (Schrader) Iltis), which has been cultivated in many parts of the world for more than 8,000 years (the United States of America, China, India, Brazil, France, Indonesia, South Africa and others). It is a polytypic species, being probably one of the greater genetic variability between the cultivated plants. There is genetic variability for practically all traits of the plant and the maintenance of this variability is due to the establishment of germplasm banks, which maintains the diversity of individualized types and under controlled conditions of races (Fornasieri Filho, 2007).

Botanically, maize belongs to the group of C4 plants, its root system is the fasciculate type reaching up to 3 m deep in the soil, while a great part of the roots are in the 0 to 30 cm layer. The stem is a full culm type, consisting of nodes and internodes, while its lanceolate leaves are inserted alternately in the stem, in addition to presenting male inflorescences, tassel, and female inflorescences and ear, and the fruit being classified as cariopse (Ritchie et al., 1993). The domestication of the maize crop occurred through visual selection in the field, considering important traits such as productivity, resistance to diseases and adaptability, among others, giving rise to varieties known nowadays. Thus, from a Gramineae with several stems, small spikelets and with few seeds, it became an erect plant, with a single culm, monoic, with larger ears containing higher seed quantity and quality.

Maize has a great adaptability, represented by varied genotypes, which allows the cultivation from Ecuador to the limit of temperate lands and from sea level to altitudes above 3,600 m, in tropical, subtropical and

temperate climates (Barros and Calado, 2014). It is possible that the maize crop may be the one with the greatest genetic variability among the cultivated plants, since there are genetic differences for practically all the traits of plants, being the maintenance of this variability, kept in germplasm banks, which maintains the diversity of individualized types and under controlled conditions of races (Fornasieri Filho, 2007).

Commercially, there are varieties and maize hybrids on the market. Hybrids are suitable for production of systems that use high technology, being single hybrid, single modified hybrid, triple hybrid, triple modified hybrid and double hybrid. While the varieties are recommended for less technical plantings, being obtained by natural pollination through the selection of groups of plants with desirable characteristics, presenting some variability, but with common genetic characteristics (Cruz et al., 2010).

In socioeconomic terms, maize has an undisputed role in the world, due to its exceptional position among exploited agricultural species (Môro and Fritsche, 2015). According to projections, by 2022, maize grain for animal feed will be the most traded in international markets, accounting for 80% of world trade. This position is consolidated due to maize being the most produced cereal in the world, fundamental for animal and human feed, as well as indispensable and driving of several agroindustrial complexes in function of their productive potential, chemical composition, and nutritional value. Demand for the crop has been particularly high in China, driven mainly by animal growth and industrial processes, which account for over 90% of the country's maize imports (USDA, 2017).

Among the nutrients most required by the maize crop is the N, which is important at the initial stage of development of the plant (second week after emergence), when it is with four leaves fully expanded. It is at this stage that the developing root system already shows a considerable percentage of absorbent hairs and differentiated branches and the addition of N stimulates its proliferation with consequent development of the aerial part. Also at this stage the process of floral differentiation begins, which originates the beginnings of the panicle and ear, as well as, it defines the production potential. This implies the necessity of the availability of at least 30 kg ha^{-1} of N at this stage in order not to limit this physiological event (Fancelli, 1997).

Worldwide, it is estimated that maize will reach 1,038.80 million tons of grain produced in the 2017/18 season. The United States, China, and Brazil are the world's largest producers, respectively, totaling a combined production of 672 million tons (USDA, 2017). Over the last five years, the USA maize production has grown by more than 110 million tons at an average yield of 11,000 kg ha^{-1} (USDA, 2017), surpassing the total Brazilian production, which increased by 25 million tons since 2010 (CONAB, 2017), more than 30% of the total produced by the country (FAO, 2017). From the data presented, it can be inferred that even the crop presenting high productive potential is evidenced by grain yields of up to 16,000 kg ha^{-1} in some countries not only in experimental conditions and also by technified farmers (Cantarella, 1993). Their productivity is complex and depends on the interaction between genetic, environmental factors (Argenta et al., 2001) and well-defined management techniques.

The projections in the coming years are that, there will be a substantial increase in the use of fertilizers in Brazil for attending to the intensification of agriculture and the recovery of degraded areas. Considering Brazilian fertilizer production is insufficient to meet national demand, mostly imported from out of the country, as in the period from January to September 2017, 75% (19,182 tons) of fertilizers usage in Brazilian agriculture came from abroad, indicating an increase of 10.3% in relation to the same period of 2016. There were important nutritional growths in nitrogenous fertilizers of 9.2%, phosphates of 23.6% and potassium of 6.6% (ANDA, 2017).

Biological nitrogen fixation (BNF)

Nitrogen is essential for the proper functioning of plants, as it participates in the composition of the related amino acids, protein, chlorophyll, photosynthesis and many essential enzymes for cell maintenance and development as well as it is present in the processes of ionic absorption, respiration, multiplication and differentiation of cellular and genetic inheritance which are essential for the growth and development of the aerial part and the root system (Marschner, 1995; Epstein and Bloom, 2006; Malavolta, 2006; Grassi Filho, 2010; Taiz and Zeiger, 2013). It is known, for example, that N represents about 40% of the total cost of production of the maize crop (Barros Neto, 2008) and about 50% of this applied nitrogen undergoes the action of ammonia volatilization, denitrification, erosion, microbial immobilization and leaching (Reis Júnior et al., 2010) into the water until it reaches the water table, rivers and lagoons, consequently polluting the environments (Lewis et al., 1984).

BNF involves the transformation of atmospheric nitrogen (N_2) to forms assimilable by the plant: ammonium (NH^{4+}) or nitrate (NO^{3-}) through dinitrogenases enzymes existent in diazotrophic bacteria present in the soil (Novakowiski et al., 2011). This process provides nitrogen compounds directly to plants through associations, or when organisms die and release them into the environment, providing the necessary nitrogen for plant development (Lindemann and Glover, 2003).

According to Rudnik et al. (1997), BNF is a process related to the need of the environment and the fixing species, because of enzyme nitrogenase, which is responsible for the reduction of N_2, is inactive in the presence of ammonia. BNF is a significant process in the

agricultural sector, with the biological process contributing most of the fixed N. It is estimated that it provides about 175 million tons of N to the biosphere, or 65% of the total which makes it the second most important biological process on the planet after photosynthesis, along with organic decomposition (Moreira and Siqueira, 2006). However, only biological nitrogen fixation is not able to provide all the necessary N to the development of crops that demand a larger amount of this nutrient, so nutritional supplementation with nitrogen fertilizer formulations is necessary. In this way, it is important to know well the history of the planting area, as well as the predecessor crop, in order to define more accurately the N doses, sources and parceling to be applied (Portugal et al., 2017).

Environments of degraded areas, with poor soils, substrates devoid of organic matter, can be stimulated by BNF, when compared with areas with climax vegetation, provided with rich substrates in organic matter, because the cycling that occurs in these environments guarantees the preservation of metabolism and growth rate (Moreira et al., 2010). BNF is a process that depends on several factors. In order for the bacterium to establish a positive interaction with the plant, it is indispensable to use selected *A. brasilense* strains (Hungria, 2011) capable to compete with the microorganisms already present in the soil. Another factor to be taken into account is the choice of the genotype to be inoculated since the beneficial relationship between the hybrid and the bacterium is determined by the quality of the exudates released by the roots of the plant (Nehl et al., 1996). This phenomenon is known as chemotaxis, where each genotype releases a different amount of exudate with different chemical composition, which may or may not be attractive and serve as a carbon source (malate, pyruvate, succinate, and fructose) for the inoculated bacteria (Quadros, 2009).

As for the survival of this microorganism, it is known that *A. brasilense* has a low ability to survive for prolonged periods of time in most soils. The physicochemical conditions of the soil and the absence of the host plant can directly affect the population of the bacteria (Bashan et al., 1995). However, in unfavorable situations, these bacteria develop protection mechanisms such as cysts formation, poly-β-hydroxybutyrate, and melanin production, favoring their survival (Del Gallo and Fendirik, 1994).

The inoculation with diazotrophic bacteria can alter the root system morphology, a number of radicels and root diameter, probably due to the production of growth promoting substances (auxins, gibberellins, and cytokinins), and not only by BNF (Cavallet et al., 2000). The production of phytohormones helps the growth of plants, and can modify the morphology of the roots, which allows a greater volume of soil exploration and a higher nutrient uptake (Silva et al., 2004), greater tolerance to salinity, dryness (Bashan et al., 2004) and plant pathogens (Correa et al., 2008), resulting in more productive plants (Hungria, 2011). The inoculation with *Azospirillum* is carried out similarly to inoculate soybean seeds with *Bradyrhizobium*. The commercial product can be applied in solid form (as peat) or in liquid form. Also, it is necessary to be cautious of the temperature conditions, not leaving exposed to the sun and without joint application with agrochemicals, since they are living microorganisms (Hungria et al., 2010).

The most common inoculant application method is via seeds. In a study by Portugal et al. (2017) and according to Hungria (2011), seed inoculation associated with the addition of 24 kg ha^{-1} of N at sowing and 30 kg ha^{-1} of N at the flowering stage enable average yields around 7,000 kg ha^{-1}. However, seeding furrow inoculation has been studied as a way of avoiding toxicity of the products used in the treatment of seeds on the bacterium, since some chemicals can disorganize the flagellum used by *A. brasilense* in association with the plant (Croes et al., 1993). According to Basi et al. (2011), the application of *A. brasilense* (Abv5 and Abv6 strains) provided an increase in maize productivity, and the inoculation through the sowing groove did not differ from that in the seeds, showing an efficient application method. However, the selection of strains for inoculant manufacturing still needs a lot of research. There are currently technological packages using plant varieties and efficient bacterial strains, which can supply more than 50% of the N necessary to the plant (Bárbaro et al., 2008).

Plant growth-promoting bacteria: *Azospirillum* spp.

An alternative to achieve high yields of maize, with lower consumption of nitrogen fertilizers, is the inoculation of the crop with bacteria that have the capacity to supply nitrogen to the plants, known as plant growth-promoting bacteria (PGPB) which belong to the phylogenetic groups called diazotrophs (Moreira et al., 2010). PGPB are known as free-living bacteria in the soil, rhizosphere, rhizoplane, and phyllosphere that are beneficial to plants. PGPB endophytes residing within the plant have also been found. They directly affect plant growth by supplying substances that are generally scarce. PGPB may aid to uptake nitrogen nutrition of crops through various mechanisms. They are able to fix atmospheric nitrogen; solubilize phosphorus and iron, and produce plant hormones such as auxins, gibberellins, cytokinins, and ethylene. In addition, they promote higher plant tolerance to stresses, such as drought, high salinity, metal toxicity and pesticide loading (Bashan and De-Bashan, 2010).

Diazotrophs comprise a broad range of prokaryotic microorganisms, including representatives of archebacteria, cyanobacteria, gram-positive and gram-negative bacteria that exhibit great morphological, physiological, genetic and phylogenetic diversity. Such diversity guarantees not only the resilience of the processes that mediate in a given ecosystem but also the

occurrence of this, in the most different terrestrial habitats (Moreira and Siqueira, 2006). This kind of bacteria can contribute to plant growth by the following characteristics: nitrogen supply, phytorium production, phosphate solubilization (Pedrinho, 2009), increase the activity of nitrate reductase when they grow endophytically in plants (Cassán et al., 2008), as well as acting as an agent for the biological control of pathogens (Correa et al., 2008). Chavarria and De Melo (2011) report that the use of microorganisms in agricultural practices has become increasing since nitrogen fertilization represents an important element in production costs.

The loss of diversity of soil microorganisms, especially diazotrophs, can alter the population structure of other organisms located along the trophic chain. Vital soil processes such as the decomposition of organic matter and the cycling of nutrients can suffer impacts taking the agricultural system to higher dependence on fertilizers. In this context, the knowledge of the phenotypic diversity and genetic structure of the populations present in the rhizosphere can help in the understanding of how the variations in the environment may be influencing the functionality of these populations.

The diazotrophic bacteria, the most studied PGPB, belonging to the *Azospirillum* genus do not form a symbiosis with the host plant (Bashan and Bashan, 2005), and *Azospirillum* spp. is among the most important bacteria involved in the fixation of N_2 in grasses (Cáceres, 1982). This bacterium is characterized by its rod shape, which are commonly uniflagellated, gram-negative, with characteristic vibratory movement and mixed flagellar pattern (Hall and Krieg, 1984). These microorganisms fit into the group of facultative endophytic diazotrophs, as they colonize both the interior of the roots, where their cells can penetrate into intercellular spaces and lodge, as well as in the external part of the roots, being found in the mucigel present in the rhizosphere of plants and occur frequently in tropical and subtropical soil (Bashan and Levanony, 1990; Baldani et al., 1997).

The *Azospirillum* genus, when inoculated, may not achieve the similar efficiency of the rhizobia-leguminous symbioses in the soil. The contribution of N fixed to Gramineae is around 25 to 50 kg N ha^{-1} $year^{-1}$, equivalent to the average supply of approximately 17% of crop demand (Moreira et al., 2010). Several studies have been carried out to identify microorganisms that have a symbiosis with Gramineae, as occurs in the soybean crop with the bacterium *Bradyrhizobium japonicum*. However, the bacterium *A. brasilense* has a great response potential in association with maize cultivation. The interest in the use of this development-promoting bacterium capable of contributing to plant nutrition has increased and tends to increase in the coming years, due to the high financial value invested annually with fertilizers and in relation to the search for sustainable agriculture (Hungria et al., 2010).

These bacteria have a wide ecological distribution, being found in association with monocotyledonous and dicotyledonous plants (Magalhães and Döbereiner, 1984; Döbereiner and Pedrosa, 1987; Lange and Moreira, 2002). It has been investigated that the effect of *Azospirillum* spp. not only on crop yield but also on the physiological causes the possibly of increase in yield (Bárbaro et al., 2008). It is possible to classify this bacteria as rhizocompetent bacteria, because the survival of this genus in the soil, in the absence of host plants, is related to different physiological mechanisms of protection (Bashan and Levanony, 1990; Del Gallo and Fendrik, 1994; Moreira et al., 2010), they are: melanin production, poly-β-hydroxybutyrate (PHB) and polysaccharides (Del Gallo and Fendrik, 1994), formation of cysts (cell aggregates) and change in cell shape.

The *Azospirillum* genus can act on the vegetative growth through the reduction of nitrate in the roots of the plants (Döbereiner et al., 1995; Cassán et al., 2008). Among the effects of the association between these bacteria and the plants are the biological nitrogen fixation capacity (Fukami et al., 2016), solubilization of inorganic phosphate, production of hormones such as auxins and cytokinins (Tien et al., 1979), gibberellins (Bottini et al., 1989), regulation of ethylene biosynthesis (Strzelczyk et al., 1994), as well as a variety of other bioactive molecules (Perrig et al., 2007); the solubilization of phosphates (Rodriguez et al., 2004); the biological control of pathogens (Correa et al., 2008); and the increase of plant resistance to different abiotic stresses (Yang et al., 2009). One of the most striking effects of inoculation with *A. brasilense* on root morphology is represented by the root hair proliferation, making them more voluminous, and consequently able to absorb larger amounts of water and nutrients (Saikia et al., 2012). The association of diazotrophic bacteria of the *Azospirillum* genus culminates with the increase of maize crop yield (Bashan and De-Bashan, 2010).

Other physiological responses caused by inoculation with *Azospirillum* include the improvement in photo-synthetic parameters of leaves, including chlorophyll content and stomatal conductance, higher proline content in aerial part and roots, improvement in water potential, increase in water content of apoplast, higher cell wall elasticity, higher biomass production and higher plant height (Barassi et al., 2008).

Worldwide, the majority of inoculation evaluating experiments with *Azospirillum* spp. in the maize crop showed increases in grain yield (Kennedy et al., 2004; Kannan and Ponmurugan, 2010). In Brazil, Hungria et al. (2010) when inoculating selected species of *A. brasilense* and *A. lipoferum* in maize and wheat, found increases of 26 and 30% in grain yield of these crops, respectively, as well as increases in P and K uptake by plants. Increases in maize yield were also obtained by Cavallet et al. (2000), Novakowisk et al. (2011), Martins et al. (2012) and Araújo et al. (2014) with the inoculation of

Azospirillum spp in the treatment of seeds, as in the sowing furrow or in foliar application. However, positive responses to increase in productivity are not always obtained with inoculation of the seeds with *Azospirillum* spp. as reported by Campos et al. (1999) in oat and wheat crops and by Müller et al. (2012) with the inoculation of *A.brasilense* in the sowing furrow and the treatment of seeds in the maize crop.

Farinelli et al. (2012) evaluated the agronomic viability of the use of the inoculant (*A. brasilense*) in the treatment of seeds in the maize crop, associated to nitrogen topdressing (0, 90 and 120 kg ha^{-1}). They verified that seed inoculation promoted improvements in the morphological and productive traits of maize and that the highest average of grain yield was achieved with the inoculant powder associated with the application of 120 kg ha^{-1} of N in topdressing. Vazquez et al. (2012) evaluated the effects of *A. brasilense* (liquid, peaty and control without inoculant) and the N rates in topdressing (0, 30, 60 and 120 kg ha^{-1}) on the development of the plant and productivity of maize grains. The researchers found that the use of *A. brasilense* based liquid and peaty inoculant did not interfere with the agronomic traits and grain yield of maize and the fertilization with N applied in topdressing resulted in a linear increase in grain yield and the higher applied dose was not sufficient to obtain the maximum response.

Duarte et al. (2012) evaluated the agronomic performance of two maize hybrids (DKB390YG and 30F35H) as a function of seed inoculation with *Azospirillum* spp. ABV 5 + ABV6 strains and N rates in topdressing (0, 30, 60, 90, 120 and 150 kg ha^{-1}). They concluded that the effects of nitrogen fertilization and inoculation with *Azospirillum* on grain nutrition and grain yield of maize depended on the genetic material, with a positive response of DKB 390YG to the inoculation and a higher response of 30F35H to nitrogen fertilization compared to DKB 390YG. The researchers observed that inoculation increased leaf N concentration, but did not provide partial substitution of nitrogen fertilization in maize crop.

Marini et al. (2015) evaluated the efficiency of inoculation of *A. brasilense* based commercial product via seed treatment (100 ml ha^{-1} of inoculant at a concentration of 2.0×10^8 UFC ml^{-1}), in association with different levels of N topdressing fertilization (0, 40, 80, 120 and 160 kg ha^{-1}) via urea, applied between the V_4 and V_6 stages, in two maize genotypes (30F53 and CD386). They verified that the inoculation provided increases of 11 and 12%, for leaf area and dry matter of maize aerial part, respectively. There was a differentiated response of maize hybrids to most of the analyzed variables. Grain yield data were adjusted to the cubic model, obtaining a higher value in the dose of 160 kg ha^{-1} of N by the 30F53 hybrid, with a linear effect increasing as a function of the N doses applied in the hybrid CD386, with an increase in yield of 14.6 kg ha^{-1} for each kg of N applied to the soil.

Laboratory results indicate that the beneficial effect of *Azospirillum* is probably due to obtaining plants with longer roots and larger seedlings, which present a faster initial growth. In field experiments, more roots (54%), higher dry matter in the aerial part (28%) and higher grain yield (7.1% - average of 221 places) were observed, mainly due to the higher number of grains, since there was no change in mean grain weight (Hungria, 2011). The results of studies with inoculation of this microorganism in maize are contradictory. Apparently, what is verified is that there are different ways to carry out the inoculation, with the main ones directly in the seeds, in the furrow of sowing or in the soil when the plant is already in development. Among the causes of variation of available results, it can be inferred that there may be an effect of the fungicides and insecticides applied to the commercial seeds on *Azospirillum*, the culture phase influences the response, or that there is a differential response of the genotypes used.

Considering the aforementioned, obtaining technical information on the application efficiency of inoculants based on *A. brasilense* via seed treatment, spraying in the interior of seeding furrow or foliar spraying (reaching the neck region), in the V_4 stage, can promote the reduction in the use of nitrogen fertilizers in maize crop, with increases in morphological, agronomic and grain yield components.

In Brazil, it is estimated that the use of inoculants containing selected strains of *A. brasilense* can result in an estimated saving of US$ 2 billion per year, considering fertilizer transport costs (Hungria et al., 2010). Consideration should also be given to the benefits of less environmental pollution resulting from the production and use of nitrogen fertilizers as well as the reduction in the emission of greenhouse gases. Thus, researches involving bacteria of the *Azospirillum* genus is developed by plant breeders, because these microorganisms can associate and provide benefits to crops of great economic importance, such as maize, sorghum, wheat, sugarcane, among others. However, the interaction of maize genotypes with the strains of bacteria of the *Azospirillum* genus is not yet fully elucidated, so research in this sense has been growing steadily in the world.

Genotype × inoculation interaction

Studies related to the interaction between genotypes × inoculation demonstrate that there is a differentiated response of the genotypes when they are inoculated with diazotrophic bacteria. Reis Júnior et al. (2000) pointed out that when BNF is related with non-leguminous species, the effect of plant genotype on N fixation is expressive. Thus, identifying, selecting and using less demanding genotypes for the N element are important tools (Revolti, 2014).

Usually, inoculation with *A. brasilense* provides an increase of dry mass, N accumulation in plants and grain yield, especially if the association is between bacteria and unimproved genotypes and under conditions of low N availability (Okon and Vanderleyden, 1997). In addition to these factors, the nutritional state of the plant, the quality of the exudates, the existence of competing microorganisms and the choice of the adapted strain to each region in terms of climate, management system and cultivars, are also factors that can influence the interaction between maize plant and bacterium and affect the efficiency of BNF (Quadros, 2009). According to Bartchechen et al. (2010), research involving *Azospirillum* in maize indicates that the interaction between the bacterium and the plant varies according to the cultivar, edaphoclimatic conditions, and methodologies of conduction of the trials. These methodologies mainly involve: a) forms of inoculation: seed coating, sowing furrow, application via leaf or plant spray; and b) experimental designs, control of pathogens and pests, and vegetative stage of the plant at the time of inoculation.

However, these bacteria naturally exist in most soils and present wide genetic diversity (Ardakani et al., 2011), making it necessary to use efficient strains in BNF and in the production of growth and development hormones capable to compete with native bacteria as well as to select maize genotypes responsive or suitable for this association (Basi, 2013). Thus, following the Brazilian legislation for inoculants, established by the Brazilian Ministry of Agriculture, Livestock and Supply, Embrapa Soybean researchers led by Hungria (2011) found that Ab-V4, Ab-V5, Ab-V6, and Ab- V7 showed higher soil survival, higher growth promotion and adaptation to technologies used in maize and wheat crops. Following the same study, maize yield increases up to 30% in relation to the control not inoculated with the bacteria. This fact justifies the reason why inoculant manufacturers opt for the Ab-V5 and Ab-V6 strains in their products intended for maize and wheat crops.

In practical terms, there are studies in which 85% of the experiments involving maize and bacteria of the *Azospirillum* genus responded positively, with an average productivity increase of 472 kg ha^{-1} (Díaz-Zorita and Fernandez, 2008). Several experiments conducted in Latin America during the last decades, have indicated in the majority, plant growth and/or productivity of the crops studied (Cassán and García de Salamone, 2008). Costa et al. (2015) studied the inoculation with *A. brasilense* in seeds and nitrogen doses in maize crop in Cerrado region and verified that the use of the bacterium promoted higher plant height, culm diameter, leaf chlorophyll index, culm and root dry mass, ear insertion height, thousand grain weight, and grain yield. Similarly, Cunha et al. (2014) also found positive results when inoculating maize seeds with the bacterium, obtaining an increase in productivity and reduction of nitrogen topdressing application by 16%. Morais et al. (2016), when testing several doses of inoculant containing *A. brasilense* applied to maize sowing, verified that the dose of 200 ml ha^{-1} promoted an increase in grain yield. Verona et al. (2010) observed that the inoculation provided greater culm diameter and greater weight in relation to the aerial part dry mass even in water stress. It is known that in addition to the leaves, most of the reserves produced by the plant are stored in the stalks, making this ratio of greater aerial part mass and larger culm diameter produce better storage conditions and a possible higher final production, since these reserves are indispensable for the good development of the plant, mainly in the reproductive phase, to supply the drains represented by the ears.

The benefits of inoculation of *A. brasilense* can be verified in other cultures. Sala et al. (2008) inoculated wheat seeds (*Triticum aestivum hard* L. and *Triticum durum* L.), providing 0, 60 and 120 kg ha^{-1} of urea (70% at sowing and 30%, 30 days after sowing) and they could see that there is an interaction of the endophytic bacteria response with nitrogen fertilization. As a result of the research, the highest cumulative amount of N was obtained with the inoculation of the *A. brasilense* IAC-AT-8 strain and with the addition of 60 kg ha^{-1} of N when compared to the control. They were also able to prove that when the dose of N increased from 60 to 120 kg ha^{-1}, there was a linear decrease in the nutrient utilization efficiency index.

However, not all of the necessary N in the maize crop provided the bacteria. It is an alternative that allows the producer to reduce the use of nitrogenous fertilizers achieving an economy equal to or greater than that found in legumes, which can be self-sufficient in N (Döbereiner, 1992). García de Salomone and Döbereiner (1996) evaluated different maize genotypes inoculated with *Azospirillum* and obtained different responses regarding the inoculation under the yield in the production, noting that there are variations in the interactions between maize genotypes and diazotrophic bacteria.

Scientific advances regarding the use of *Azospirillum* sp. in maize crop

A summary of the main results obtained from inoculation with *Azospirillum* sp. in recent years is shown in Table 1.

FINAL CONSIDERATIONS

Although there are plans to set up new industries and open up new areas for mineral exploration, the situation over the next ten years is quite critical. Thus, the use of Plant Growth-Promoting Bacteria that assist in the biological fixation of nitrogen is of great value for maize and other grasses, as it is an excellent economic

Table 1. Main recent results obtained from inoculation with *Azospirillum* spp. in the corn crop.

Species / methodology adopted	Inoculation effects	References
Azospirillum lipoferum strain (Accession no. GQ255950)	Mitigation the deleterious effects of drought on maize. Benefits in corn crop in normal as well as drought stress conditions.	Bano et al., 2013.
Seed inoculation with Ab-V5 and Ab-V6 strains of *A. brasilense*	Increase in maize plant height, yield of maize grains and plant's dry matter when compared to control.	Braccini et al., 2012
Liquid inoculant carryng *A. brasilense* Ab-V5	The grain production was increased by 29% in the treatment with *A. brasilense* and nitrogen compared to nitrogen fertilization alone.	Ferreira et al., 2013
Seed inoculation with *A. brasilense* and five rates of N.	Decreasing of Fe concentration in leaves and increase of corn grain yield.	Galindo et al., 2016
Seed inoculation with diferente doses os *A. brasilense* strains Ab-V5 and Ab-V6.	The dose of 200 mL ha^{-1} *Azospirillum* was noteworthy for grain production.	Morais et al., 2016
Seed inoculation with *A. brasilense* Ab-V5 strain coinoculated with *Rhizobium tropici*	Seed inoculation with *A. brasilense* as well as their co-inoculation with *Rhizobium tropici* in the absence of N fertilization was efficient to increase plant growth.	Picazevicz et al., 2017.
Inoculation by pelleting with a mixture of *A. brasilense* Az39 and Az30 and *A. lipoferum* Sp7	The number of seeds per ear was increased ca. 2-fold in the inoculated plants. The dry weight of seeds (kg ha^{-1}) was also increased by 59%.	Fulchieri and Frioni, 1994
Three doses of seed inoculation with *A. brasilense* Ab-V5 and Ab-V6 associated with presence and absence of N fertilization	Half the dose of N fertilizer combined with 150 g per 25kg of seeds of A. brasilense in peat formulation provided significantly superior results in agronomic performance of maize, particularly regarding grain yield, thousand seed weight and dry biomass of both shoot and root.	Garcia et al., 2017
Experiments *in vitro* and bioassays were evaluated studying the capacity of *Azospirillum* sp. and *Pseudomonas* sp. to degrade glyphosate residues both in vitro and in vivo in maize plants (*Zea mays* L.) at different growth stages.	In bioassays, inoculation with both bacteria improved germination and root emergence, primary root growth, root hair development and coleoptile growth in seeds previously treated with the herbicide. Foliar inoculation with *Azospirillum* sp. and *Pseudomonas*sp. in glyphosate-treated plants improved root and shoot biomass and increased foliar area, photosynthetic pigments and phytohormone content as well, thus increasing maize yield in the field while concomitantly decreasing herbicide accumulation in leaves and grains.	Travaglia et al., 2015
Efficiency of *Azospirillum brasilense* MTCC125 flocculated cells with standard grown cells under in vitro conditions and in association with maize (*Zea mays* L.) under field conditions.	Field studies with *A. brasilense* flocculated cells conducted under normal irrigated conditions and by withholding irrigation at 25, 50, and 75% available water-holding capacity (AWHC) showed a significant increase in plant height (19%), plant dry weight (16%), grain yield (31%), stover yield (17%) and nitrogen uptake (18%) compared with standard grown cell treatment.	Joe et al., 2012
Corn seeds were inoculated with a commercial product based on the Ab-V5 and Ab-V6 strains of *A. brasilense*	Inoculation with *A. brasilense* provided increases of 11 and 12% in leaf area and shoot dry matter, respectively.	Marini et al., 2015

Table 1. Contd.

Inoculation with the active strain of *Azospirillum brasilense* (strain 65B) Inoculation was performed just before sowing: bacterium suspension was mixed with maize seeds and used for spraying the field.	Inoculation of maize with *A. brasilense* bacteria contributed to an increase of that plant vigour and yield.	Swędrzyńska and Sawicka, 2000
Seven strains of *A. brasilense* (Ab-V1, Ab-V2, Ab-V4, Ab-V5, Ab-V6, Ab-V7 and Ab-V8) isolated from maize plants and two of *A. lipoferum* (Al-V1 and Al-V2) were aplicated to seeds as peat-based inoculants.	*A. brasilense* strains Ab-V4, Ab-V5, Ab-V6 and Ab-V7 increased grain yields of maize by 662–823 kg ha^{-1}, or 24-30%, in relation to non-inoculated controls.	Hungria et al., 2010
Many strains of *A. brasilense* and *A. lipoferum* have been used to inoculate cultivars of different cultivars of species of plants, in including maize, in more than ten countries.	The data indicates 60-70% occurrence of success with statistically significant increases in yield of the order of 5-30%.	Okon and Labandera-Gonzalez, 1994.
The present study in pots was performed to investigate the effect of inoculation of individual strains (and a mixture) of *Azospirillum* spp., and their nitrate reductase negative (NR-) mutants, on the growth of four of these maize genotypes.	Two maize genotypes produced similar increases in grain yield when they were inoculated with a mixture of *Azospirillum* spp. strains or fertilized with the equivalent of 100 kg N ha^{-1}. The two genotypes showed a large increase in total N accumulation, suggesting that the response was due to increased N acquisition, but not due to bacterial nitrate reductase as the NR- mutants generally caused plant responses similar to those of the parent strains.	De Salamone et al., 1996
Association of doses of nitrogen fertilization with and without inoculation with *A. brasilense* strains Ab-V5 and Ab-V6.	There was a significant increase in both number and mass of commercial corn cobs with A. brasilense inoculation as compared with treatment without inoculation. The association of inoculation with *A. brasilense* and nitrogen increase more than 30% the corn cobs production.	Araújo et al., 2014
Four methods of inoculation with *A. brasilense* (Ab-V5 and Ab-V6) were compared: (1) standard seed inoculation – control treatment; (2) inoculation in the planting furrow at sowing; (3) leaf spray inoculation at the V2.5 stage of the maize plant growth cycle or 3rd tiller for wheat ; and (4) spray inoculation on the soil surface at the V2.5 stage of the maize plant growth cycle or 3rd tiller for wheat.	All inoculation techniques increased the abundance of diazotrophic bacteria in plant tissues, and foliar spray improved colonization of leaves, while soil inoculations favored root and rhizosphere colonization. In field experiments, inoculation with *A. brasilense* allowed for a 25 % reduction in the need for N fertilizers.	Fukami et al., 2016

opportunity to be able to increase the efficiency of nutrient absorption, in addition to providing environmental benefits associated with reduced fertilizer use. However, the efficiency in the use of diazotrophic bacteria in maize is related to the all management involved during the development of the plant, since there is a need to know the physiological characteristics and water needs, nutritional, pest control and crop diseases, so that the actual influence of the growth-promoting bacteria present in the rhizosphere on the plants can be obtained. In addition, the total nitrogen supply of the crop will not only be supplied by the microorganisms, it is necessary to stagger the topdressing fertilization at the recommended doses for the crop, as shown in several types of research.

On this way, considering the existence of a wide range of available genotypes of maize, either commercially or in research institutes, there is a great importance to know the interaction of the genotype under study with the inoculation form of diazotrophic bacteria, in order to

identify, select and use less demanding genotypes for the N element. This happens because, as occurs with *A. brasilense* and several microorganisms, there are genetic variations within the same species, which demands the development of research aimed to evaluate and relate more closely maize genotypes under study with the degrees of association of the existing strains. So it will be possible to delineate genetic breeding programs in an efficient way in order to develop cultivars more responsive to the inoculation with diazotrophic bacteria. Thus, in addition to the low cost for farmers, the use of beneficial bacteria containing *Azospirillum* contributes to the environment and may be the subject of future negotiations on carbon credits trading. The prospects are also that, in the coming years, the agronomic efficiency of inoculation with *Azospirillum* can be confirmed with other Gramineae.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest

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Review

Propagation of Pecan (*Carya illinoensis*): A review

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The Pecan (*Carya illinoensis*) is the most important species that belongs to the *Carya* genus. It is cultivated mainly for its nut, which is rich in oils and proteins, and for its good quality wood. Pecan is conventionally propagated by budding or grafting onto rootstocks obtained by open pollination. However, these techniques are not very efficient due to low propagation rates, poor survival and difficult establishment. Therefore, *in vitro* propagation of pecan can play a very important role in rapid multiplication of cultivars with desirable traits and production of healthy and disease-free plants in a short time. In the present review, the improvements over the years in the propagation of pecans, the refinements of protocols for obtaining high shoot multiplication and regeneration through organogenesis and/or somatic embryogenesis is discussed. Some basics of genetic transformation and its possible benefits are also discussed.

Key words: Pecan, *in vitro* root induction, shoot multiplication, regeneration, micropropagation, somatic embryogenesis, genetic transformation.

INTRODUCTION

Distribution and production

The Pecan is a species native to North America, which is the leading producer in the world. It is however also cultivated in Australia, Brazil, Canada, Mexico, Israel and South Africa. In 2015/2016, the world production of Pecans was more than a total of 101,000 metric tons (kernel basis), that is 5% more than in 2005/2006. Production of pecans is clearly led by the United States and Mexico, which account for 92% of the world production. These two countries' production in 2015/16 was 52,889 and 40,824 metric tons, respectively and was followed by South Africa with 5,380 metric tons and Australia with 1,716 metric tons (International Nut and Dried Fruit Council Foundation, 2016). In South Africa, the Southern Lowveld is the biggest pecan production area. Other important areas are White River, Tzaneen, Louis Trichardt /Levubu, KwaZulu-Natal, the Vaalharts irrigation scheme, the Middle veld around Pretoria and some parts along the Orange River (De Villiers and Joubert, 2008) (Figure 1).

Classification and biology

In systematic botany, the Pecan is classified under the

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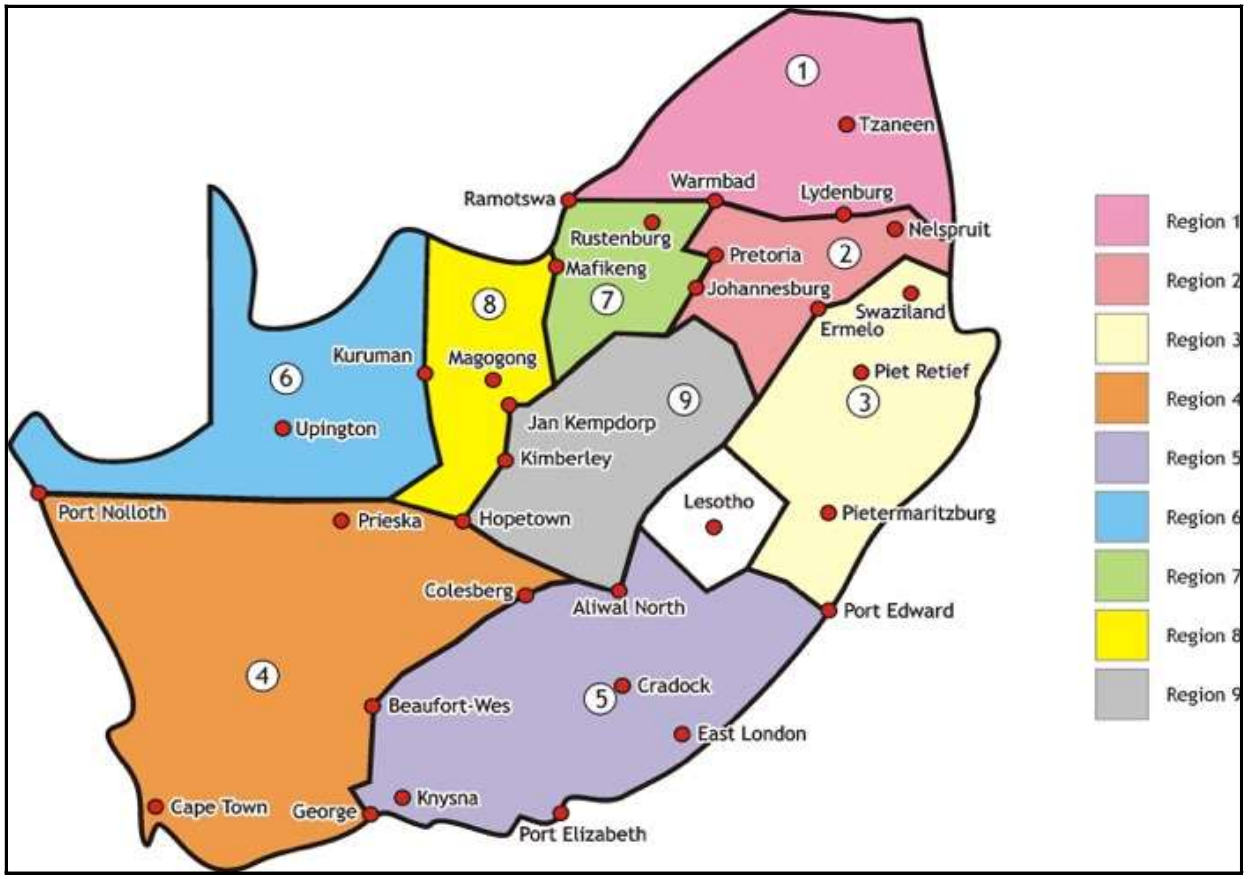

Figure 1. Different South African pecan nut production regions.

Kingdom *Plantae*; Class, *Magnoliopsida*; Order, *Juglandales*; Family, Juglandaceae and Genus, Carya. The *Carya* genus has approximately 25 species, in which *Carya illinoensis* represents the most economically viable nut crop. Pecan can reach, in its natural habitat, a maximum height of 55 m and a canopy of more than 2 m in diameter. The Pecan root system can be divided into 3 classes (Woodroof, 1934): 1) Taproot, which grows downward, and its depth of penetration may vary in relation to the type and structure of the soil, and the moisture conditions; 2) Lateral roots, which have a horizontal development and generally remain superficial; 3) Fibrous roots that grow in all directions from laterals and are constantly dying and replaced by new roots. It is a deciduous tree with alternate and compounded leaves, whose numbers of leaflets can vary from 9 to 17 (Reed and Davidson, 1954). The leaves can vary in length and color depending on the cultivar (Figure 2).

It is a monoecious species, with staminate and pistillate flowers on the same tree. The male flowers are pendulous and grouped in catkins and grow on one-year-old branches. Generally, the pollens produced by the male flowers are abundant every year. The female flowers are organized in terminal spikes and grow on the current season's growth. The pistillate flowers are apetalous with bilobed stigmas and surrounded by four foliar bracts. The female flowers can be present in adequate amounts or they can be scarce if the yield was high the previous year, due to the alternate bearing phenomenon typical of woody fruit plants.

Shedding of the flowers may occur in the early spring season mainly for three reasons: (1) Rudimentary flowers located near the shoot tip; (2) Normal flowers that were not pollinated and (3) Pollinated flowers in which nutlets did not develop because the food reserves were depleted during early growth or because of unfavorable moisture conditions. Another flower fall may also occur in late summer and is caused by incomplete fertilization (fruit set) (Byford, 2005). Pollination is typically by wind (anemophilous pollination). Pecan is a hetero-dichogamous species, that is, another dehiscence and pistil receptivity do not coincide. In fact, some cultivars are protandrous (which means the male flowers develop before the female ones), and others are protogynous (where the stigma receptivity precedes the maturity of the pollen). Dichogamy promotes cross-pollination; although a short period of overlapping exists self-pollination is also possible in some cultivars. Therefore, for adequate

Figure 2. Pecan orchard cv. Wichita located in Hartswater, Northern Cape, South Africa.

pollination, it is crucial to choose compatible cultivars that synchronize with each other in the production of mature pollen when the stigma is receptive (Vendrame and Wetzstein, 2005). Pecan fruit is a nut, which consists of a kernel enclosed by the shell. The shape and dimension of the nut varies from cultivar to cultivar and the maturation occurs in the autumn of the same season (Peterson, 1990). The nut can be considered as a storage organ. In fact, it stores minerals, carbohydrates, oils, amino acids and proteins that will serve the future embryo for respiration, germination and even in the early life stages of the seedling until it becomes self-sufficient.

Nut development starts with pollination and can be divided into two distinct phases: With phase I, which occurs from pollination to shell hardening and phase II that occurs from shell hardening until the shuck splits (Herrera, 1990). Harvesting time depends on the area of cultivation. In South Africa, pecan nuts are usually ripe from April to July, depending on the cultivar. As soon as the nut is physiologically ripe, the green husk becomes dry, cracks open and the nut drops out (Anonymous, 2000). The nuts are collected manually or mechanically, using a hydraulic trunk shaker and a mechanical harvester.

Uses

The nut is the main economic product used as food for humans and wildlife (Harlow et al., 1991); the wood is also used mainly for the manufacturing of floors, furniture and veneer, it being of good quality. Pecan is also used as an ornamental specimen in the decoration of parks and gardens. The main chemical constituents of pecan nuts are lipids, which make up 73% of the total (primarily oils), followed by proteins (9.4%), carbohydrates (3.9%), water (3%) and a relatively small number of other components. Sometimes, alterations of a particular minor component may affect the quality of the nuts (Kays, 1990). The oil extracted from the kernel is edible and is used to produce medicine and essential oils. The quality of Pecan nuts in postharvest depends on many factors and varies according to the intended use of the product. Generally, the most important external parameters are nut size, color and external appearance. The color of the testa is the major external quality attribute and light-colored kernels are preferred. Most of the color development occurs on the tree during maturation after the onset of dehiscence (Kays and Wilson, 1977), followed by a progressive synthesis of pigments after harvest (Senter, 1976). The external quality of the kernels affects the assessment of their overall quality. The absence of insect damage and the absence of breakage are very important.

Flavor is the most important internal quality attribute used by consumers. However, this evaluation can only be made after purchase, due to the way in which the nuts are sold (sealed packages). Fresh Pecans have a

distinctive aroma and taste, and for this reason are often consumed without roasting.

Kernel fill is also another important quality. It occurs in the last stage of development and can vary with location, cultivar and year of production. Generally, poorly filled nuts do not have visual attractiveness, a good flavor quality and the textural properties of high quality nuts (Kays, 1990).

The storage conditions of the nuts play a key role in nut quality because it influences the moisture content of the nut. Under high temperature, relative humid environments the kernels become progressively more spongy and moist, but if stored under low temperatures and low humidity, the kernels become excessively crisp and brittle (Kays, 1990).

Conventional methods of propagation

The first commercial Pecan orchards date back to the nineteenth century. They were established by sowing seeds collected from mother trees with desirable characteristics such as nut size, a resistance to scab disease, early maturity and high yields (Taylor 1906, 1907). These open-pollinated half-sib populations existed until clonal propagation of superior genotypes led to the widespread use of true cultivars through the improvement of budding and grafting techniques. Conventionally, Pecan trees are propagated by budding or grafting a scion of a selected clone (cultivar) onto rootstocks obtained from seed. Budding (patch budding or ring budding) is done during the vegetative growth phase in summer, while grafting (3-flap grafting) is done during the dormant winter season (Woodroof, 1979).

ROOTSTOCK PROPAGATION

The seeds chosen to produce rootstocks of a particular cultivar should adhere to four essential conditions: 1) Uniformity, with a well-developed kernel; 2) Readily available, to have a continuous supply each year; 3) Vigorous seedlings; 4) Resistance against seedling diseases (Conner, University of Georgia). Careful consideration should be given to the choice of rootstock and cultivar, which are used to establish an orchard. Due to the undersupply of trees, many growers grow their own rootstocks using seeds and when seedlings reach a sufficient diameter (1.5 to 2 cm), they graft or bud a superior Pecan nut cultivar on to it (Reid, 2010).

Seed

Three important parameters are used to select seed to propagate rootstocks namely: price, kernel percentage and uniformity. Usually, small nuts are selected (Andersen, 2004). However, Pecan seeds are generally not ready for germination, being in a state of dormancy. However, in some cases, premature germination can occur while the nuts are still attached to the tree. This phenomenon, called vivipary, occurs when the seeds go through a stage of rest, which is controlled by an inhibitor hormone called abscisic acid (ABA). Leaves will not produce ABA because of a heavy fruit load, causing mature seeds to germinate while still attached to the tree, due to a low ABA concentration (McEachern, 2010).

Dormancy

Dormancy in deciduous fruit trees is a natural mechanism, which is necessary to enable the tree to overcome adverse climatic conditions during winter (Faust et al., 1997). Over the years, there have been several definitions of dormancy. The most universally accepted definition of dormancy was formulated by Lang (1987), who introduced the terms eco-, para- and endo-dormancy. Eco-dormancy refers to the environment (eco), para-dormancy refers to physical or biochemical external signals that are sent to the affected structure (para), and endo-dormancy refers to physiological factors inside the affected structure (endo).

For plant propagation in nurseries, dormancy is a barrier in the production of seedlings because it extends the time of germination after sowing and the seeds are also exposed to predators and unfavorable weather conditions. Uneven germination results in seedlings that differ in size and consequently increase the production cost of trees in the nursery (Poletto et al., 2016). Therefore, the main challenge is obtaining a faster germination rate and increasing the uniformity of the seedlings. Stratification is a process in which dry seeds are soaked in water and then stored in a cold, moist condition for 90 to 120 days to break dormancy. Immediately after harvesting, Pecan seed should be stored at low temperatures (1 to 5°C) to maintain viability, break dormancy and ensure germination. Stratification is basically the representation of what happens in nature. Dormancy is gradually broken during the winter because the nuts lie on the leaf litter and is subjected to several cycles of humidity and cold temperatures. The artificial stratification process begins by soaking the nuts in running water within a tank for a variable period of 1 to 3 days. Afterwards, it is placed in media such as peat moss, cedar shavings, potting soil or saw dust to capture the excess water and maintain the humidity. Then, the nuts are stored at a temperature of 1.7 to 7.2°C, until planting.

It is recommended to stratify the nuts for at least 10 weeks to have a fast germination rate (Sparks et al., 1974). Several reports are available in literature regarding the stratification conditions of Pecan seeds (Bonner, 1976a; van Staden and Dimalla, 1976; Dimalla

and van Staden, 1977, 1978; Adams and Thielges, 1978; Goff et al., 1992).

Pre-germination treatments

Shu-fang et al. (2011) performed a germination test on Pecan seeds using different storage times, various soaking methods, applying phyto-hormones and different seed stratification methods and seeds stored for more than two months, at 3 to 5°C germinated the best. As the storage time increased, the germination rate decreased. The germination rate improved remarkably when the seeds were treated with phyto-hormones and stratified. The seeds that were soaked in phyto-hormones for 8 days and stratified indoors for 35 days, resulted in a 91% germination. Bonner (1976b) stated that germination without prior stratification is greatly enhanced by soaking the seeds in gibberellic acid. Recently, Poletto et al. (2016) executed a study to overcome Pecan seed dormancy using different methods.

The seed were stored for a period of 30, 60 and 90 days at either room temperature (controlled treatments) or at 4°C. Half of the seeds stored at 4°C were also scarified by using sandpaper. The results obtained showed that the highest Pecan emergence speed index (ESI) and the best seedling development were observed when seeds were stored at 4°C as well as scarified.

Seed viability was significantly reduced when stored at room temperature, irrespective of the storage time. Bilan and Foster (1970) also assessed the effect of various chemical treatments upon germination of stratified and unstratified Pecan nuts. Results showed that chemical treatments did not have any significant effect on germination for stratified and unstratified seeds. They also stated that stratification is the only method to speed up the germination of Pecan seeds. Casales et al. (2017) investigated the effect of different chemical seed treatments (2500 or 5000 ppm KNO_3; 500 or 1000 ppm GA_3; 1% HCl; 2% Dormex and 98% H_2SO_4; control: no chemical treatment) and two temperatures (25 and 28°C) on dormancy breaking and germination of Pecan seeds cv. Ukulinga. A significantly higher germination percentage was obtained at 28°C (55%) than at 25°C (41.3%). Shelled seeds treated with 5000 ppm KNO_3 or 1000 ppm GA_3 had a 100% germination rate. However, shelled seeds not treated with any chemicals also resulted in 100% germination.

Effect of moisture and temperature on seed germination

Water and temperature, separately or together, are the most important factors affecting the germination percentage and germination rate (Shaban, 2013). On the one hand, temperature plays a pivotal role in determining the periodicity of seed germination and the distribution of species (Guan et al., 2009); on the other hand, water is an essential requirement for germination. In fact, water allows the activation of a series of enzymes, translocation and the use of food storage (Shaban, 2013).

Generally, the temperate-region seeds (such as Pecan) require lower temperatures than tropical region seeds, and wild plant species have lower temperature requirements than domesticated plants. High-quality seeds can germinate under wider temperature ranges than low-quality seeds (Shaban, 2013). After the harvest, a period of dry storage is required for Pecan nuts to reduce the kernel moisture percentage from 20% to 6-4% before being stored for the long term (McEachern, 2010).

According to King and Roberts (1980), Pecan is included in the list of species with recalcitrant or short-lived seeds. This group belongs to the species whose seeds retain viability for a short period of time, and most of them cannot tolerate moisture below 25%. However, seed longevity can be increased with proper handling and storage (Hartmann et al., 2002). Van Staden and Dimalla (1976) defined Pecan seeds as non-dormant seeds because the seeds can germinate at any time after harvest, if incubated under favorable conditions. Although the nut shell is freely permeable to water and gases, the germination is delayed by mechanically restricting the radicle to elongate (Smith et al., 1997).

However, this problem can be overcome by incubating the seeds at a temperature of between 30 to 35°C. At these temperatures, the germination of seedlings is uniform, rapid and is completed within 20 days. Dimalla and van Staden (1977) also reported that incubated Pecan seeds at 30°C showed higher levels of endogenous cytokines and gibberellins than those seeds that were incubated at 20°C. These results suggested that endogenous hormones play an important role in the mobilization of lipid food reserves to supply the energy required for germination.

Sowing in seedbeds and containers

In nut tree nursery, two methods will normally be followed for the propagation of trees for subsequent use in orchard: 1) Sowing in seedbeds or 2) Sowing in containers.

Seedbeds provide a high seedling production, but it is more difficult to harvest the trees for transplanting in the orchard. A soil analysis is needed before sowing to evaluate the fertility of the seedbed and rectifying imbalances that may occur. Fertilization with a slow-release fertilizer along the row is usually performed during the beginning of summer. Budded or grafted trees should be dug for transplanting in orchards in the spring of the following year (Reid, 2010). Seeds should be sown 10 to 15 cm apart in the row and 7 to 10 cm deep. Sowing depth should be shallower in clay soils than in

sandy soils (Wells, 2014). Seedbeds must be weed-free and well watered.

Another way to grow Pecan seedlings in a nursery is in containers. Different sizes and shapes are available, but it is advisable to choose containers with an "open bottom". Roots are generally air-pruned by placing the pots on a screen wire bench. Using this method prevents the circling of the taproot in the container and promotes a fibrous root system. It is advisable to use a growth mixture that allows the free flow of water through the pot. A slow-release fertilizer that can provide both macronutrients and micronutrients can be added to promote root system growth.

A mix of three parts ground pine bark, one-part peat moss and one-part coarse sand is recommended. All potting mixes should be sterile (Wells, 2014). Keever et al. (1986) investigated the effect of container size and shape, root pruning and fertilization rate on the growth of Pecan seedlings, with the aim to reduce the time required to reach the budding stage. Results showed that seedling Pecan height growth was greater in 38 L containers (68.6 cm) and 19 L containers (61.7 cm) compared to shallow 19 L containers (49.3 cm) and 11 L containers (50.3 cm). Nevertheless, all the seedlings reached the budding stage in the first growing season. They also found that root pruning at transplanting did not affect the top growth but increased root branching and total root growth as well as the increment rates of a complete fertilizer.

Zhu et al. (2017) executed an experiment on one-year-old Pecan container seedlings under sub- and overhead irrigation, with the aim to determine water use efficiency (WUE), vegetative growth, photosynthesis and the nutrient status of the Pecan seedlings. The results showed that sub-irrigation can conserve up to 62% of the irrigation water and improve water use efficiency (WUE) by 193% compared to overhead irrigation. Seedling height and root collar diameter increased by 11.7 and 41.5% respectively. The net photosynthetic rate, stomata conductance and transpiration rate also improved significantly. The nitrogen and potassium content of the roots, stems and leaves increased as well as the phosphorus content of the leaves under sub-irrigation treatment compared to overhead irrigation.

Benucci et al. (2012) evaluated the possibility to obtain formations of mycorrhizae of European truffle species (*Tuber aestivum*, *T. borchii* and *T. macrosporum*) on the roots of pecan seedlings. Roots of Pecan seedlings were inoculated with the truffle spores and grown in a greenhouse for 10 months. *T. borchii* and *T. aestivum* spores produced well-formed ectomycorrhizae on the seedling roots with a colonization percentage of 62 and 42%, respectively. No ectomycorrhizae of *T. macrosporum* were formed on the roots. Marozzi et al. (2017) specified that Pecan seedlings were inoculated with spores of black truffles (*T. melanosporum* and *T. brumale*), which are economically important in Europe. Mycorrhization on Pecan roots were assessed over a 2-year period. In the first year, *T. melanosporum* and *T. brumale* produced ectomycorrhizae and 37.3 and 34.5% colonization of the roots were obtained, respectively. After 24 months, the percentage of colonization for *T. brumale* increased to 49.4% and decreased for *T. melanosporum* to 10.5%. In both works described above, the mycorrhization of Pecan seedlings was aimed at obtaining an extra income (due to truffles) along with the future fruit of the Pecans. In the nursery, the mycorrhization of Pecan seedlings might be performed routinely to help them to survive in adverse weather conditions (Smith and Read, 2008), to improve the absorption of mineral elements, especially nitrogen (Chalot and Brun, 1998; Dighton, 2009) but also to tolerate soils with high concentrations of salts, heavy metals (Blaudez et al., 2000; Sell et al., 2005; Colpaert et al., 2011) and organic pollutants (Dighton, 2009).

CUTTINGS

Greater uniformity is obtained when rootstocks are propagated by means of cuttings due to the absence of genetic variation. It is inexpensive, rapid and simple and does not require special techniques (Chiu, 1977). Over the years, indole-3-acetic acid (IAA) and other synthetic auxins have been used to promote the rooting of cuttings (Cooper, 1935; Went, 1935). However, the response of the cuttings to the auxin treatment is not the same in all plant species. In addition, it has been shown that softwood cuttings respond better to auxins, compared to hardwood cuttings (Hess, 1959, 1962). This difference is attributed to the presence of substances other than auxins, which can stimulate or inhibit rooting (Went, 1934; Cooper, 1938; Spiegel, 1954). The production of rooting promoters or inhibitors is not consistent throughout the year but instead, their production fluctuates with the season. Therefore, the root-ability of the cuttings seems to be associated with substances produced inside the plants, e.g., rooting co-factors (Hess, 1960; Tognoni et al., 1977). Rooting experiments, using Pecan stem cuttings, gave highly variable results. Some attempts (Stoutemeyer, 1938; Romberg, 1942; Gossard, 1944; Sparks and Pokorny, 1966; Whatley et al., 1966) gave poor results. Even the use of auxins, such as indole-3-butyric acid (IBA) to induce rooting, gave inconsistent results as reported in literature (Wolstenholme and Allan, 1975; Brutsch et al., 1977). The main factors affecting the rooting of cuttings were harvest time, thickness and origin of the cuttings and genetic factors.

Hardwood cuttings

In Pecan hardwood cuttings, the time of collection seems to be the most important factor to induce rooting, as

reported by McEachern (1973). In fact, he affirmed that the optimum period for collecting Pecan hardwood cuttings seems to be in the middle of the dormant season or after they have accumulated 200 to 400 h of field chilling (7°C). Smith et al. (1974) used juvenile (softwood) and mature (hardwood) cuttings collected during the mid dormant season. Juvenile wood dipped in 10000 ppm IBA, gave a 100% rooting, while mature wood rooted at 85% under the same conditions.

In another work, Taylor and Odom (1970) used hardwood and softwood Pecan cuttings, which were exposed to various preconditioning treatments prior to propagation. In particular, hardwood cuttings, 15 cm long and 1 cm in diameter, were collected in winter and divided into 2 groups. In the first group, cuttings were stored for 42 days in moist peat moss at 4°C. In the second group, cuttings were stored at 3°C for 30 days with the basal half of the cuttings submerged in moist peat moss and maintained at 20°C. The controls were directly inserted into the propagation medium at the time of application without any treatment.

Softwood cuttings were exposed to preconditioning treatments in the spring, immediately before bud break. For this group, etiolated shoots (15 cm long and 0.5 cm in diameter) were used, which were girdled or not. Control cuttings were not etiolated or girdled. The medium used was a mixture of perlite and peat moss in the ratio 1:1. Softwood cuttings received intermittent mist, and all the cuttings remained in the propagation bed for 75 days in the greenhouse. The etiolated and girdled softwood cuttings obtained a 15% rooting. These cuttings showed a vigorous root system and retained their leaves. Hardwood and softwood cuttings that were not girdled, did not initiate roots. The presence or absence of endogenous roots, promoting or inhibiting compounds, was determined by using the mung bean rooting bio-assay. Data obtained showed that the Pecan, regardless of type of cutting or preconditioning treatment, contains 3 or 4 distinct areas of root promotion.

Endogenous rooting inhibitors were present in all the treatment groups, except for those hardwood cuttings that were subjected to cool storage. A compound with similar chemical characteristics to juglone, which is associated with rooting inhibitory activity, was also found in the leaves and stems. Wolstenholme (1976) obtained Pecan regeneration by the stub. If the strongest shoot is trained as the new tree, while the others are removed, it will grow faster and more vigorous. Allan et al. (1980) carried out an experiment using hardwood Pecan cuttings collected from vigorous 'Barton', 'Desirable' and 'Shawnee' top-worked trees. Two experiments were conducted.

The first experiment was to study the effect of girdling and cutting thickness (10, 15 and 20 mm) on root-ability, while the second experiment was to determine the effect of cold treatment before or after treating cuttings with 1% IBA for 5 s. The best rooting (90%) was obtained with thick basal cuttings irrespective of whether they were girdled or not. Good survival percentages were obtained with all the basal cuttings (70 to 82%). The percentage was much lower with median cuttings (18 to 25%) and worthless for thin terminal cuttings. The best combination of IBA/cold treatment that gave the best rooting percentage was the control (cuttings directly placed in mist beds after a basal dip for 5 s with 1% IBA) (about 65%). Over 60% of the rooting was also obtained by the two or four week's cold treatment at 4°C followed by the IBA treatment.

The survival percentage of the cuttings was significantly better when the two weeks cold treatment was applied before the IBA treatment (48%). No significant difference in rooting percentage was found among the cultivars, although 'Shawnee' survived the best. Gustafson and Miles (1978) investigated the effect of apical buds and cultivar on the rooting of hardwood Pecan cuttings taken from adventitious sources during the winter. After cuttings were treated with IBA and a fungicide, cuttings were placed in an insulated propagation box and maintained at 3°C filled with (1 peat: 1 perlite: 1 vermiculite) and kept at 23 to 25°C. Results showed that adventitious cuttings rooted better than visible bud cuttings, especially when removing the apical bud (90 and 25%, respectively). Among the cultivars, Coy and Greenriver had the highest rooting percentage (37.5% and 30%, respectively), while 'Major' and 'Indiana' had moderate rooting (17.5 and 10%, respectively). Treatments with IBA and fungicide, or both, were beneficial because they reduced dieback from the proximal end of the cutting.

Spencer (1980) stated that the problem of establishing the cuttings, once they are rooted, can possibly be overcome by taking hardwood cuttings in summer, before the buds are dormant, and subjecting them to basal heat for rapid root development. Huang et al. (2006) used hardwood cuttings of three-year-old Pecan seedlings. Among the concentrations tested, the best rooting was obtained using 200 ppm NAA (α-naphthaleneacetic) and 100-500 ppm IBA.

Li et al. (2013) studied the effects of the parent tree age and the thickness of the cuttings on the rooting capability of the Pecan. One-year old wood with a diameter of 0.5, 0.5 - 0.8 and ≥ 0.8 cm were collected from 2, 5, 16 and 25 years old parent trees. Cuttings were treated with different concentrations or combinations of IBA and NAA. Callus and rooting percentages of the cuttings from 2-year-old parent trees were 87.7 and 14.4%, respectively, significantly higher than the other tree ages. The best results were observed from the cuttings with a diameter of ≥ 0.8 cm, callus and rooting percentages were 33.1 and 9.7%, respectively. The treatment of IBA 1.0 g l^{-1} + NAA 0.25 g l^{-1} exhibited the best results after treating the cuttings with the thickness of ≥ 0.8 cm, and the callus and rooting percentages were 43.0 and 14.6%, respectively. Under the same ages of the parent trees, the callus and rooting percentages of the cuttings were positively correlated to the thickness of the cutting.

Zhang et al. (2015) used hardwood cuttings collected from 1-year-old Pecan trees to investigate the influence of auxins (0.03, 0.06 or 0.09% IAA or IBA and 0.06, 0.09 or 0.12% NAA) and different combinations of media and air temperatures on their rooting ability. Cuttings (12 cm long) were treated with different concentrations of auxins at room temperature for 4 h. The basal ends of each cutting were soaked in distilled water as a control. The rooting substrate was composed of a mixture of peat, perlite, coarse sand and silver sand. Cuttings were then planted into the root substrate (2 peat: 4 perlites: 1 coarse sand: 1 silver sand) at a media/ambient temperature as follows: 1) Media and ambient temperature both at 13 ± 2°C. 2) Media (25°C ± 2°C) and ambient (13 ± 2°C) temperature. 3) Media and ambient temperature both at 25 ± 2°C. The best result was obtained with the 25 ± 2°C media and the 13 ± 2°C ambient temperature treated with 0.09% NAA (82% rooting) or 0.06% IBA (80% rooting).

Softwood cuttings

Gossard (1944) was one of the first to obtain the rooting of Pecan softwood cuttings. Although he obtained rooting under continuous mist, in the end no plant survived transplantation. Shreve (1974) used six 1-year-old Pecan seedlings to induce the rooting of softwood cuttings. From the six seedlings, all the visible buds were removed from three cuttings, and all the terminal buds were removed from the stems of the other three cuttings, to force growth from lateral visible buds. The cuttings were planted in pots of 1 peat: 1 perlite. Twelve cuttings (2 from each seedling) were set (6 from visible buds and 6 from adventitious shoots) and maintained under intermittent mist and sprayed with a 4-4-8 Bordeaux mixture. Rooting was obtained from the six cuttings from adventitious shoots after 15 days, and shoot growth started after 35 days. Cuttings from the visible buds formed roots in 30 to 70 days, but none of them developed shoots.

LAYERING

The purpose of the Layering Technique is to induce rooting from a stem of the mother plant. When the new growing plant can survive on its own, it will be cut off from the mother plant (Anonymous, 2007). Gossard (1941) reported that it is possible to obtain roots from a Pecan with success by trench-layering the budded or grafted trees and by air-layering the old trees in a marcot in conjunction with an IBA treatment. Due to the unavailability of clonal Pecan rootstocks, studies were initiated by Abou-Taleb et al. (1992) to evaluate the effectiveness of air-layering, stooling and trench layering techniques for propagation and field survival of clonal Pecan rootstocks and to obtain an estimation of the relative responsiveness of genotypes.

Air-layering

Air-layering is another technique to obtain clonal Pecan rootstocks. It is performed with a sharp knife, as two parallel cuts are made about 2 cm apart around the stem and through the bark and cambium layer. The two parallel cuts relate to one long cut. The ring of bark is removed to leave the inner woody tissue exposed. The newly bared ring is scraped to remove the cambial tissue, which is to prevent a bridge of callus tissue from forming. At this point, the rooting hormone is applied and the moss will be wrapped and covered with plastic or aluminum foil and held in place with twist ties or electrician's tape (Beckford, University of Florida). This process should be completed during rainy seasons, when the humidity is the highest (Anonymous, 2007). Litchi, Guava, Macadamia, Mango and Pecan (Pokorny and Sparks, 1967; Abou-Taleb et al., 1992) are all propagated with this method.

Mound (stool) layering or stooling

Mound layering is an old method of propagation, initially established to mass propagate apple clonal rootstock, but subsequently it was also used for other fruit tree species such as Quince, Currants, Gooseberries and Pecan (Carlson and Tukey, 1955; Brase and Way, 1959; Duarte and Medina, 1971; Medina, 1981; Garner, 1988). With this method the shoots are cut back to ground level and soil or rooting medium is mounded around them to induce rooting at the base. Stool shoots will be separated from the parent plant if they have sufficient roots by the end of the growing season. Rooted shoots are cut at their bases and delivered to customers to transplant into the nursery as "rooted liners" (Hartmann et al., 2002).

BUDDING AND GRAFTING

In Pecan propagation there has been important improvements over the years using tissue culture and cuttings. The commercial Pecan industry is still dependent on propagation by grafting and budding. Grafting and budding provide a success rate of more than 75% (Nesbitt et al., 2002). However, the disadvantages of these techniques are that the seedlings require 2 to 3 seasons of growth before it reaches an acceptable stem diameter to be grafted (Zhang et al., 2015).

Zhang et al. (2015) performed grafting using 1-month-old, open-pollinated 'Shaoxing' Pecan seedlings for evaluation of grafting as a technique to reduce the production time of grafted trees. Germinated seeds with a hypocotyl less than 0.5 cm were selected for the

experiment. The diameter of the epicotyls was 2 to 3 mm, the stems were 15 cm long and the 1-year-old scions 'Caddo' and 'Desirable' Pecans were used. Each scion had at least 4 healthy buds and was 2 to 3 mm in diameter to match the diameter of the seedling epicotyl. Scions cut in the center were sealed in polyethylene bags and marked as "terminal scions" and "base scions" respectively and stored at 5°C until used for grafting. Buddy tape and medical tape were also compared to assess which one was the best in terms of the graft success rate. The rootstock was cut off smoothly above the epicotyl and was then split 2 cm deep with a grafting knife. The scion was obliquely cut through both sides, 2 cm from the lower end and fitted and inserted on the rootstock. The cut area and the scion were wrapped with both types of grafting tape. The completed graft was transferred to a 15 × 20 cm container with 1 or 2 buds exposed. The containers were placed in full sun, on a concrete floor, in a greenhouse. Successfully grafted seedlings began to sprout 40 to 45 days after grafting. Results showed that 'Desirable' scions had greater success than 'Caddo' scions, and earlier grafted plants had more success than plants grafted later (20-day difference). Grafting took place in late spring. Graft success percentages were 87.13% for 'Desirable' and 70.51% for 'Caddo'.

In a study conducted in Iran by Ajamgard et al. (2016), three different grafting methods and different grafting times were investigated. The grafting methods included Cleft grafting using hot cables, which is done five different times from late October until mid-February; Cleft grafting using polyethylene bags, and Side-stub grafting done five different times from early November until early March. Scion wood of ‘GraTex’, ‘Wichita’, ‘Choctaw’, ‘10J’ and ‘GraKing’ were grafted onto two-year old seedling rootstocks that were 1 to 1.5 cm in diameter. The best grafting time was from late January until late February, using Cleft grafting with hot cables. For Cleft grafting with polyethylene bags the best time was from mid-February until late March. The highest percentage of graft success was achieved using ‘Wichita’ scions (92%), and the lowest ‘10J’ (less than 10%). The Side-stub grafting method was unsuccessful and grafting success for different cultivars was below 20%.

Patch budding

Patch budding is generally used to propagate Pecan nursery stock which simply involves removing a portion of bark with a dormant bud from the scion and fitting them into a space cut into the bark of the rootstock. When the bud starts to grow, the shoot above the bud will be pruned for the grafted bud to become the primary shoot (Wells, 2014). Patch budding is usually performed in late summer but can also be done in the spring. The rootstock and scion wood should have the same diameter, from 1.5 to 2.5 cm. Scion wood consisting of the current season's shoots should be vigorous (Wells, 2014).

Whip grafting

Whip grafting is generally performed with seedling trees and nursery stock with a diameter up to 2.5 cm. It may be done in late winter when the buds are still dormant. The rootstock and scion wood should have the same diameter. The scions should be vigorous and have a length of at least 18 to 25 cm (Wells, 2014). Using a sharp knife, an oblique cut is performed in both the rootstock and scion so that the two cuts are face to face and overlaid perfectly. Afterwards, the cut areas and the scion should be wrapped with parafilm to seal the cuts and protect the scion. Successful grafts start to grow in 3 to 4 weeks (Wells, 2014).

Four flap grafts (banana graft)

The four-flap graft is one of the easiest grafts to use. It can be used with smaller sized rootstock to graft more than one cultivar onto the same rootstock (Anonymous, 2010). It may be done in early spring after the bark begins to slip. Scions should be collected in late winter, and should have a maximum diameter of 2.5 cm. The rootstock used should be a healthy 1-year old seedling with well-developed buds (Wells, 2014). Grafting should be done at a height of 45 to 60 cm from the ground, on a selected point of the rootstock without damage and no bud scars, and the rootstock should be cut straight across with sharp pruning shears.

Afterwards, a small lightweight rubber band is wrapped around the rootstock leaving a 7.5 to 10 cm space from the top cut (Carroll, Oklahoma State University). The rubber band is useful during the grafting procedure, as it should fit perfectly with moderate pressure on the rootstock. Lateral growth on the rootstock should be removed to approximately 15 cm. Four vertical cuts of 4 to 5 cm long and equally spaced (quartered) must be done around the circumference of the rootstock. Cuts are made through the bark only.

The scion should be smooth, straight and slightly larger than the rootstock, with a cut made to about 15 cm in length with 2 or 3 buds remaining. With a sharp knife, the scion is cut on 4 sides, starting the cut about 2.5 to 5 cm from the bottom end. The end should be square-shaped. The four flaps of the bark are pulled down so as not to touch the inside of the flaps, 4 to 5 cm of rootstock is now exposed. The exposed rootstock is cut with sharp pruning shears, taking care not to damage the four flaps. The scion is inserted upright on the rootstock and the four flaps are pulled in place to cover the cut surfaces on the scion, and the rubber band is moved up around the flaps to secure them in place (Carrol, Oklahoma State University).

The cut areas are wrapped with masking tape or

grafting tape to make sure that it is firm but does not pull too tightly. The tape is covered with a piece of aluminum foil with the shiny side to the inside, to reflect heat from the graft. The corner of a polyethylene bag is clipped and carefully slipped down over the rootstock with the scion protruding through the bag (Stafne, 2015). The bag is tied to the scion approximately 2.5 cm above the foil and 2.5 to 5 cm below the foil. With any type of tape, the polyethylene bag is kept secure in place to stop the graft from drying out and to protect it from rainfall. Buds begin to break through three to four weeks after grafting. Once the grafting is performed, the grafted scion is kept at a reduced growth throughout the first summer by removing growing tips. This operation must be done several times during the season to stimulate an increment in diameter of the trunk and a better tree establishment (Stafne, 2015).

Bark graft

The bark graft is an effective way to propagate Pecan cultivars onto rootstocks of 5 to 10 cm in diameter (Reid, 2010). It is advisable to select a point on the rootstock above the first whorl of branches to perform the grafting. Bark grafting should be done in spring, about 2 to 3 weeks after growth begins (Wells, 2014). The top of the rootstock is cut with a saw and the outer portion of the bark is removed with a sharp knife to the point where the scion is inserted. The scion should have at least 3 buds and a diameter of 1 cm. The scion is carved down to less than half its original thickness and a shallow cut is made on the back of the scion angled to one side (Reid, 2010). The scion should have a wedge shape after making the second cut, and a triangular shape after making the third cut. It will be ready for grafting after making a chisel point at the end of the bud stick.

Afterwards, the bark of the rootstock is lifted away from the wood with a sharp knife and the scion is inserted between the bark and the wood of the rootstock. The scion is tapped down until the shoulder of the deep cut touches the wood of the rootstock. A staple gun can be used to secure the graft union on smaller trees, or brad nails can be used to secure the bark graft on larger trees (Reid, 2010). Once the grafting is performed, the graft union is wrapped with aluminum foil and a polyethylene bag the same as the four-flap graft.

Top-work

Generally, Pecan trees with a diameter between 7.5 and 30 cm and a height of 1.40 m above the ground are top-worked. It is advisable to leave one limb below the graft to provide food and to shade the trunk (Stafne, 2016). The limbs are selected to promote the development of a well-balanced crown. The limbs are sawn from the bottom of the limb until the saw begins to bind, while the final cut is done from the top side. This is done to prevent splitting (Stafne, 2016). The main limb, since it is the most important single graft, is cut and grafted leaving 30 to 40% of the limbs uncut for 1 or 2 years. All suckers or new growth are pruned from any place except the grafts to force new growth from the scion. The scion of the desired variety should be collected in late winter, while the buds are dormant, from young trees which produce vigorous growth. The scion is cut into 15 cm sticks and tied into bundles of 25. Both extremities of the sticks are covered with wax or paraffin to prevent dehydration. The sticks are then stored in peat moss or moist newspaper in the refrigerator until the time for use (Stafne, 2016).

The most common graft used in top-working Pecan trees is the inlay bark graft which has been successfully used when other systems have failed because of heat, drought and wind (McEachern et al., 1992). Top-working is generally used to replace old trees or cultivars that have become commercially unacceptable for the Pecan industry (Sparks, 1990). However, top-working can require up to 30 grafts per tree and long-term aftercare. For this reason, it is considered cost-prohibitive.

Therefore, Yates and Sparks (1992) executed a grafting of 'Desirable' Pecan scion wood onto the lateral roots of a 70-year-old 'Van Deman' seedling rootstock, with the aim to obtain an acceptable cultivar that would produce faster than nursery-grown trees.

Two methods of grafting were performed. The first was a bark graft, positioned on the root either above or below the soil level, while the second was an inlay graft, positioned below the soil line. Results showed that the most successful method of grafting was the modified bark graft positioned beneath the soil line. Survival was higher for grafts treated with 1 to 2% IBA than those without IBA, and the time of grafting also influenced the success rate. The most suitable time for making grafts was in late spring 6-8 weeks after bud break. Root bark thickness also affected graft survival. The survival percentage of grafts was highest (74%) on roots with a bark thickness of 8.1 to 10.0 mm.

MICRO PROPAGATION

With the advent of *in vitro* cultures, the problem of genetic variability was overcome because micro propagation has the enormous advantage of generating many genetically identical plants (clones) in a short time, which cannot be obtained with conventional propagation methods.

Stages involved in micro propagation

In micro propagation, it is possible to identify five basic stages (from 0 to IV). These stages describe, not only the techniques applied in tissue culture, but also the changing

environmental conditions of the crop (Miller and Murashige, 1976).

Stage 0: Selection and preparation of the mother plant

The quality of the explants and subsequent *in vitro* responses of the explants are significantly influenced by the plant health and physiological condition of the mother plant (Debergh and Maene, 1981; Read, 1988). Before *in vitro* establishment of a crop, attention should be paid to the selection and maintenance of the mother plants used as sources of the explants. The use of appropriate agronomic practices such as adequate fertilization allows you to select bigger explants, which will have a more rapid *in vitro* response.

Stage I: Initiation of aseptic cultures

The aim of this stage is to initiate and establish a pathogen-free culture of terminal or lateral meristems. The primary explants excised from the mother plants are subjected to sterilization of the external surface. The high presence of organisms, such as bacteria or fungi, affects the survival of explants, their growth and their subsequent proliferation.

Sterilization of explants

Sterilization is the most delicate moment because it affects the success or failure of the establishment of the explants *in vitro*. It is often difficult to sterilize the surface of the plant material because the use of products such as ethanol, sodium hypochlorite and mercuric chloride, can irreversibly damage the explant. Therefore, the explant tissue influences both the type of sterilizing agent and the exposure time (Mahmoud and Al-Ani, 2016). For Pecans, sterilization procedures include ethanol, sodium hypochlorite, and in some cases, antibiotics and/or fungicides. The sterilization of the stem segments gave mixed results. The sterilization of the buds excised from mature plants gave unsatisfactory results or has required laborious treatments. In contrast, low levels of contamination have been obtained using immature seeds as explants (Wetzstein et al., 1996).

Table 1 shows the detailed protocols adopted for several explant tissues of Pecans by several workers.

Stage II: Shoot multiplication and seed germination

Stage II is characterized by repeated cycles of proliferation of auxiliary buds from apical or lateral shoots, cultured on medium containing high levels of cytokinin to inactivate the apical dominance of the terminal bud. The number of possible subcultures, which can be made in this stage, depends on the species or cultivar and on its ability to maintain an acceptable rate of proliferation and, at the same time, a minimum level of genetic variability (Kurtz et al., 1991).

The first attempts of *in vitro* propagation of Pecans date back to the early '80s when Knox and Smith (1981) and Wood (1982) tried to establish proliferating shoots derived from nodal stem segment explants from seedlings. However, no plants were established in soil due to the lack of roots present on the *in vitro* elongated shoots. More precisely, Knox and Smith (1981) used Knox and Smith basal medium (KS), supplemented with 0.1 to 0.7 mg l^{-1} IBA and 0.1 to 2 mg l^{-1} BAP (6-benzylaminopurine) for shoot elongation, and 1 mg l^{-1} IBA and 1 mg l^{-1} phloroglucinol for rooting. The shoots, after 4 days on root-inducing medium, were transferred to the same basal medium supplemented with activated charcoal and washed with 3 g/l acid. Although shoot elongation was satisfactory, roots produced were fleshy, like a tap root, and in the end no plants survived in the soil.

Wood (1982) obtained rooting and contamination problems using nodal stem segments as explants. He used woody plant medium (WPM) (Lloyd and McCown, 1980), supplemented with 4 mg l^{-1} BAP to obtain shoot proliferation. The best shoot elongation was obtained by transferring the explants on WPM with 0.1 mg l^{-1} BAP and 2 to 8 mg l^{-1} GA_3. However, the subculture of the axillary shoots and the rooting was unsuccessful.

Phillips and Ramirez (1983) and Ramirez-Martinez (1983) used different types of Pecan explants, but positive results were obtained only with apical and auxiliary buds from 50-year-old plants. They also tested different media, Murashige and Skoog (MS, 1962), KS and BDS (basal nutrient medium of Dunstan and Short, 1977), different auxin sources (Picloram and IBA), different carbohydrate sources (sucrose, glucose and fructose) and activated charcoal and ascorbic acid as anti-oxidants. Among all the combinations tested, the BDS medium with 0.2 to 0.5 mg l^{-1} Picloram and 2 to 10 mg l^{-1} BAP was the best. From the results obtained, they stated that buds from 50-years-old trees do not lend themselves well to tissue culture. Cortes-Olivares et al. (1990b) used auxiliary buds from trees of different ages (3 to 4, 9 to 10 and 16 to 17 years). After sterilization, the explants were placed on BDS medium with 0.51 mM ascorbic acid, 4.4 µM BAP and 0.4 µM Picloram under 16-h photoperiod and sub cultured every 30 days. The explants were subsequently cultured in a rooting medium, which consisted of the same BDS supplemented with 14.8 µM IBA, for 4 weeks. Although contamination was quite high, shoot multiplication and rooting were successfully obtained (between 0.3 and 6 shoots per explant). Normal-appearing roots formed on 40% of these shoots, resulting in complete plantlets. Renukdas et al.

Table 1. Sterilization procedures used in pecan tissue culture.

Explant	Sterilization procedure	Reference
Nodal segments from seedlings	20% Clorox + 0.35% Tween 20 - min 4 Rinses with sterile double distilled water	Knox and Smith (1981)
Nodal segments from seedlings	Distilled water + 0.5% Tween 20 1% Sodium hypochlorite - 20 min 4 Rinses with sterile distilled water 200 mg l^{-1} Streptomycin (in medium before autoclaving) 40 mg l^{-1} Filter sterilized Pimarcin (in medium before autoclaving)	Wood (1982)
Nodal segments from seedlings	0.525% NaOCl - 10 min	Hansen and Lazarte (1984)
Buds from mature trees	95% Ethanol - 5 min (vacuum) 50% Commercial bleach - 15 min (vacuum) 1% Benlate - 15 min (vacuum) Sterile water - 15 min (vacuum)	Phillips and Ramirez (1983)
Apical and axillary buds from mature trees	Four-step disinfestation procedure including pretreatment with anti-oxidants and vacuum/benomyl treatment	Ramirez-Martinez (1983)
Axillary buds from grafted trees	1% Citric acid (anti-oxidant) 95% Ethanol briefly Sodium hypochlorite + 2 drops of detergent - 7 min 2 Rinses with sterile deionized water 1% Benomyl under vacuum - 7 min (late season material only) Wash in 1% citric acid (late season material only)	Corte-Olivares et al. (1990b)
Buds from mature trees	Wash in running distilled water - 5 min 70% ethanol with continuous stirring - 2 min Several rinses with sterile distilled water 5.25% Sodium hypochlorite (NaOCl 3% v/v) + 0.1% Tween 20 - 2 min 7-8 Rinses with sterile distilled water	Haroon (2011)
Buds, leaves and embryos	Solution of Foca detergent + Tween 80 - 2 min 70% Ethanol - 3 min 10% Sodium hypochlorite - 15 min	Ávila-Treviño et al. (2013)
Intact seeds	95% Ethanol - 20 min Air dried under aseptic hood	Yates and Reilly (1990)
Unshelled mature seeds	95% Ethanol - 5 min 5.25% Sodium hypochlorite + 0.1% Tween 20 to 3 to 7 h Sterile water rinses, held in final rinse 2 h MS medium + 1.5% agar - 30 days, followed by transfer to medium with 0.7% agar - 4 weeks	Obeidy and Smith (1990, 1993)
Unshelled mature seeds	Wash with sterile water - 10 min Carbendazim solution (1 g l-1) overnight Wash with sterile water 70% Ethanol - 30 min 3 Rinses with sterile water 2.83% (w/v) Sodium hypochlorite - 30 min 5 Rinses with sterile water Break the shell with a nutcracker in sterile condition to extract the embryos	Renukdas et al. (2010)

Table 1. Contd.

Mature embryos	2.83% (w/v) Sodium hypochlorite - 10 min 5 Rinses with sterile water - 5 min each	Renukdas et al. (2010)
Immature intact seeds	70% Ethanol dip 10% Roccal dip 100% Clorox (5.25% sodium hypochlorite) - 5 min Sterile water rinse 0.01 N HCl rinse 3 Sterile water rinses	Merkle et al. (1987) Wetzstein et al. (1988, 1990)
Immature intact seeds	70% Ethanol dip - 30 s 10% Roccal dip - 1 min Repeat first 2 steps 100% Clorox - 7 min 3 Sterile water rinse - 3 min each 0.01 N HCl rinse	Wetzstein et al. (1989) Rodriguez and Wetzstein (1994)
Immature seeds	Wash under tap water and bleach solution - 30 min 70% Ethanol - 1 min 15% Sodium hypochlorite - 20 min + 0.01% Tween 20 3-5 Rinses with distilled water	Payghamzadeh and Karemitabar (2010)
Immature embryonic axes	Intact fruits immersed in 70% ethanol - 20 min 3 Rinses in sterile water	Yates and Wood (1989)
Immature zygotic embryo axis	95% Ethanol briefly 2.6% Sodium hypochlorite (50% bleach) - 7 min 2 Sterile deionized water rinses	Corte-Olivares et al. (1990a)
Shelled mature seeds	70% Ethanol with continuous stirring - 2 min 5.25% Sodium hypochlorite (NaOCl 3% v/v) + 0.1% Tween 20 - 2 min 7-8 Rinses with sterile distilled water	Haroon (2011)

(2010) used nodal explants from cultivars Desirable and Cape Fear. The explants were cultured on modified liquid WPM, supplemented with 2% glucose and different concentrations of BAP (0.44 to 44.39 µM). After 3 weeks of culture, at least 9 multiple shoots per explant were induced on modified WPM containing 13.32 µM BAP. Subsequently, the multiple shoots were separated and successfully rooted in liquid WPM containing 49.20 µM IBA. The efficiency of rooting for both cultivars was over 90%.

Pecan seeds can be used as explants in tissue culture due to the ease of sterilization and a lower risk of contamination as compared to other explants such as buds, leaves and nodal segments (Figures 3 and 4). There are few reports in literature that describe the sterilization process of Pecan seeds and their subsequent cultivation into a solidified medium. Yates and Wood (1989) used immature embryos, which were excised during kernel development from seeds of Pecan Cultivars Desirable and Stuart. After sterilization, immature seeds were placed on a basal medium with four combinations of cytokinin and auxin. After 4 weeks of darkness at 25°C, the explants were transferred to a basal medium without plant growth regulators. Normal plants were obtained, with no problems of contamination. Haroon (2011) noticed that the best medium for *in vitro* germination of Pecan seeds was a MS formulation supplemented with 4 µM BAP. Formation of multiple shoots was also observed from intact nodal regions of developing seedlings. After reaching a sufficient length (3 to 4 cm), the explants were transferred to the two different rooting media, DKW (Driver and Kuniyuki, 1984) or MS supplemented with different combinations of growth regulators (IAA, NAA and IBA). The medium which provided the best root induction was MS supplemented with a combination of 4 µM IBA and 4 µM

Figure 3. *In vitro* germination of pecan seed ('Ukulinga').

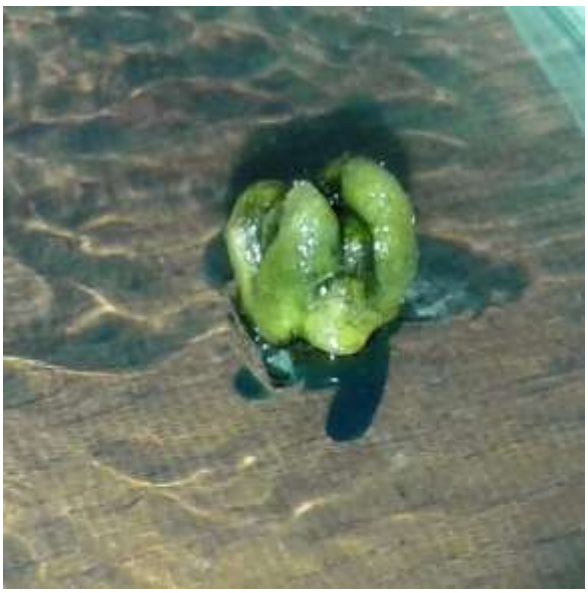

Figure 4. *In vitro* culture of pecan bud ('Ukulinga').

NAA. Browning of the growth medium is the result of polyphenol oxidation exuded from the cut end of the explants. Generally, it occurs at the initial stage of culture but can be overcome by adding substances such as ascorbic acid, citric acid, polyvinyl pyrrolidone (PVP) and activated charcoal to the growth medium. This phenomenon is widely reported in literature, not only for Pecan, but also for other nut crops such as Chestnut (Osterc et al., 2005), Walnut (Payghamzadeh and Kazemitabar, 2011) and Pistachio (Barghchi and Alderson, 1985). For *in vitro* germination of Pecan for instance, testa is generally removed, since it produces phenols that can inhibit the growth of callus (Haroon, 2011). Therefore, it is advisable to add ascorbic acid or other compounds to the medium, and if browning occurs, the explants must be transferred to new fresh medium. This is also valid for *in vitro* buds' culture. *In vitro* shoot-tip necrosis (STN), also known as apical necrosis or non-pathogenic dieback, is a common physiological disorder in micropropagation of many plants. The symptoms result from the senescence and death of tissues in the apical bud, which subsequently proceeds basipetally.

Several methods were tested for alleviating shoot necrosis in literature such as, shortening of the culture period, altering the media salt strength, use of various plant growth regulators, different levels of sucrose, fructose, silver nitrate and increasing the concentration of calcium chloride (Kishore et al., 2015; Nezami et al., 2015; Thakur and Kanwar, 2011; Chiruvella et al., 2011; Bairu et al., 2009; Abousalim and Mantell, 1994). Chiruvella et al. (2011) reported a positive synergism between activated charcoal and higher levels of calcium that facilitated the recovery of more than 98% of the shoots affected by STN in *Soymida febrifuga*. The important role of adenine sulfate (AdS) as adjuvant to alleviate *in vitro* STN was recorded by Naaz et al. (2014) in *Syzigium cumini*. On Pecan, Ávila-Treviño et al. (2013) used activated charcoal (1%), polyvinyl pyrrolidone (0.1%), silver nitrate (1%), citric acid (150 mg l^{-1}) and ascorbic acid (100 mg l^{-1}) to successfully control necrosis in both light and dark conditions. Tissue necrosis of Pecan explants was reduced by 75 and 83% by adding activated charcoal and silver nitrate, respectively.

Stage III: Rooting of microshoots

At this stage, proliferated microshoots obtained in Stage II were transferred to a rooting medium, which is different from the shoot multiplication medium, particularly in its hormonal and salt compositions. This stage is very important to obtain plantlets with well-developed rooting systems to facilitate their establishment in the soil. Several research projects were done to enhance the rooting rate of Pecans. The rooting of micro shoots was accomplished both under *in vitro* and *ex vitro* conditions. Hansen and Lazarte (1984) obtained rooting in both *in vitro* and *ex vitro*. They used nodal segments from seedlings as explants and cultured it on WPM medium supplemented with 20 g l^{-1} glucose and 0.3, 1 or 3 mg l^{-1} BAP for 1 day, and then transferred it to WPM basal medium. Afterwards, the cultures were subjected to a darkness condition for 2 weeks and then kept under a 16-

h photoperiod. *In vitro* rooting was obtained using WPM with glucose and 1, 3 or 10 mg l^{-1} IBA for 6 to 10 days. *Ex vitro* rooting was obtained using WPM with 20 g l^{-1} glucose and 10 mg l^{-1} IBA for 10 days, followed by a transfer to peat pellets and watered with half-strength WPM minerals. Up to 63% of the rooting was obtained and the plantlets were successfully established in soil. Hassanen and Gabr (2013) developed an efficient method for *in vitro* rooting of Pecans. Nodal segments were cultured on WPM medium containing 3 mg l^{-1} BAP for multiplication. After 4 weeks of culture, the shoots were cultured on ¼ and ½ strength WPM supplemented with IBA (0, 1, 2 and 3 mg l^{-1}) and 1.5 mg l^{-1} $AgNO_3$. In addition, 20 g l^{-1} sucrose, 1 g l^{-1} activated charcoal and 3 g l^{-1} phytagel were added to the medium. The best rooting percentage (75%) was obtained on a ½ strength WPM medium supplemented with 3 mg l^{-1} IBA and 1.5 mg l^{-1} $AgNO_3$.

Stage IV: Acclimatization and field establishment

The positive outcome of the *in vitro* buds' culture, or nodal segments, depends on the ability to transfer the plantlets from a controlled tissue culture laboratory to a glasshouse, for it to acclimatize. This means that to acclimatize or prepare *in vitro* plantlets, it needs to be exposed to a significantly lower relative humidity and a higher light intensity. During the acclimatization, plants change from a heterotrophic to an autotrophic state (Preece and Sutter, 1991). Unfortunately, this step is not immediate. To overcome these limitations, plantlets are transferred into a well drained substrate and kept at high levels of humidity, reduced light intensity and a temperature between 20 and 27°C. Plants are normally acclimatized by gradually reducing the relative humidity over a period, between 1 and 4 weeks.

Applied *in vitro* technologies

Organogenesis

Organogenesis is a process that belongs exclusively to the plant kingdom and consists of the production of unipolar structures (shoots or roots), starting from non-meristematic cell aggregates, or plant tissues, through the formation of meristematic adventitious centers called meristemoids. Obeidy and Smith (1993) induced organogenesis from cotyledon segments to form adventitious roots in a medium with 50 µM NAA. A regeneration medium with 20 and 5 µM IBA stimulated prolific auxiliary shoot production from the embryonic axis without causing cotyledon abscission. Thirty percent of the microshoots rooted on auxin-free medium after a pretreatment in dark conditions on a medium with 20 µM IBA. Rooted plantlets were successfully transferred to soil. Payghamzadeh and Karemitabar (2010) conducted organogenesis studies using immature embryos of Pecan as explants. After disinfection, immature fruits were cultured on a modified DKW basal medium. For immature embryo culture, a high frequency of plantlets was obtained on modified basal medium, supplemented with 1 mg l^{-1} BAP, 0.05 mg l^{-1} IBA and 2 mg l^{-1} GA_3 and dark culture conditions. Callus can be defined as an amorphous mass of undifferentiated tissue with thin walled parenchyma cells developing from proliferating cells of parent tissue (Dodds and Robert, 1985). Callus formation in tissue culture can be initiated using a small portion of plant tissue due to the use of phytohormones (auxins and cytokinin); it is induced to produce calli (Skoog and Armstrong, 1970; Letham, 1974; Akiyoshi et al., 1983). In micropropagation, callus culture is a very important technique for developing clonal populations, plant regeneration and genetic manipulation in both monocotyledon and dicotyledonous plants (Reinert and Bajaj, 1976). Rodriguez and Wetzstein (1994) used immature seeds of Pecan to induce embryogenesis, as well as the entity of callus produced in relation to the type and concentration of auxin. Callus formation was greater in cultures induced on 2,4-D compared to NAA. The higher levels of both auxins formed greater amounts of callus than lower levels. Payghamzadhe and Kazemitabar (2010) also used immature seeds of Pecan as explants for organogenesis studies. They found that the presence of different concentrations of BAP and IBA induced callus formation frequently and it was also inhibited by adding GA_3 in the culture medium.

Somatic embryogenesis

Somatic embryogenesis has been considered as one of the most important invasions in the tissue culture sector. It has several applications such as mass clonal propagation, genetic transformation and use in studies of embryo development. Due to somatic embryogenesis, it is possible to propagate clonal Pecan rootstocks introducing genes of commercial interest such as dwarfing for size control, enhanced nutrient uptake, alternate bearing control, salinity tolerance, nematode resistance and growth uniformity (Wetzstein et al., 1996). The first studies of somatic embryogenesis date back to 1987, when Merkle et al. (1987) obtained somatic embryos using immature nuts as explants. These immature zygotic embryos were cultured on modified WPM medium with 2 mg l^{-1} 2,4-D and 0.25 mg l^{-1} BAP. A low embryogenic frequency was obtained (2%) and they stated that the developmental stage of explants can be a limiting factor on the induction of somatic embryos. Other studies have been followed over time with the aim to increase embryogenic frequency. Wetzstein et al. (1988) obtained up to 40% of embryogenic frequency using different explants. Other subsequent studies were

focused on other factors that can be responsible for a different frequency and embryogenic response such as cultivar, explant sampling date, source of explants, duration on conditioning medium (Wetzstein et al., 1989) and auxin type and concentration (Wetzstein et al., 1990). An embryogenic frequency of 85% was obtained by Wetzstein et al. (1989) using immature Pecan seeds cv. Stuart, collected 15 weeks after pollination, which is considered the optimum stage for embryogenic induction. Wetzstein et al. (1990) performed a cold treatment followed by a desiccation treatment to improve the rooting of somatic embryos. Yates and Reilly (1990) used immature Pecan seeds collected from eight cultivars at different stages of development. They found that the embryogenic response was different among cultivars and is genotype-dependent. In the same year, Corte-Olivares et al. (1990a) obtained somatic embryos, although with a low frequency (3.5%), using zygotic embryo axes from cvs. Western Schley and Wichita. Mathews and Wetzstein (1993) stated that adding 29.4 µM silver nitrate to the germination medium (WPM) and the application of 100 µM BAP to the embryo shoot apex, promotes a higher frequency of plant conversion. Rodriguez and Wetzstein (1994) stated that the use of NAA rather than 2,4-D as an auxin source, enhanced the embryos development by providing embryos with a stronger defined shoot apex. Burns and Wetzstein (1995) used aggregates of globular and pre-globular stage somatic embryos derived from a liquid culture of Pecan. A mild dehydration of the embryo aggregates up to 49% moisture and was applied for 3 to 4 weeks, promoting a complete development of somatic embryos and preventing repetitive embryogenesis. Kumar and Sharma (2005) obtained somatic embryos using cotyledon explants of Walnut and Pecan, cryo-preserved the embryos using non-toxic cryoprotectants (dimethyl sulfoxide, DMSO), glycerol and ethylene glycol) and assessed their survival percentage. DMSO had the highest survival rate (5%) followed by glycerol (1.5%) and ethylene glycol (3%) pre-treatment. They also noticed that high levels of sucrose decreased survival rate, and in Pecan, visible browning occurred.

Synthetic seed technology

For Pecans, no information is available in literature about the use of synthetic seed technology as an important propagation tool. Therefore, it would be interesting to test the response of unimodal Pecan micro cuttings to encapsulation, not only as an alternative propagation method, but also for the storage and exchange of plant material with national or international micro propagation laboratories.

To produce synthetic seeds, the procedure adopted includes three steps: coating, complexation and washing. The coating is a single process performed by inserting the propagule, excised from *in vitro* cultures, into a gel or encapsulating solution for a few seconds. Generally, sodium alginate is the most widely used substance for this operation, as it has a moderate viscosity, low toxicity for explants, low cost and biocompatible characteristics. It is also widely used since it better protects encapsulated explants against mechanical risks, depending on their concentration, viscosity or commercial type, as well as complexation conditions (Casales, 2013). For the complexation, which gives hardness to the capsules, coated alginate explants are dipped in a calcium chloride solution for 30 - 40 min. An ion exchange process occurs during this phase, following the substitution of sodium (Na^{+}) ion with calcium (Ca^{++}) with a calcium alginate formation (Ara et al., 2000; Redenbaugh and Walker, 1990). Thus, the coating acquires the consistency necessary to ensure protection against mechanical damage and the risk of dehydration. Hardening of the calcium alginate capsules is influenced by the sodium alginate and calcium chloride concentration and may also vary by the time of complexation. Finally, the third step consists of several subsequent rinses in sterile endosperm to remove toxic residues of chloride and sodium. After washing, such encapsulated propagules can be stored or transferred to the sowing medium.

Several research groups have begun to use non-embryogenic explants (unipolar) obtained through *in vitro* direct organogenesis, or through the proliferation of auxiliary buds. Regarding the use of unipolar explants for encapsulation, uninodals are generally used, these are portions of 3 - 4 mm with terminal buds or lateral buds and cut off during or at the end of a subculture; generally, they are called micro cuttings. In these explants, the absence of root primordium is often associated with the inability to form adventitious roots spontaneously, representing a major obstacle to obtain synthetic seed conversions (Casales, 2013). The poor conversions observed in some species to produce synthetic seeds by using unipolar explants, such as citrus or mulberry, is attributable to a variety of factors (genotype, inadequate nutritional formulations linked to artificial endosperm or seed, ineffective procedures to induce rooting in micro cuttings), which strongly restricts its use (Casales et al., 2011, 2015). When the most appropriate encapsulation protocol for each genotype is found, even using biotization, with the insertion of Arbuscular Mycorrhizal Fungi (AMF) or Plant Growth Promoting Bacteria (PGPB) into synthetic seed, researchers will probably be able to take advantage of this biotechnological tool in the nursery sector.

Genetic transformation

Genetic transformation techniques allow the breeder to insert a gene into the genome of the plant of interest with valuable agronomic characters, obtaining new genotypes

in a single generation. Genetic engineering is particularly useful in the genetic improvement of woody plants, since it accelerates the timing of releasing cultivars, overcoming the high level of heterozygosity and shortens the length of the juvenile period that characterizes them. The main goals of genetic engineering for woody plants are the introduction of the resistance to biotic and abiotic stress and the vegetative and productive control of the trees, especially in relation to aspects relating to the quality of the fruit. Unfortunately for Pecan, there is little information relative to its genome (Thompson and Romberg, 1985; Marquard, 1991). Therefore, it leads to first identifying the traits of the genome containing the desired genes and then starting a genetic transformation program. Only in this way, will combining micro-propagation and genetic transformation be possible to obtain clonal rootstocks of Pecan with superior characteristics. Burns et al. (1991) used somatic embryo technology for gene transformation in Pecan, albeit with limited success. It studied the levels of kanamycin for a selection of cultures bombarded with foreign DNA, containing the genes for β-glucuronidase activity and kanamycin resistance. Although a stable GUS (beta-glucuronidase gene) expression was obtained, the variation in gene expression and embryo chimerism were limiting factors. A gene transfer mediated by *Agrobacterium* was performed by McGranahan et al. (1993). Somatic embryos derived from open-pollinated seed of 'Elliott', 'Wichita' and 'Schley' were co-cultivated with the *Agrobacterium* strain EHA 101/pCGN 7001, which contains marker genes for β-glucuronidase activity and a resistance to kanamycin. Although transgenic clones were obtained, plant regeneration was limited.

SUMMARY AND CONCLUSIONS

Over the years, Pecan propagation techniques have profoundly changed due to the help of various methodologies that have improved both qualitative and economic efficiency. Techniques such as mist propagation and basal heating of hardwood and/or softwood Pecan cuttings, associated with treatments with auxins such as IBA have certainly enhanced the recalcitrant behavior to emit roots of this species. The attempts to micro propagate Pecans through organogenesis have been limited due to a low regeneration frequency, poor rooting and high rates of contaminations. All these factors have strongly precluded crop improvement and clonal propagation. On the other hand, somatic embryogenesis, using immature nuts as explants, is a very efficient method in terms of obtaining both high multiplication rates and plant regeneration. Trees grown in open fields obtained by somatic embryogenesis show a genetic stability and have kept the original characters of a cultivar. Genetic engineering techniques (gene transfer mediated by *Agrobacterium*, direct insertion of DNA into protoplasts and biolistic methods), when properly applied together with molecular biology techniques and efficient tissue culture protocols, will contribute enormously to improve the efficiency of the Pecan industry with the elimination of barriers imposed by conventional genetic improvement programs.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

2,4-D, 2,4-Dichlorophenoxyacetic acid; **BAP,** 6-benzyl aminopurine; **IBA,** indole-3-butyric-acid; **NAA,** α-naphthalene acetic acid; **TDZ,** Thidiazuron; **IAA**, indole-3 acetic acid; **GA_3**, Gibberellic acid; **ABA**, abscisic acid; **DKW**, Driver and Kuniyuki Walnut medium; **MS**, Murashige and Skoog; **KS**, Knox and Smith basal medium; **BDS**, basal nutrient medium of Dunstan and Short; **WPM**, woody plant medium; **PVP**, polyvinyl pyrrolidone; **DMSO**, dimethyl sulfoxide; **PGR**, plant growth regulators.

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Full Length Research Paper

Development of *in vitro* propagation protocol for some recalcitrant cassava (*Manihot esculenta* Crantz) genotypes in Sierra Leone

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A study on rapid propagation of cassava through tissue culture was conducted with three elite cassava genotypes: *Slicass 6, Slicass 11* and *Cocoa* from Sierra-Leone. They showed slow growth in Murashige & Skoog (MS) basal medium which was proven to be optimal for a vast number of cassava accessions. Prior to mutation induction, a large population needs to be produced for mutagen susceptibility test and for mutant population development. The ultimate objective of this study was to investigate the effects of plant growth regulators on the shoot development of three cassava genotypes. *In vivo* shoot tips were sterilized and sub-cultured on MS media supplemented with six combinations of plant growth regulators (PGRs) at different concentrations. The results showed that from all media used, the MS medium with 1.0 mg/L α-naphthalene acetic acid (NAA) showed the best response for rooting (5.50), fresh weight (0.29 g), root number (10.00) and plantlet height (3.81 cm), while 0.1 mg/L 6-benzylaminopurine (BAP) was found to be more favourable to shoot development of leaves (6.38). The highest plant height and fresh weight were 3.81 cm and 0.29 g, respectively for Cocoa at 1.0 mg/L α-naphthalene acetic acid (NAA), 10.00 roots for *Slicass 6* at 1.0 mg/L, 6.37 leaf numbers for *Slicass 11* at 0.1 mg/L 6-benzylaminopurine (BAP) and 5.6 at 1.0 and 1.5 mg/L α-naphthalene acetic acid (NAA). These observations indicate that a supplement of 0.1 mg/L 6-benzylaminopurine (BAP) in MS medium can be useful in propagation of recalcitrant cassava and low concentration of α-naphthalene acetic acid (NAA) will be beneficial in root induction prior to acclimatization with promotion in recovery of the *ex vitro* plants before field assessment.

Key words: Cassava*, Manihot esculenta,* propagation, shoot tip, 6-benzylaminopurine (BAP), α-naphthalene acetic acid (NAA).

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is one of the major food crops for over 800 million people in sub-Saharan Africa (Taye, 2009) and the second most important staple food in Sierra Leone (Olsen and Schaal, 2001). In tropical

countries like Sierra Leone, cassava constitutes the most important source of energy in the diet of people (Burns et al., 2010; Perez et al., 2011). In addition, about 70% of the world cassava root is used for human consumption and the remaining is used for animal feed and industrial products, such as starch, glucose and alcohol (El-Sharkawy, 2004). In many African countries of which Sierra Leone is not exempted, the young leaves of cassava are also consumed as a vegetable to provide proteins, calcium, iron and vitamins, supplementing predominantly starchy diets in poor communities (Fregene et al., 2000). The world annual production of cassava is estimated at 241 million tonnes of fresh roots mostly from smallholder farmers in Africa and Asia (Bull et al., 2011).

According to the Food and Agricultural Organization/Ministry of Agriculture Forestry and Food Security Crop Survey Report of 2003, land area under cassava cultivation in Sierra Leone was 99,484 ha yielding a total of 479,458 metric tonnes with average yield of 4.8 mt/ha (FAO/MAFFS, 2003).

In Sierra Leone, one of the major constraints responsible for low production of cassava is the widespread cultivation of inherently low-yielding local varieties that are also highly susceptible to diseases (African cassava mosaic disease) and high pest incidence (Grasshopper) (Samura et al., 2017; Mansaray et al., 2012). Recently, the government of Sierra Leone has given top research priority with special emphasis on cassava promotion to develop superior cultivars and their production expansion. Hence, there is urgent need for disease free and high-quality cassava planting materials in the production system. To achieve this aim, the Sierra Leone Agricultural Research Institute (SLARI) released fourteen cassava varieties, but limitations are the low propagation of the crop due to conventional methods of production and systemic infections that prevent the progress in substituting susceptible varieties. As a result, tissue culture techniques could be a viable option to resolve these difficulties.

Santana et al. (2009) reported that plant tissue culture technique has been acknowledged as a powerful tool for studying and solving basic and applied problems of cassava production and productivity. Moreover, Loyola-Vargas et al. (2006) indicated that plant tissue culture technique is quicker and requires less space as compared to conventional methods of preparing cassava cuttings. Similarly, Le et al. (2007) had long established the tissue culture technique to be one of the realistic and efficient means for supplying large volumes of true-to-type clean planting materials of cassava within limited period. This is also an advantage for the production of large size population as required in mutation breeding.

The multiplication of shoots requires optimum concentration of plant growth regulators (PGRs) in Murashige & Skoog (MS) medium, which appears to vary among materials with different botanical origins (including 2008). Acedo (2009) and Konan et al. (2006) showed that multiplication of cassava shoot could be enhanced with a relatively higher concentration of cytokinins, while rooting is boosted by the use of auxin. Kane (2005) also reported cytokinins, 6-benzylaminopurine (BAP) and kinetin (Kin), and auxin, α-Naphthalene acetic acid (NAA) as the most widely used and effective PGRs for shoot multiplication and root induction. Despite these good reports, no work has so far been done to develop an *in vitro* mass propagation procedure for these selected cassava genotypes (*Slicass 6, Slicass 11* and *Cocoa*) in Sierra Leone. Thus, the development of optimal protocol is needed to ensure fast mass propagation prior to improvement through mutation induction and spreading of the improved cassava genotypes to increase cassava production in the country. This study aimed to investigate the responses of the three cassava genotypes to plant growth regulators on shoot propagation.

MATERIALS AND METHODS

Three selected most desirable cassava genotypes (*Slicass 6, Slicass 11* and *Cocoa*) were acquired from Sierra Leone Agricultural Research Institute (SLARI). The two improved *Slicass* genotypes were selected due to the following characteristics: early maturity, high yielding and resistance to pests and diseases in comparison with other genotypes tested, while the *Cocoa* (Local) genotype was chosen due to its palatability, poundability and farmer's preference across Sierra Leone. Each of the three cassava genotypes with 20 cm long stem cuttings possessing 7 to 8 nodes were transferred and grown in the glasshouse of FAO/IAEA Plant Breeding and Genetics Laboratory (PBGL), Seibersdorf, in Austria to provide shoots as donor material to culture.

Establishment of donor plant

About 20 cm long stem cuttings with 2 to 4 nodes of all three varieties were collected from the Njala Agricultural Research Center research fields and taken to the IAEA Seibersdorf glasshouse for establishment. The cuttings were planted in plastic containers of 4 L volume filled with surface sterilized soil mixture of manure, Seibersdorf soil and sand. The donor plants were established in the glasshouse of the Plant Breeding and Genetic Laboratory at a temperature of 25±2°C.

Media preparation

A liter of the medium was prepared by weighing 4.4 g of Murashige and Skoog (1962), MS basal powder in a beaker of 700 ml distilled water stirred on hot plate with a string ball, 1 ml/L MS vitamin and 3% sucrose for which six media combinations were supplemented

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Table 1. Effect of different hormonal concentrations and control on *in vitro* plantlet height (cm) at 4 weeks of culture of the three cassava genotypes.

Hormone	Genotypes			
	Slicass 6	***Slicass 11***	***Cocoa***	**Mean**
BAP 0.05 mg/L	1.04	1.00	1.29	1.11
BAP 0.1 mg/L	3.13	2.65	3.30	3.03
NAA 1.0 mg/L	3.67	3.20	3.81	3.56
NAA 1.5 mg/L	2.45	2.65	3.05	2.72
BAP 0.1 mg/L + NAA 1.5 mg/L + GA_3 1.0 mg/L	2.41	2.06	1.93	2.13
Control (0.0 mg/L)	1.77	2.09	2.16	2.00
Mean	2.41	2.27	2.59	
LSD genotype		0.14		
LSD hormone		0.20		
LSD genotype x hormone		0.36		
CV (%)		8.9		

with 0.05 mg/L BAP, 0.1 mg/L BAP, 1 mg/L NAA, 1.5 mg/L NAA, 0.1 mg/L BAP + 1.5 mg/L NAA + 1.0 mg/LGA_3 and control MS without PGR. As gelling agent, 1.8 g/L gelrite was used and pH adjusted to 5.8 before autoclaving. The autoclaved media were kept in the cold room before use.

Surface sterilization and initiation of explants

Sprouted shoots over 2 cm long were harvested from the three cassava genotypes. The de-leaved buds were washed thoroughly using running tap water in order to clean off debris and taken to lamina flow bench for surface sterilization and initiation. The explants were initiated *in vitro* after surface sterilization with 70% ethanol for 30 s and instantly rinsed with sterile distilled water, 20% commercial bleach (Clorox) for 20 min, shaken intermittently and at least rinsed three times with sterile distilled water. The dead edges were excised for Clorox damage. The sterilized explants were cut into 1 to 2 nodes and transplanted into test tube containing 12 ml of solid propagation medium, Murashige and Skoog (1962) medium supplemented with 3% sucrose (w/v), 1.8% gelrite (w/v) and different concentrations and combinations of plant growth regulators. The cultured explants were incubated in a controlled growth room at 22±2°C for 16 h photoperiod and sub-cultured at 4 weeks on the same initiation medium.

Data collection

After a period of 4 weeks, various growth parameters such as number of leaves/plantlet (nodes), plantlet height (cm), fresh weight/plantlet (g), root induction and number/plantlet were evaluated.

Data analysis

Data was subjected to analysis of variance using Genstat 12.1 statistical package and means were separated according to Student-Newman-Keuls multiple-range test (SNK) at 5% level of probability.

RESULTS AND DISCUSSION

Five culture media supplemented with cytokinin or auxin were used in the improvement of tissue culture initiation of the three Sierra Leonean cassava cultivars in comparison with MS medium used by Owoseni and Ogunnusi (2006) in over 20 cassava accessions from Nigeria. The values from analysis of variance of the hormones and genotypes indicated that the F-probability of all the five parameters studied were highly significant with regards to the different media used. The interactive effects of genotype with hormone was significant for plantlet height, number of roots, root induction and formation and plantlet weight, while number of leaves (or number of nodes) was significantly different. However, genotype showed significant difference for root number, weight of plantlet and leaf number.

Effect of hormones on *in vitro* plantlet height of the different cassava genotypes

The MS medium supplemented with BAP and NAA either alone or in combination with GA_3 showed that there was significant (P<.001) difference between treatments used. Among the various concentrations, treatment 1.0 mg/L NAA showed the greatest plant height of 3.67, 3.20 and 3.81 cm, respectively, for *Slicass 6, Slicass 11* and *Cocoa.* Whereas, the analysis of variance showed that MS medium with NAA (1.0 and 1.5 mg/L) alone produced better results in response to plant height when compared with the other treatments (Table 1). 0.1 mg/L BAP exhibited the second better growth for *Slicass 6* and *Cocoa* with respectively, 3.13 and 3.30 cm as plantlet height as compared to other plant growth regulators. However, the *Slicass 11* observed second better plantlet height of 2.65 with 0.1 mg/L BAP and 1.5 mg/L NAA. The combinations of BAP + NAA + GA_3 cultured in MS medium elicited optimal responses with an average mean plant height of 2.13 cm as compared to only 0.05 mg/L BAP (1.11 cm). On the contrary, MS medium supplemented with 0.0 mg/L (control) concentrations

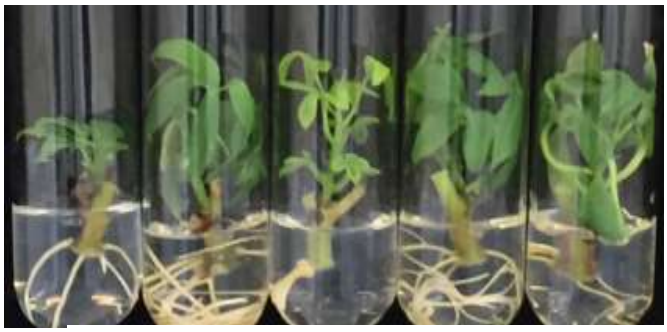

Figure 1. Efficacy of different concentrations of plant growth hormones on growth parameters of *Slicass 11* cassava genotype (4 WAP), A: Control; B: 1.0 mg/L NAA; C: 0.05 mg/L BAP; D: 1.5 mg/L NAA; E: 0.1 mg/L BAP.

Table 2. Influence of different hormonal concentrations and control on *in vitro* rooting (%)/plantlet at 4 weeks of culture of the three cassava genotypes.

Hormone	Genotypes			
	Slicass 6	***Slicass 11***	***Cocoa***	**Mean**
BAP 0.05 mg/L	4.00	7.67	7.00	6.22
BAP 0.1 mg/L	4.00	7.67	7.00	6.22
NAA 1.0 mg/L	55.00	55.00	55.00	55.00
NAA 1.5 mg/L	55.00	55.00	55.00	55.00
BAP 0.1 mg/L + NAA 1.5 mg/L + GA3 1.0 mg/L	10.0	10.0	10.0	10.0
Control (0.0 mg/L)	25.00	24.78	24.78	24.85
Mean	25.50	26.68	26.46	
LSD genotype		0.02		
LSD hormone		0.02		
LSD genotype x hormone		0.34		
CV (%)		2.7		

produced greater plant height than 0.05 mg/L BAP which produced the shortest plant height.

This improvement in plantlet height could be attributed to addition of the phytohormone. Cytokinin and auxin (alone or together) are known to stimulate cell division and reduce lateral bud dormancy which is relevant to the plant tissue culture (Davies, 2004). The increase in plant height is also due to the effects of phytohormone influencing initiation of cell division with cell growth and expansion. The results of the present experiment were found to corroborate the findings of Davies (2004), that cytokinin influences cell division in order to broaden the area of the tissues and plantlet height. However, medium without phytohormone (control) produces taller plant height as compared to 0.05 mg/L BAP media. Similar results were reported by Mapayi et al. (2013) where MS medium (without growth regulator) showed better growth for three cassava genotypes evaluated for micro-propagation.

Figure 1 is visual appearance of lower root number, stunted stem and fewer leaves in the control treatment (A); NAA 1.0 and 1.5 mg/L (Figure 1B and D) showed maximum root number and turgid stem growth. Furthermore, BAP 0.05 mg/L (Figure 1C) produced callus roots, thin stem and tiny yellowish green leaves. Thus, BAP 0.1 mg/L produced greater number of leaves and larger stem, but inhibited root formation.

Effect of hormones on *in vitro* rooting percentage/plantlet of the different cassava genotypes

The analysis of variance indicated that *in vitro* rooting percentage was influenced considerably by different concentrations of culture medium used in the experiment (Table 2). The results showed that there was increasing formation on rooting percentage at various hormonal

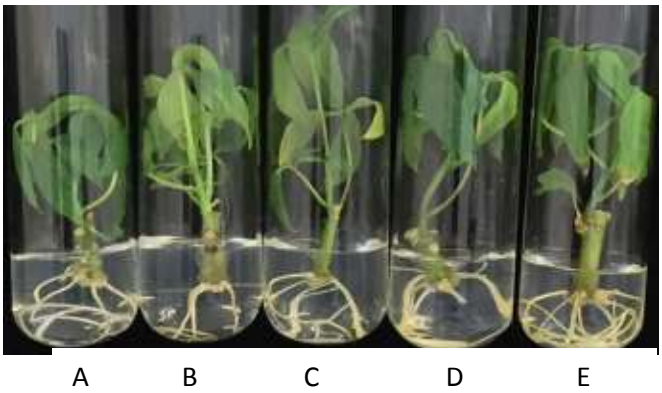

Figure 2. Efficacy of different concentrations of plant growth hormones on growth parameters of *Slicass 6* cassava genotype (4 WAP), A: 1.5 mg/L NAA; B: BAP 0.1 mg/L; C: Control; D: BAP 0.05 mg/L; E: 1.0 mg/L NAA.

Table 3. F-probability values of hormonal effect on cassava genotypes.

Factors	Number of leaves plantlet^{-1}	Plantlet height (cm)	Number of roots plantlet^{-1}	Root induction and formation plantlet^{-1}	Plantlet weight (g)
Genotype	0.91	<.001	0.304	<.001	0.458
Hormone	<.001	<.001	<.001	<.001	<.001
Genotype x Hormone	0.38	0.002	<.001	<.001	0.038

concentrations which was significant at P<0.001 level of significance. The percentage of rooting was remarkably highest on MS media supplemented with 1.0 and 1.5 mg/L NAA hence, best for rooting in the cassava genotypes studied, followed by hormone free medium (control) than the other treatments (Table 2). The results of the present study conformed to the findings of Demeke et al. (2014) that regenerated cassava shoots produced an average of 6.14 roots within a 0.5 mg/l NAA. Furthermore, Chen et al. (2003) reported that in *Dioscorea zingiberensis*, half strength MS with NAA (2.0 mg/L) was the best rooting medium. Similar results in yam were also obtained by Behera et al. (2010). From this study, smaller rooting was observed among the genotypes *Slicass 6, Slicass 11* and *Cocoa* in the 0.1 and 0.05 mg/L BAP medium during 4 weeks of culture. Thus, the addition of BAP, especially at high doses in the MS medium deduced strongly, the formation of roots (Faye et al., 2015).

The experimental results on NAA 1.0 (E) and 1.5 mg/L (A) produced relatively higher number of roots, percent rooting, bigger stems and lower number of leaves (Figure 2). In addition, BAP 0.1 mg/L (Figure 2B) and 0.05 mg/L (Figure 2D) showed greater number of leaves, lower rooting percentage and root number. Non-plant regulators media (Figure 2C) produced higher number of secondary roots, normal stem growth and profuse leaves.

Effect of hormones on *in vitro* root number per plantlet of the different cassava genotypes

The effect of different concentrations of BAP, NAA and in combination with GA_3 on the number of roots/plantlet was found to be statistically significant at P<0.001 level of significance (Table 3). The number of roots varied with various concentrations of BAP, NAA and in combination with GA_3 (Table 4). A good number of roots were achieved at 1.0 mg/L NAA with an average mean of 7.72 followed by only 1.5 mg/L NAA (6.39) which was superior to the other treatments. But the highest number of roots was significantly produced by 1.0 mg/L NAA (10.00) with the Slicass6 cultivar followed by Cocoa cultivar with 7.03 roots per plantlets. The use of NAA (1 to 1.5 mg/L) relatively produced higher number of roots as compared to BAP (0.05 to 0.1 mg/L). These results confirm the report of Fan et al. (2011) that NAA (0 to 2.0 mg/L) proved to be effective on root development in cassava. Also, Kane (2005) similarly reported that NAA (0.01 to 10 mg/L) is the most widely used and effective plant growth regulators for root induction. Cacai et al. (2012) has shown that kinetin induces more roots than BAP. On the contrary, 0.00 mg/L (control) produced higher number of roots as compared to BAP (0.1 and 0.05 mg/L) in all three cultivars together with the results of the three cassava genotypes assessed by Mapayi et al. (2013).

Table 4. Effect of different hormonal concentrations and control on *in vitro* root number/plantlet at 4 weeks of culture of the three cassava genotypes.

Hormone	Genotypes			
	Slicass 6	***Slicass 11***	***Cocoa***	**Mean**
BAP 0.05 mg/L	0.40	2.30	1.53	1.41
BAP 0.1 mg/L	0.40	2.23	1.53	1.38
NAA 1.0 mg/L	10.00	6.13	7.03	7.72
NAA 1.5 mg/L	6.83	6.37	5.97	6.39
BAP 0.1 mg/L + NAA 1.5 mg/L + GA_3 1.0 mg/L	6.57	6.10	5.50	6.05
Control 0.0 mg/L	4.58	6.52	5.18	5.42
Mean	4.79	4.94	4.45	
LSD genotype		0.64		
LSD hormone		0.91		
LSD genotype x hormone		1.5		
CV (%)		20.1		

Table 5. Effect of different hormonal concentrations and control on *in vitro* leaf number/plantlet at 4 weeks of culture of the three cassava genotypes.

Hormone	Genotypes			
	Slicass 6	***Slicass 11***	***Cocoa***	**Mean**
BAP 0.05 mg/L	5.57	6.27	5.77	5.87
BAP 0.1 mg/L	6.17	6.37	5.70	6.08
NAA 1.0 mg/L	4.08	4.27	4.20	4.18
NAA 1.5 mg/L	2.70	2.63	3.47	2.93
BAP 0.1 mg/L + NAA 1.5 mg/L + GA_3 1.0 mg/L	4.53	5.63	4.93	5.03
Control (0.0 mg/L)	5.90	4.47	5.63	5.33
Mean	4.82	4.94	4.95	
LSD genotype		0.66		
LSD hormone		0.94		
LSD genotype x hormone		1.63		
CV (%)		20.1		

The use of BAP (0.1 and 0.05 mg/L) showed no significant difference on root number as compared to the use of NAA (1.0 and 1.5 mg/L) among the three cassava genotypes. The lowest number of roots was produced by 0.1 mg/L BAP treatment. This indicates that the addition of cytokinin like BAP in growth medium has suppression effects, whereas auxin (NAA) promoted the root induction in comparison with control condition. The present results corroborate the findings of Gubbuk and Pekmezci (2001).

Effect of hormones on *in vitro* leaf number/plantlet of the different cassava genotypes

As shown in Table 5, plantlets cultured in medium with BAP produced more leaves (or also corresponded to the visible nodes) than those cultured in medium containing either NAA only or in combination with BAP and GA_3. Significant differences in number of leaves were observed among genotypes grown on different hormonal treatments. Comparison of effects of different culture media on leaf number after 4 weeks revealed that 0.1 mg/L BAP showed the highest (6.37) number of leaves in *Slicass 11* cultivar followed by 0.05 mg/L BAP (6.27), whereas, 0.1 mg/L BAP was effective with the *Slicass 6* cultivar. However, no significant difference was observed in two concentrations of BAP with response of *Cocoa* cultivar. These results are confirmed by Ahanhanzo et al. (2008) who reported that explants cultured in media containing BAP produced highest number of leaves in all the cassava genotypes. On the contrary, NAA appeared to be unfavourable for the development of leaves in all genotypes. Therefore, number of leaves per plantlet was only 2.63 after four weeks of culture in the medium, 1.5 mg/L NAA in the *Slicass 11* cultivar.

The responses of *Cocoa* cassava genotype cultured on

Figure 3. Efficacy of different concentrations of plant growth hormones on growth parameters of *Cocoa* cassava genotype (4 WAP), A: Control; B: 1.0 mg/L NAA; C: 0.05 mg/L BAP; D: 1.5 mg/L NAA E: 0.1 mg/L BAP.

Table 6. Influence of different hormonal concentrations and control on *in vitro* fresh weight (g)/plantlets at 4 weeks of culture of the three cassava genotypes.

Hormone	Genotypes			
	Slicass 6	***Slicass 11***	***Cocoa***	**Mean**
BAP 0.05 mg/L	0.07	0.07	0.09	0.07
BAP 0.1 mg/L	0.21	0.19	0.13	0.18
NAA 1.0 mg/L	0.28	0.25	0.29	0.27
NAA 1.5 mg/L	0.21	0.19	0.17	0.19
BAP 0.1 mg/L + NAA 1.5 mg/L + GA3 1.0 mg/L	0.17	0.18	0.18	0.18
Control (0.0 mg/L)	0.12	0.14	0.14	0.14
Mean	0.18	0.17	0.17	
LSD genotype		0.01		
LSD hormone		0.02		
LSD genotype x hormone		0.02		
CV (%)		15.3		

MS media supplemented with BAP 0.05 mg/L (Figure 3A) produced little root with some callosity at the base of the stem but had greater number of leaves (Figure 3). For BAP 0.1 mg/L (B) concentrations, few leaf number and short internodes was produced, thus it will be difficult to subculture. The hormone concentrations of NAA 1.5 (Figure 3C) and 1.0 mg/L (Figure 3D) produced maximum number of roots, rooting percentage, turgid stems but fewer leaved number. Control had stunted growth with few numbers of root and leaves (Figure 3E).

Effect of hormones on *in vitro* fresh weight/plantlet of the different cassava genotypes

The data indicated that increase in fresh weight/plantlet was significantly ($P < 0.05$) better at higher concentrations (Table 6). Treatments with 1.0 mg/L NAA showed the best results in terms of fresh weight followed by treatments with1.5 mg/L NAA. The interactive effect of BAP 0.1 + NAA 1.5 + GA_3 1.0 mg/L significantly ($P<.001$) resulted in high fresh weight as compared to 0.05 mg/L BAP and control (0.00 mg/L). Similar results were reported by Hussein (2012), whereas, supplementing NAA to increasing concentration of BA (from 0.2 to 0.4 mg/L) resulted in higher fresh weight. This could be attributed to increased cytosolic calcium concentration resulting from enhanced uptake from the media due to the use of higher amount of NAA (Ngomuo et al., 2013).

Conclusion

Supplementing the growth medium with various hormonal concentrations and control each with Murashige & Skoog basal medium, showed *in vitro* responses of plantlet

height, root induction, root number, leaf number, and fresh weight among three recalcitrant cultivars, *Slicass 6*, *Slicass 11* and *Cocoa*. In all the media, the MS medium with 1.0 mg/L NAA showed the best response to percent rooting, fresh plantlet weight, root number and plantlet height, while 0.1 mg/L BAP was found to be more favourable for normal development of the leaves which is beneficial in shoot multiplication. Therefore, the addition of cytokinin like BAP at 0.1 mg/L is recommended for the propagation of recalcitrant cassava genotype, whereas, the auxin, NAA is optimal for root development prior to plantlets acclimatization, to improve the recovery of *ex vitro* plantlets for field evaluation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Genetic diversity and relatedness of oil palm progenies determined by microsatellite and agronomic markers

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Genetic diversity in oil palm has been usually determined using agro-morphological markers (traits) before the advent of DNA-based markers including microsatellite markers, which have the advantage of being environmentally independent. The current study aimed to compare genetic diversity and relationships among 10 Nigerian Institute for Oil Palm Research (NIFOR) *dura* × *tenera* oil palm progenies using simple sequence repeats (SSR) and agronomic markers. 114 individual palms representing the NIFOR progenies were screened for polymorphism at five agronomic traits and 16 SSR loci. Coefficients of variation and genetic diversity parameters were calculated to compare the trait-based variation with genetic (SSR) diversity. Hierarchical clustering and principal coordinate analyses (PCA) were performed with agronomic and molecular datasets generated from the progenies. Agronomic traits showed wide range of variation from 5.6% for oil-to-mesocarp ratio to 40.5% for bunch number. The SSR markers deployed showed 100% polymorphism and high genetic diversity (H_e = 0.661, H_o = 0.580) among the progenies. While SSR data discriminated the progenies with respect to pedigree or shared ancestry of the parents, the clustering pattern based on agronomic data predominantly reflected the differences in agronomic traits. Results of this study suggest that agronomic trait data are insufficient in selecting parents for crossing and that genotypic data are more informative.

Key words: Agronomic traits, *Elaeis guineensis*, genetic diversity, Nigerian Institute for Oil Palm Research (NIFOR), simple sequence repeats (SSR) markers.

INTRODUCTION

The African oil palm (*Elaeis guineensis* Jacq.) is the most productive oil bearing crop yielding more than five times oil per hectare of any annual oil crop. In West Africa in general, and particularly in Nigeria, palm oil is the most valuable natural oil in the local diets both as crude red palm oil and as refined palm oil; olein (Corley and Tinker, 2003). The oil palm industry is a major source of employment and income to a substantial proportion of

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Nigerian populace (Omoti, 2009). The world palm oil production in 2016 was 58.72 million tons, with a projected yield increase of up to 6.93%. Nigeria's stagnated 970,000 metric tons of annual palm oil production for the past 6 years from an estimated 2.6 million hectares of oil palm production area, ranks first in the African oil palm belt and fifth in the world after Indonesia, Malaysia, Thailand and Colombia (United States Department of Agriculture (USDA) Statistics, 2017). Notwithstanding its fifth position, the country's average yield is low compared to those of South East Asian palm oil producing countries. In addition, the demand for vegetable oil in Nigeria outstrips domestic supply leading to a deficit of about 500,000 metric tons. Continuous yield improvement to cope with the ever increasing demand for palm oil and its products is currently on-going at the Nigerian Institute for Oil Palm Research (NIFOR), and substantial progress has already been made in this direction. Presently, the commercial variety *(tenera*) yields 20 to 25 mt of fresh fruit bunch ha^{-1} $year^{-1}$ and 3 to 3.5 tons oil ha^{-1} $year^{-1}$ in mature plantations (Okwuagwu et al., 2005). Therefore, replanting old areas with improved varieties in conjunction with proper management and cultural practices would be more than enough in boosting oil palm productivity in Nigeria.

Germplasm collections of oil palm and related richness in new genes are valuable sources of traits of agronomic importance for subsequent development of new improved varieties. The on-going oil palm Main Breeding Programme carried out by NIFOR is presently testing several oil palm genotypes for high yield and adaptation to different agro-ecological areas.

Genetic diversity in crop species can be determined indirectly by phenotypic markers (agro-morphological and biochemical markers) and genotypic (DNA-based) markers (Mohammadi and Prasanna, 2003). Several of these agro-morphological trait based studies have assessed genetic diversity in oil palm populations (Kushairi et al., 1999; Ataga et al., 2005; Okwuagwu et al., 2008, 2011). However, this approach is limited by the long juvenile phase, confounding effects of developmental stage of the plant, long term field evaluation, and vulnerability to environmental effects. Currently, a range of molecular marker techniques are available for measuring genetic diversity. They assess genetic variation at the DNA level and are renowned to be less environment-dependent. The most routinely used are restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and microsatellites or simple sequence repeats (SSRs) (Purba et al., 2000; Rajanaidu et al., 2000; Maizura et al., 2006; Bakoumé et al., 2015).

Microsatellites or SSRs markers provide an important source of polymorphism because they are neutral and widely distributed throughout species genome, thus suitable in assessing genetic diversity within populations. Though SSRs are considered selectively neutral, they have selective value or are linked to genes of selective value. Therefore, it has been assumed here that the fraction of genetic diversity detected by the SSRs can be reflected by the variation revealed by related agronomic traits if any. Several works on genetic diversity, progeny legitimacy test, ortet-ramets relationship, and genetic mapping using microsatellite markers, have been reported in oil palm (Billotte et al., 2001, 2005; Bakoumé et al., 2007, 2011; 2015; Singh et al., 2008; Cochard et al., 2009; Arias et al., 2012; 2013; Okoye et al., 2016). This work aimed at establishing if the amount of genetic variation and structure of genetic variation detected in oil palm parental materials and progenies of the NIFOR Main Breeding Programme by SSR markers agree with those revealed by highly heritable agronomic traits of interest for oil palm breeding and for oil palm industry.

MATERIALS AND METHODS

Plant material

Ten *dura* × *tenera* (D × T) oil palm progenies of the NIFOR second cycle Main Breeding Programme were evaluated in this study. The progenies were derived from 11 bi-parental crosses comprising four NIFOR Deli *dura* (DD), two *dura* (AD), and five *tenera* (T) parents collected from Nigerian oil palm groves and maintained at NIFOR. A total of 114 oil palms that is, 8 to17 palms per progeny whose agronomic traits were recorded were used for SSR analysis (Table 1).

Agronomic data collection

The highly heritable agronomic traits recorded per individual palm basis included: (i) number of harvested bunches (BN), (ii) average bunch weight (ABW), (iii) mesocarp to fruit ratio (M/F), (iv) oil to mesocarp ratio (O/M), and (v) palm height. Average bunch weight (ABW) was obtained as the ratio of fresh fruit bunch (FFB) yield to BN. Mesocarp to fruit ratio and oil to mesocarp ratio was evaluated through fresh fruit bunch analyses according to standard method outlined by Blaak et al. (1963). Seven years (1999 to 2005) mature yield data from the NIFOR oil palm Main Breeding Programme were used for this study. Palm height was measured from the ground level to the base of the leaf or leaf subtending the last harvested bunch for mature palms.

DNA extraction and quantification

DNA was isolated from the 114 oil palm samples at the Bioscience Centre, International Institute of Tropical Agriculture (IITA) Ibadan, using CTAB DNA isolation protocol of Doyle and Doyle (1990), with minor modifications. DNA quantification was determined by electrophoresis and NANODROP® (ND-1000) Spectrophotometer (Thermo Fisher Scientific Inc., Denver). All DNA samples were stored at -20°C until microsatellite analysis at the Genomics Unit of Advanced Biotechnology and Breeding Centre (ABBC), Malaysian Palm Oil Board (MPOB) Selangor, Malaysia. The DNA samples were diluted to an optimum concentration of 25 ng/µl by addition of sterile distilled water or appropriate amount of TE (Tris-EDTA) buffer and stored at 4°C until polymerase chain reaction (PCR) amplification.

Table 1. Progenies derived from 11 NIFOR oil palm breeding parent trees.

S/N	Cross	Progeny code	Number of sample	*Dura* parent/pedigree	*Tenera* parent/pedigree
1	DD1 × T2	DT1	12	NIFOR (ex Serdang Avenue Malaysia)	Calabar
2	DD1 × T6	DT2	17	NIFOR (ex Serdang Avenue Malaysia)	Umuabi OP
3	T1 × DD2	DT3	14	NIFOR (ex Serdang Ave. X IRHO – Pobe)	Ufuma (ex Aba)
4	T3 × DD3	DT4	10	Ulu Remis Deli × ex Sabah	Aba (ex Calabar)
5	DD3 × T5	DT5	9	Ulu Remis Deli × ex Sabah	Ufuma
6	DD4 × T2	DT6	12	Ecuador Deli	Calabar
7	DD4 × T6	DT7	13	Ecuador Deli	Umuabi OP
8	AD1 × T6	DT8	9	Calabar	Umuabi OP
9	AD3 × T6	DT9	8	Ufuma	Umuabi OP
10	AD3 × T5	DT10	10	Ufuma	Ufuma
	Total		**114**		

S/N, Serial number.

Microsatellite analysis

A total of 16 microsatellite markers comprising nine markers developed at the Genomics Unit of ABBC-MPOB and seven from the French Centre de Coopérationen Recherche Agronomique pour le Développement (CIRAD) were used for the PCR amplification. MPOB SSRs were developed from the oil palm expressed sequence tags (ESTs) and genomic sequences reported by Singh et al. (2008) and Ting et al. (2010). SSRs developed from *E. guineensis* ESTs, *E. guineensis* genomic sequences, and *E. oleifera* genomic sequences were labeled sEg, sMg, and sMo, respectively. SSR primer sequences from CIRAD were downloaded from the Trop GENE database (http://tropgenedb.cirad.fr/html/oilpalm Marker. html) and originally labeled as mEgCIR (Billotte et al., 2005). Details of the 16 SSR markers are presented in Table 2. The amplifications of microsatellite loci with fluorescently labeled polymerase chain reaction (PCR) primers were performed as described by Ting et al. (2010) in a Perkin Elmer 9700 thermocycler (Life Technologies, Thermo-Fisher Scientific, USA). PCR products were analyzed on an ABI 3730 Genetic Analyzer and sized using GeneMapper® 4.1 (Applied Biosystems Inc., Foster City, CA, USA) and GS LIZ 500 size standard. Electropherogram profiles (sample plots) were generated and genotype data for all the SSR markers was exported as data table for scoring. The scoring of the genotype data was performed manually with reference to allele and peak size.

Data analyses

For the agronomic data, progeny means as well as population mean, range, and standard error of mean were determined using GenStat software. Coefficients of variation were calculated within progeny for each of the 5 agronomic traits. Clustering of progenies was performed using an average linkage algorithm (UPGMA, Unweighted Pair Group Method with Arithmetic mean) based on Manhattan dissimilarity coefficients (MD) (Sokal and Michener, 1958). Principal coordinate analysis (PCoA) (Gower, 1966) was performed to better depict the relatedness among progenies or individual genotypes. The pair-wise comparisons of progenies and PCoA were facilitated by DARwin 6.0.4 programme (Perrier and Jacquemoud-Collet, 2006). For the microsatellite data, the genotype data of the 10 D × T progenies at all SSR loci were used to assess the number of alleles (A_o), percentage of polymorphic alleles (%P), observed (H_o) and expected (H_e) heterozygosities using Genetic Analysis in Excel (GenAlEx) version 6.5 (Peakall and Smouse, 2006, 2012). Rogers' dissimilarity coefficients (Rogers, 1972) were calculated and the dissimilarity coefficient matrices were again subjected to cluster analysis and PCoA to explore and establish similarity or dissimilarity among D × T progenies. All genetic distance calculations and construction of dendrograms were performed using PowerMarker v3.25 (Liu and Muse, 2005) and MEGA software v4.0 (Tamura et al., 2007), respectively.

RESULTS

Variability in progenies of NIFOR parents of the main breeding programme revealed by SSR markers and agronomic traits

The genetic diversity parameters in progenies of NIFOR parents of the Main Breeding Programme using SSR markers and agronomic markers are given in Table 3. Among the five traits evaluated, wider range of variation was observed across the ten different oil palm progenies for most of the traits. The number of harvested bunch (BN) was in the range of two to 14. The minimum (3 kg/p/yr) and maximum (26.3 kg/p/yr) average bunch weight (ABW) was observed in DT5 and DT10 progenies, respectively. The annual growth rate (height) was maximum (90 cm/yr) in DT10 and minimum (31.9 cm/yr) in DT7. Mesocarp to fruit ratio varied from 40 in DT2 to 92.5 in DT9 while oil to mesocarp ratio (O/M) was as small as 45.1 in DT10 and as large as 65.8 in DT2, with a mean of 57.23. The coefficients of variation (CV) for the progenies with respect to the agronomic traits revealed wide range of values. BN was found to be the most variable character for all the progenies evaluated. The CV% ranged from 27.76% in DT1 to 59.29% in DT5. This is followed by height (8.23% in DT8 to 31.94% in DT10) and ABW (22.64% in DT9 to 39.47% in DT7), respectively. The CV within the progenies was moderate or low for M/F (16.63% in DT8 to 26.17% in DT2) and O/M (3.339% in DT1 to 7.231% in DT4).

Based on SSR markers derived from oil palm, the level polymorphism was 100% in all the D × T progenies

Table 2. Microsatellite primer pairs used for population genetic analysis of oil palm

S/N	SSR LOCI	Linkage group	Ta (°C)	Primer sequence	SSR repeat motif	Expected fragment size (BP)	Accession number
1	mEgCIR3813	1	52	F-CATACCCTGCTTATCTTTC R- GTAGATACCCGTTAGTTGAC	(GA)19	167	AJ578734
2	mEgCIR0793	2	56	F-GTACTTCGCAACTATTCCTTTTCTT R- AGTTGATCGTGGTGCCTGAC	(GA)15	149	AJ578545
3	mEgCIR0425	3	58	F-AGCAAGAGCAAGAGCAGAACT R- CTTGGGGGCTTCGCTATC	(CCG)9	232	AJ578521
4	sMg00156	4	50	F-GGTGTCATAACTTCGTTGTTGCT R- ATGCTCAAAAGTGGGTTTCTCTC	(CT)15	237	Pr010615888*
5	mEgCIR3828	5	50	F-AGCCAGATGGAAATACAC R- GTGCGATAAAGAGGAGAGT	(GA)23	282	AJ578738
6	sEg00154	6	57	F-TCCCCCAATACTCATCATGC R- TGATCGACGGTTGTCACATT	(CAG)5	238	EY410356**
7	sMo00102	7	53	F-ATGAGATGGGACAAATCAAAC R- ACCATACCAACTAGAGAACTAAACA	(AG)11	235	Pr010615939*
8	sMg00228	8	54	F-CACGTATATGAGCAGGATTTGA R- CTCCAAACCAACTAGAGCTGA	(AT)25	205	Pr010615913*
9	sMg00016	9	52	F-GCGATTCCGGTTATCTTTAG R- GAGTTTTTGCTGTGATGATTAG	(GA)13	274	Pr010615861*
10	mEgCIR3519	10	52	F-CCACTGCTTCAAATTTACTAG R- GCGTCCAAAACATAAATCAC	(GA)15(GT)8	236	AJ578672
11	sMg00120	11	54	F-GATCAATGCGAGAAATCAGG R- GATCATGCTTATCCTTTCCAAGT	(AT)11	152	Pr010615881*
12	mEgCIR0790	12	52	F-TTGGTGGTCCTTTTGAATATC R- ACAAACCCAGCACTTAAAATAAC	(GA)19	215	AJ578544
13	sEg00151	13	57	F-ATCACAACAGCAGCAGCATC R- CGCATCAAGAAACATGGAGA	(CAG)8	219	EY411661**
14	sMg00179	14	54	F-AACCCCTTTTTCATGCTCTAA R- CTGATTTTGGAATCAGAGGTG	(AAAAG)6	214	Pr010615893*
15	sMg00087	15	58	F-CACCTAAAAACGGCAAGGAAC R- GGAGGAGAGAAATGGAAGACG	(AG)19AA(AG)	212	Pr010615880*
16	mEgCIR3745	16	52	F-GGAAGTCTTGATGTTGAAAG R- ATCAAGCAGTCGCATAATAC	(GA)18	260	AJ578718

*Probe Unique Identifiers (PUIDs) of NCBI Probe Database. **Accession numbers of NCBI GenBank

evaluated. The number of alleles per progeny (A_o) varied from 3.2 (DT9) to 4.1 (DT10), with a population mean value of 3.770. Observed (H_o) and expected (H_e) heterozygosities were greater than 0.5 in all but one progeny, DT3 (H_o=0.448) and DT4 (H_e=0.488), respectively with an average $\overline{H_o}$ of 0.580 and H_e of 0.661. Progeny DT1 recorded the highest H_o (0.662) and H_e (0.614).

Table 3. Diversity in progenies of NIFOR parents of the main breeding programme using SSR markers and agronomic markers.

Progeny	SSR markers				Agronomic markers									
					BN		ABW		M/F		O/M		Height	
	Å	%P	H_o	H_e	Mean	CV (%)	Mean (kg)	CV (%)	Mean (%)	CV (%)	Mean (%)	CV (%)	Mean (cm/yr)	CV (%)
DT1	4.000	100	0.662	0.614	5.909	27.76	11.582	35.70	63.12	23.14	57.38	3.339	61.46	13.59
DT2	3.900	100	0.622	0.537	6.059	35.24	9.495	31.04	63.39	26.17	57.98	5.764	51.62	19.61
DT3	3.500	100	0.448	0.517	5.929	44.61	12.82	29.50	68.36	22.97	56.54	5.280	66.62	11.16
DT4	3.500	100	0.532	0.488	4.1	42.17	14.98	33.20	61.37	22.87	57.00	7.231	77.19	12.75
DT5	3.900	100	0.510	0.561	5.333	59.29	16.36	31.70	67.88	19.78	58.03	7.143	70.72	15.96
DT6	3.800	100	0.580	0.568	5.417	29.93	11.28	33.09	60.27	24.04	57.20	6.515	47.05	16.55
DT7	4.400	100	0.642	0.576	6.154	34.37	8.3	39.47	68.9	22.73	56.90	5.429	44.47	17.03
DT8	3.400	100	0.520	0.526	7.444	46.59	10.27	27.44	68.51	16.63	57.03	4.654	50.26	8.23
DT9	3.200	100	0.538	0.512	4	32.73	16.62	22.64	70.81	23.71	60.84	4.448	61.03	13.26
DT10	4.100	100	0.607	0.573	5	52.49	11.27	38.56	70.0	24.70	53.99	5.764	59.79	31.94
Total Popul.														
Mean	7.6	100	0.580	0.661	5.611	40.52	11.917	32.23	65.953	22.67	57.23	5.56	58.22	16.01
Minimum					2		3		40		45.1		31.92	
Maximum					14		26.3		92.5		65.8		90.12	
SE	0.476		0.041	0.034	0.228		0.427		1.409		0.341		0.156	

Å=Number of allele; H_o=Observed heterozygosity; H_e=Expected heterozygosity; %P=Percentage of polymorphic loci; BN=Number of harvested bunches; ABW=Average bunch weight; M/F=Mesocarp to fruit ratio; O/M=Oil to mesocarp ratio; CV=Coefficient of variation; SE=Standard error of mean. Popul: Population.

Relatedness of progenies of NIFOR parents of the main breeding programme revealed by agronomic markers and SSR markers

Using the mean values of the 5 agronomic markers (number of bunches (BN), average bunch weight (ABW) (kg/p/year), mesocarp to fruit ratio (M/F), oil to mesocarp ratio (O/M), and height (cm/yr)), Manhattan dissimilarity coefficients (MD) were calculated by pair-wise comparisons of progenies using DARwin software. Manhattan dissimilarity coefficients ranged from 0.145 to 0.617 with an average of 0.4. The UPGMA based dendrogram grouped the progenies in two major clusters (Figure 1). The progeny DT9 was clearly differentiated from all other progenies in Group I. Pedigree analysis of DT9 revealed that T6 and AD3, the Umuabi *tenera* and Ufuma *dura* parents are involved in the development of this progeny. DT9 is characterized by high ABW, M/F, and O/M that separated it from the rest of the progenies. Group II was constituted by progenies classified either by different agronomic traits or pedigree. Five additional sub-clusters varying from 1 to 3 progenies per sub-cluster were observed within this group. Sub-clusters IIA and IIB comprised only one progeny DT4 and DT10, respectively. These progenies are unique for palms with high increment in height (tall palms) and high M/F, respectively. Progeny DT3 and DT5 fell in sub-cluster IIC due to the common origin of the male parents (T1 and T5); both parents are from Ufuma. Similarly in sub-cluster IID, DT7 and DT8 share the same male parent (T6) from Umuabi. Finally, sub-cluster IIE included progenies sharing same female parent (DD1) and male parent (T2).

Clustering pattern of 10 D x T progenies based on the SSR markers (Figure 2) was not entirely different from the patterns obtained from agronomic markers. The UPGMA dendrogram based on the Rogers' dissimilarity among the progenies showed two main clusters. Out of 10 progenies, 9 were grouped into a single cluster with four sub-clusters. The first cluster contained progeny DT9 derived from Ufuma *dura* (AD3) and Umuabi *tenera* (T6) parents. The second main cluster was of four sub-clusters IIA, IIB, IIC and IID. Sub-clusters IIC and IID contained progenies which either share one of the parents directly or through the ancestry. For instance, DT5, DT10, DT8, and DT7 share the same male parent (T6) from Umuabi while DT5 share the same origin with DT10. Both the male parent of DT5 (T5) and the female parent of DT10 (AD3) are from the same origin/geographical location, Ufuma. Also, DT1 and DT2 in sub-cluster IID share the same female parent (DD1); a NIFOR Deli from Serdang Avenue, Malaysia. The inclusion of DT4 in this sub-cluster was probably because of the common Calabar origin of the male parent (T3) and T2 in DT1. DT6 and DT3 were distinct sub-clusters IIA and IIB respectively.

Principal coordinate analysis

To visualize the similarity or dissimilarity among

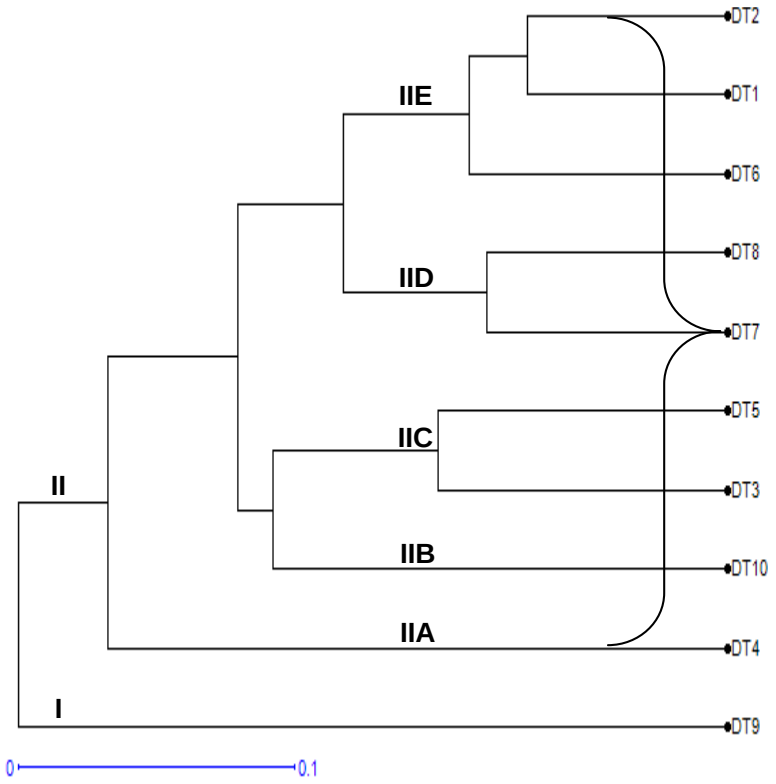

Figure 1. Dendrogram based on Manhattan dissimilarity coefficients demonstrating association among 10 DxT NIFOR oil palm progenies.

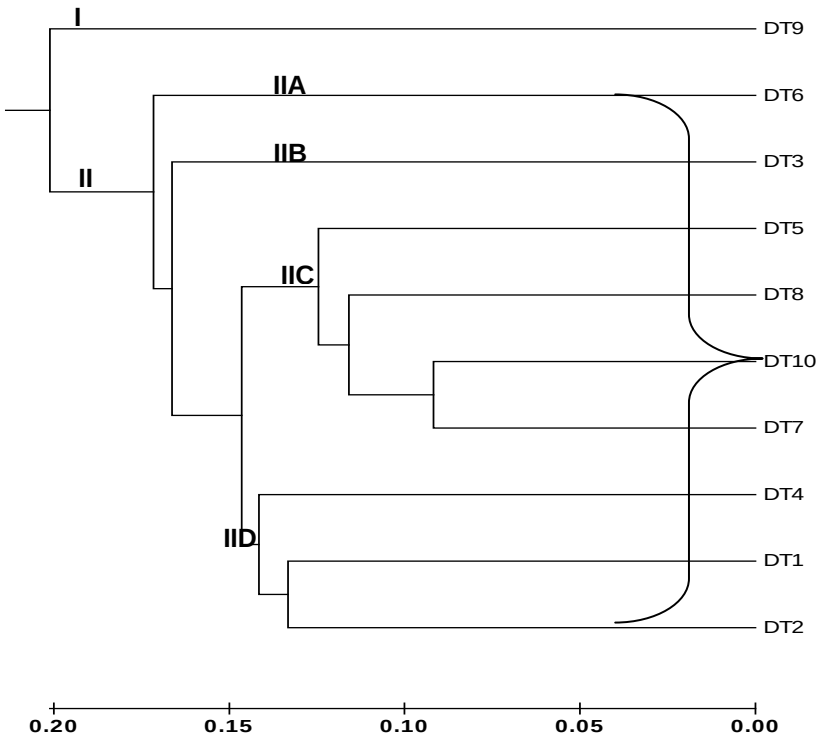

Figure 2. Dendrogram based on UPGMA clustering of 10 D x T NIFOR oil palm progenies based on Rogers' dissimilarity.

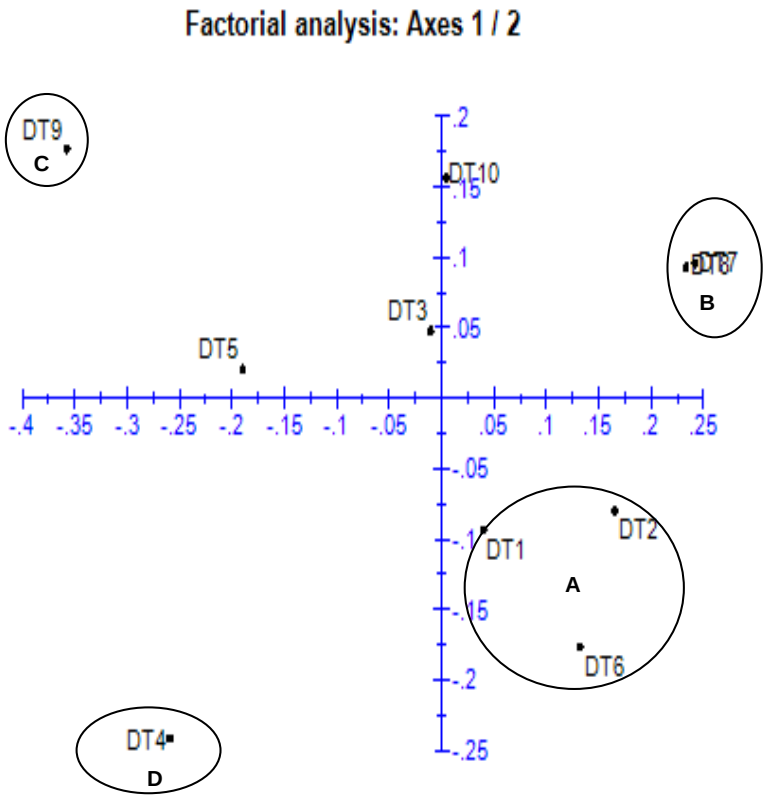

Figure 3. Principal Coordinates Analysis using genetic distance matrix based on 5 agronomic traits.

progenies or individual genotypes, principal coordinate analysis (PCoA) was performed using DARwin software version 6.0.4 programme (Figures 3 and 4). The PCoA analysis further validated the positions and grouping of progenies. PCoA based on genetic distance matrix of agronomic data (Figure 3) explained 74.17% of the diversity on the first two principal components (PC1 = 50.66% and PC2 = 23.51%). There was, however, a tendency that the progenies clustered according to their agronomic performance or shared ancestry. The derivatives of the Deli *dura* (DT1, DT2 and DT6) were distributed in one cluster (A) based on high BN and average O/M while DT7 and DT8 were grouped together in cluster B with respect to high BN and M/F, average O/M and plant height. Noteworthy is the shared pedigree of either a common male or female parent among the progenies in clusters A and B. Progenies DT9 and DT4 were separated from the rest of the progenies in clusters C and D. DT9 exhibited the highest ABW, M/F and O/M compared to the very tall palms in progeny DT4. The rest of the progenies were scattered as individuals or smaller groups on the basis of intermediate agronomic traits very much similar to the dendogram.

In comparison to the grouping based on agronomic data, the grouping of progenies derived from SSR marker data is observed to be more incisive and compelling (Figure 4). The only progeny (DT9) derived from Ufuma *dura* (AD3) and Umuabi *tenera* (T6) was solely placed in the first cluster (A) while the progenies (DT1, DT3, and DT6) were grouped in cluster B. DT1 and DT3 share the same female Serdang Avenue Deli grandparent (19 × 65) and DT6 share the same male parent (T2) with DT1. Cluster C contain progenies with common male parent (T6) as well as some of the progenies (DT8 and DT10) in cluster D. DT4 and DT5 sharing same female parent (DD3) were also grouped together with DT8 and DT10 in cluster D. These two sets of progenies were classified together due to the common Ufuma origin of the male parent (T5) in DT5 and female parent (AD3) in DT10. The PCoA based on molecular data is better in discriminating related progenies of common origin and parentage.

Simple correlation between phenotypic variation, estimated by Manhattan distances using all agronomic characters and SSR marker based distance matrices was low (r = 0.2989) and non-significant.

DISCUSSION

It is important to advance NIFOR oil palm breeding to meet the growing domestic demand for palm oil and its products. In view of the successes of the conventional

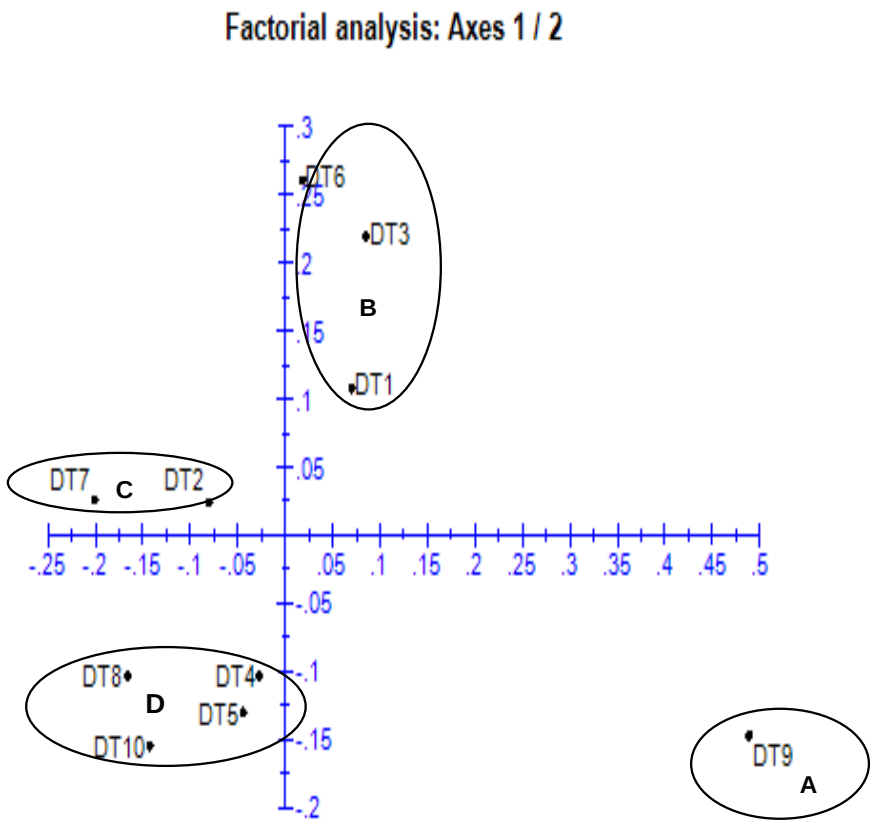

Figure 4. Principal Coordinates Analysis using SSR marker based similarity coefficient matrix of 10 NIFOR oil palm D x T progenies.

breeding methodology over the years, oil palm hybrid breeding remains a choice method. Better understanding on the genetic diversity provides possibilities for breeders to select desired individuals for the plant improvement, to produce more performing progenies that assemble the good parental characteristics. In the present study, oil palm progenies screened with SSR markers have been subjected to analysis of variations for agronomic traits.

Agronomic and microsatellite variability in the 10 D × T progenies

The results of this investigation showed that the 10 NIFOR D × T oil palm progenies were variable for most of the evaluated agronomic traits and microsatellite markers. Among the progenies, substantial variation was observed for number of harvested bunch (2 to 14), average bunch weight (3 to 26.3 $kg^{-1}/year^{-1}$), and annual growth rate (height = 31.92 to 90.12 cm/yr). Similar results on wide range of phenotypic variability of agronomic traits have been reported in the evaluation of introgressed progenies of Nigerian origin in MPOB by Noh et al. (2014). The coefficient of variation (CV) values of these traits varied from 27.76 to 59.29% between DT1 and DT5, 22.64 to 39.47% for DT9 and DT7, and 8.23 to 31.94% in favour of DT8 and DT10, respectively. The high coefficient of variation observed among the progenies for the respective traits evaluated indicates high genetic diversity existing in the NIFOR oil palm main breeding population. Differences in CV among the progenies could be explained by genotype, environment, or genotype-environment interaction. The presence and importance of genotype-environment interaction in the NIFOR oil palm progenies has been mentioned by several authors (Ataga 1993; Okoye et al., 2008). The broad range in the population mean of the various traits implies great potential for grouping the oil palm progenies into various groups of poor performers and good performers. Although agronomic traits have been used for grouping the oil palm genotypes, agro-morphological traits with high heritability were considered in the present study. The Ufuma × Umuabi progeny (DT9) ranked highest for average bunch weight production with the least CV suggesting the stability of this genotype for this agronomic trait. Such significant genetic variation has also been reported (Okwuagwu and Okoye, 2006; Okwuagwu et al., 2008; Okoye et al., 2008) on agronomic traits in oil palm. Better understanding on the influence of environment on these quantitative traits would help to

group the genotypes with better accuracy. Comparatively, microsatellite analysis revealed higher genetic diversity among the 10 D × T oil palm progenies than the agronomic analysis. The percentage of polymorphic loci was very high (100%) across all the progenies, indicating the superiority of SSR markers over agronomic markers. Agronomic traits are often limited by high cost and long term field evaluation, low polymorphism and the influences of environmental factors. Thus, these traits may not adequately represent the genetic diversity among genotypes.

However, the three progenies (DT5, DT7, and DT10) with the highest CV over the five agronomic traits also exhibited high values of genetic diversity parameters demonstrating the high genetic diversity existing in the progenies. Relatively higher genetic diversity (A_o = 4.4, H_o = 0.642 and H_e = 0.576) was observed in Ecuador × Umuabi progeny (DT7) compared to the other progenies. Interestingly, this progeny also had higher values for agronomic traits such as number of harvested bunch (6.154), mesocarp to fruit ratio (68.9), and most importantly, comprising palms with the least annual growth rate (height = 44.47 cm/year). This finding could suggest a possible correlation between the extent of genetic variation within progenies and agronomic traits in oil palm. Therefore, palms from this progeny should get priority for breeding high bunch yielding and dwarf planting materials. Variable efficiencies of different marker systems for detecting genetic diversity in oil palm have been reported in oil palm using isozyme (mean A_o = 1.6; Hayati et al., 2004) and RFLP technique (mean A_o = 1.8; Maizura et al., 2006). High genetic diversity has been reported in both oil palm breeding materials and natural collections irrespective of both the country of origin and the genetic marker technique used (Bakoumé, 2016).

Relatedness of 10 D × T progenies based on agronomic markers and SSR markers

Results of Manhattan and Rogers' dissimilarity coefficients for the respective agronomic traits and SSR marker data were comparable. The highest genetic distance (highest genetic diversity) for agronomic data (0.617) and molecular data (0.4575) corresponded well to progenies DT6 (Ecuador Deli × Calabar) and DT9 (Ufuma × Umuabi). Parents of DT9 and the female parent of DT6 are new introductions in the breeding programme hence the very high genetic diversity in the progenies. Both methods of analyses classified the ten progenies into two main clusters with some disagreements in the grouping of progenies. Although not always satisfactory, Manhattan dissimilarity coefficients differentiated the genotypes predominantly on the basis of agronomic traits. This is an indication of the extent of variation across the oil palm genotypes for the quantitative traits. Similar results were reported by Arias et al. (2013) in the evaluation of 43 oil palm progenies from Angola with nine phenotypic traits and 30 SSR markers. Similarly, Rogers' dissimilarity coefficients based on SSR marker data differentiated the oil palm progenies into two different groups with respect to their pedigree or shared ancestry. This demonstrates the effectiveness of SSR markers in identifying close pedigree relationship in breeding material.

On comparing agronomic and molecular clustering patterns, molecular markers have proved to be efficient in pedigree characterization of the NIFOR oil palm progenies. The progenies clustered according to the shared pedigree of either a common male or female parent. For instance, DT8, and DT7 share the same male parent (T6) from Umuabi while DT5 and DT10 share the same male parent (T5) from Ufuma. The grouping according to pedigree or shared ancestry is supported by previous findings in oil palm using molecular markers. Norziha et al. (2008) classified 16 D × P oil palm progenies from MPOB into 4 major clusters based on pedigree information with the aid of nine microsatellite markers. However, a tendency of clustering of progenies based on either agronomic performance or pedigree was observed using agronomic markers. For example, DT7 and DT8 were grouped in sub-cluster IID based on high bunch number in addition to sharing the same male parent palm (T6) from Umuabi. Similar result regarding effectiveness of SSR markers in monitoring genetic diversity for yield component traits as well as quality traits have also been reported in oil palm (Abdullah et al., 2011; Solin et al., 2014). Both agronomic and microsatellite clusters distinguished DT9 from the other progenies, thereby establishing high genetic diversity of the genotype. Progeny DT9 was derived from a high bunch weight Ufuma *dura* parent AD3, crossed to a high BN and O/M Umuabi open pollinated *tenera* parent (T6). The more recent assessment of the NIFOR main breeding parent genotypes by Okoye et al. (2016) with 10 microsatellite markers revealed the prevalence of private alleles in the Ufuma *dura* (AD3) and Umuabi *tenera* (T6) parents. It is possible that the pedo-climatic conditions of the parents' provenances may have an adaptive value on the genotypes to justify the presence of private alleles as proposed by Zeng et al. (2004). The highest number of private alleles was reported in the *tenera* parents from Umuabi. This provenance is derived from savannah ecology and generally regarded as marginal for oil palm production with rainfall of about <2000 mm per annum and a well-drained sandy clay loam soil. The private alleles found in such a marginal environment may have an adaptive value. The palms are characterized by slow stem increment, high bunch yield, and palm wine (alcoholic beverage from oil palm sap) production. Then again, Ufuma is a rainforest with rich clayey loam soil and high rainfall (>2000 mm). This area is characterized by high yielding palms with good fruit and bunch composition traits in addition to the unusually high proportion of *tenera* (thin-shelled) palms. Additional

support on the adaptive genetic variant is likely in sub-cluster IID comprising DT1, DT2, and DT4 (Figure 2). This is similar to the results of the agronomic analysis for the Deli *dura* derived progenies (sub-cluster IIE = DT1, DT2, and DT6; Figure 1), except that DT6 is included with the other Serdang avenue Deli progenies. The similarity between phenotypic and molecular marker analysis could suggest that SSR markers may be highlighting expressed traits with adaptive significance.

The grouping of the progenies obtained through PCoA on the basis of SSR marker data did not confirm that obtained by UPGMA cluster analysis in contrast to the agronomic dataset. Besides, the clustering provided by the dendograms failed to reflect the genetic relatedness of the progenies observed in the PCoA plots. The PCoA plot based on SSR data is more revealing in the grouping of progenies with respect to their parentage or shared ancestry; for instance, the grouping of progenies DT1, DT3, and DT6 in cluster B. DT1 is associated with DT3 by a common female Serdang Avenue Deli grandparent (19 × 65) while DT6 share similar Calabar *tenera* male parent (T2) with DT1 (Figure 4). Additional support is the classification of DT7 and DT2 in cluster D, both of which contain the same male *tenera* parent (T6) from Umuabi. The genetic information based on molecular data enables the accurate grouping of genotypes sharing common lineage or genotypes developed for specific objectives. Cochard et al. (2009) also used PCoA to delineate and visualize 318 individuals from 26 origins and eight countries into three groups. With few exceptions, the agronomic based PCoA showed very little grouping according to known pedigree of the progenies (Figure 3). The grouping of DT1, DT2, and DT6 in cluster A and DT7 and DT8 in cluster B revealed some relatedness with respect to parentage of the progenies. DT7 and DT8 share the same male parent (T6) from Umuabi irrespective of their agronomic similarity in terms of mesocarp to fruit and oil to mesocarp ratios. Overall, both PCoA methods classified the 10 oil palm progenies into four groups with some deviations in the grouping pattern.

The low correlation between genetic distances calculated from the two approaches could be due to the fact that DNA markers reports genetic variation also in non-coding regions which hardly have an effect on phenotype. On the other hand, quantitative traits are influenced by environmental factors and their phenotype is a product of genotype × environment interaction. Plants may be morphologically similar, but this does not necessarily imply genetic similarity, since different genetic bases can result in similar phenotypic expression (Khan et al., 2009). A large portion of variation detected by molecular markers is non-adaptive and is, therefore, not subject to either natural or artificial selection as compared with phenotypic characters, which in addition to pressure selection are influenced by the environment (Vieira et al., 2007). Nonetheless, the low or no correspondence between variation estimated by molecular markers and agronomic characters should not be considered a limitation given the fact that genetic and morphological diversity work in different ways to determine the relationships among populations. Besides, the disparity as in the case of the present study suggests that progeny classification and selection of parents for crosses in NIFOR oil palm breeding programme should not be relied on only one method of evaluation (agronomic traits).

Conclusion

Although both agronomic and molecular analysis revealed differences in genotype clustering, they shared several common aspects, such as high diversity between DT9 and DT6 genotypes. The genotypes analyzed in the present work had been previously selected and evaluated in NIFOR breeding programme, and had exhibited good performance. Therefore, prior screening of the most divergent genotype pairs identified through both methods is suggested for evaluation of the relative agronomic performance of their hybrids to prevent inbreeding depression. In a conventional breeding programme, numerous crosses are normally performed and evaluated in field trials. According to the data described in this study, SSR-based genetic distances could be useful in selecting superior crosses between oil palm trees derived from a population with a broad genetic base. Hence, the application of SSR markers in NIFOR oil palm breeding could be instrumental in reducing the number of single-cross hybrids to be evaluated.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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