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Full Length Research Paper

Perennial soybean seeds coated with high doses of boron and zinc

Amanda Justino Acha*, Henrique Duarte Vieira and Marta Simone Mendonça Freitas

Laboratório de Fitotecnia (LFIT), Setor de Produção e Tecnologia de Sementes, Universidade Estadual do Note Fluminense (UENF), Av. Alberto Lamego, 2000 - Parque Califórnia, Campos dos Goytacazes-RJ, CEP: 28035-200, Brazil.

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The objective of this work was to study combinations of high doses of boron (B) and zinc (Zn) in the recoating of perennial soybean seeds, in order to provide these nutrients to the future plants. The physical, physiological and nutritional characteristics of the coated seeds and initial development of plants in a greenhouse were evaluated. Tests carried out in the laboratory were conducted in a completely randomized design, and the experiments completed in a greenhouse were a randomized block design. The coating with the dose 0.8 kg of H_3BO_3 + 0.8 kg of $ZnSO_4$ kg^{-1} of seeds provides the best quality coating. The combination of B and Zn in seed coating reduces the production of shoot dry matter, while the other treatments do not affect the growth variables of the plants. Plants absorb and accumulate the micronutrients added to the coating of the seeds.

Key words: Micronutrients, coater, soybean, seeds.

INTRODUCTION

The adoption of intercropping of *Poaceae* and *Fabaceae* in Brazil is viable in the pasture ecosystem due to the capacity of atmospheric nitrogen fixation. Furthermore, *Fabaceae* can influence the quality of the pasture, which can increase production of bovine milk and meat, and protect the soil (Macedo et al., 2014).

The *Neonotonia wightii* is a palatable herbaceous *Fabaceae* of high nutritional value, intercropping-friendly and of good natural reseeding. These qualities make this plant one of the most important tropical *Fabaceae* plants in the world, being highly indicated to haymaking, pasture establishment and green fertilization (Barcellos et al., 2008). Gama et al. (2011) verified that perennial soybean is persistent throughout the years, even thriving in adverse conditions, contributing to intercrop productivity. The predominant soils in Brazil are dystrophic latosols, which are naturally devoid of micronutrients, especially zinc and boron. The deficiency of these or any other micronutrient is capable of causing a reduction in the development, growth and hence crop yields, they play important roles in the metabolism of the plant. Thus, treatment of seeds with micronutrients is a viable way to provide them to the plants (Ker, 1997; Prado et al., 2008).

Perennial soybean seeds are small, and can hinder its

*Corresponding author. E-mail: amandajacha@gmail.com. Tel: (22) 27397095.

distribution when seeding. Seed coating is a solution that only increases the size and shape of seeds, but provides the possibility of adding needed nutrients, growth regulators, insecticides and fungicides. These plants require only a small amount of such products and this technique allows an effective and safe application of them.

These studies evaluated the coating of perennial soybean seeds with boric acid (B) and zinc sulfate (Zn) at different rates, in order to provide these micronutrients in enough quantity to supply the needs of a plant life cycle.

MATERIALS AND METHODS

These experiments were completed in a laboratory and greenhouse, both belonging to the State University of Northern Rio de Janeiro (in Portuguese: Universidade Estadual do Norte Fluminense Darcy Ribeiro) from 06/2015 to 09/2015. The commercial perennial soybean seeds went through chemical scarification by being immersed in previously tested concentrated sulfuric acid for 20 min, prior to coating seed for the experiments.

To coat seeds, the methodology used by Xavier et al. (2015) was adapted in a N10 Newpack coater, regulated so the chamber would spin at the speed of 90 rpm and the compressed air that activates the solution would be under 4 bar pressure during 1 s. Then, the hot-air blower was switched on at the temperature of 40ºC for 2 min.

As stuffing material for the coating, dolomite lime and activated vegetable charcoal were used in a 3:1 proportion (p/p) and 0.08:1 (p/p), respectively. Elmer's Glue-All, a polyvinyl acetate (PVA) based glue, diluted in deionized water previously boiled at 70°C, in the proportion of 1:1 (v/v) was used for the adhesive material (Mendonça et al., 2007).

The coating process was divided in portions of 50 g of seeds, each of these portions being put in the chamber inside the coater, along with another portion of stuffing material (initially 6.25 g of dolomite lime). Then the adhesive solution spray was applied three times repeatedly along with another portion of the stuffing material (6.25 g of dolomite lime) which was added over the seeds with another application of adhesive solution. Right after, the air blower (40ºC) was used for 2 min. This procedure resulted in the first coating layer. For the following layers, another jet of adhesive solution followed by another portion of stuffing material were applied; then, another jet of adhesive solution, accompanied by the second portion of stuffing material. Finally, another jet of adhesive solution was activated, before the final hot air blowing, which lasted for more 2 min. This procedure was repeated until the stuffing material was over. The portions of activated vegetable charcoal (1 g) were added after the third layer with dolomite lime, making the 4th and 5th layers from this same material. The doses of boric acid (H_3BO_3) and zinc sulfate ($ZnSO_4$) were added all at once in the sixth coating layer, between portions of dolomite lime and glue. At the end of this process, 14 coating layers were formed.

The tested treatments were: TR1) Uncoated seeds; TR2) Coated seeds, without micronutrients; TR3) 0.8 kg of H_3BO_3 + 0.8 kg of $ZnSO_4$ kg^{-1} seed; TR4) 1.0 kg of H_3BO_3 + 0.9 kg of $ZnSO_4$ kg^{-1} seed; TR5) 1.2 kg of H_3BO_3 + 1.0 kg of $ZnSO_4$ kg^{-1} seed; TR6) 1.4 kg of H_3BO_3 + 1.1 kg of $ZnSO_4$ kg^{-1} seed; TR7) 1.6 kg of H_3BO_3 + 1.2 kg of $ZnSO_4$ kg^{-1} seed; and TR8) 1.8 kg of H_3BO_3 + 1.3 kg of $ZnSO_4$ kg^{-1} seed. All treatments were determined based on the fertilizing regulation for soils, recommended for forage crops, considering a seeding of 2.5 kg of seeds.

After coating, seeds were evaluated regarding physical and physiological features and nutritional value, in laboratory and in a greenhouse.

Using the same criteria established in the rules for seed analysis (Brasil, 2009) the laboratory tests were conducted in completely randomized design, using 4 repetitions of 50 seeds for the germination test, during which the percentage of germination, dead seeds and soaked seeds was determined after 10 days. The water content, the weight of a thousand seeds and the germination speed were also measured.

To determine the maximum diameter (MAD), the minimum diameter (MID) and the contour irregularity of the seeds, four repetitions of 50 seeds from each treatment were used, to be ultimately analysed by the seed analysis equipment Graundeye®, with the results expressed in centimeters (cm).

The emergence test, which took place inside a greenhouse, was put together in plastic trays containing previously washed up sand, where 4 blocks of each treatment including 50 seeds were sown in randomized design. The test ran for 90 days; in the first 30 days, there was a daily counting to determine the emergency speed index (ESI). By the end of the test, 10 plants were selected from the portion and had its aerial parts and roots separated, with the purpose of determining their length with the assistance of a ruler.

Using the Groundeye® equipment, the scientists determined the number of ramifications and the full length of the root, accounting the length of each ramification. Further on, the aerial part and the root were stowed in paper sacks and taken to a greenhouse with an air circulation of 65°C for 72 h, so they could establish the shoot dry matter (SDM) and the root dry matter (RDM).

To determine the nutrient content in the seeds (in the aerial part and in the roots of plants developed inside the greenhouse) after a 72-h drying period (at the temperature of 65°C) the seeds were macerated and the aerial part and root milled, to only then be stocked in hermetically sealed flasks. For the analysis of the seeds, 4 repetitions of each treatment were used. However, for plant analysis (aerial part and root) there was not enough material for repetitions. Therefore, the results of the analysis were based solely on the material utilized in each treatment. To determine calcium, magnesium, boron and zinc contents the material went through nitric digestion and the analysis of the extract was made using the ICPE-9000.

Data were tested for normality and data did not require any type of transformation. Means were tested using an analysis of variance (F-test) and the Tukey's range test (at a 5% probability level) with help from the statistical program SAEG.

RESULTS AND DISCUSSION

As for the quality of the coating, the highest TSW and maximum diameter were reached in the combination of smaller doses of micronutrients of 0.8 kg of H_3BO_3.kg-^{1} of seeds + 0.8 kg of $ZnSO_4$ kg^{-1} of seeds (TR3), which enhanced the seed mass twice if compared to the uncoated seeds (TR1) (Figure 1). Thus, the first goal of the coating process, which is to enhance and modify the shape and density of the seed was reached, what should provide greater accuracy in sowing and in the application of chemicals (Mendonça et al., 2007). However, the use of larger doses of micronutrients ended up being harmful to the increase of the weight of the seeds, because as the doses grew larger, the adhesiveness of the coating material reduced. It was also noted that the coating process was not efficient when it came to correct the irregularity of seed contour, considering that there was not a significant difference in this variable ($p<0.05$) between treatments. The results can be associated with

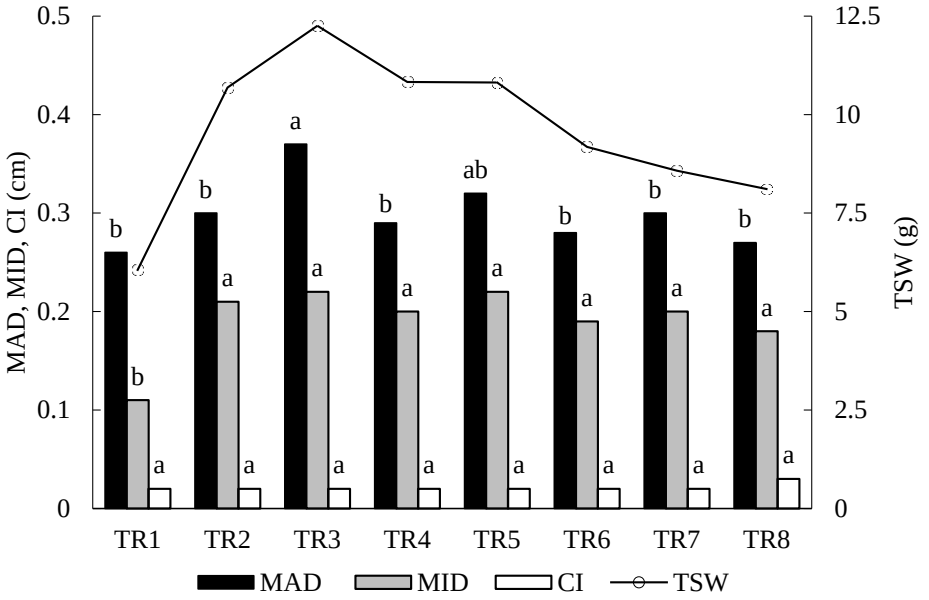

Figure 1. Maximum Diameter (MAD) and Minimun Diameter (MID), Contour Irregularity (CI) and Thousand Seed Weight (TSW). Treatments ($kg.kg^{-1}$ of seeds): TR1: Uncoated; TR2: Without micronutrients; TR3: 0.8 H_3BO_3 + 0.8 $ZnSO_4$; TR4: 1.0 H_3BO_3 + 0.9 $ZnSO_4$; TR5: 1.2 H_3BO_3 + 1.0 $ZnSO_4$; TR6: 1.4 H_3BO_3 + 1.1 $ZnSO_4$; TR7: 1.6 H_3BO_3 + 1.2 $ZnSO_4$; TR8: 1.8 H_3BO_3 + 1.3 $ZnSO_4$.

the nutritional content of the seeds, in which the combinations of B and Zn increased, causing significant reduction of dolomite lime (Ca and Mg) adhesiveness and of the micronutrients B and Zn, consequently influencing the quality of the coating.

The low adhesiveness of the material added to the coating layer may be linked to the chemical scarification to which the seeds were submitted earlier. It is believed that the sulfuric acid (H_2SO_4) responsible for taking down the impermeable barrier of the seed coat turned its surface smooth, not permitting a better coating. However, this hypothesis needs to be verified.

The granulometry of the material used throughout the coating process may also have influenced its quality. The zinc sulphate has bigger, heavier particles, while the dolomite lime and the charcoal both have finer textures; boric acid sits in the middle. During the coating process materials of finer granulometry should be preferred in the layers closer to the nucleus, so an enhancement of the seed's contact surface and weight is assured (Silva and Nakagawa, 1998). This way, it is believed that the methodology utilized in this work favoured the adherence of zinc sulphate, which was not capable of keeping the surface of the layers even, by virtue of its crystalized shape, thus reducing the adhesiveness of the other materials (Figure 2).

Another factor related to the quality of the coating is the water content of the seeds after they are coated, and in Figure 3 it is noted a certain reduction of humidity in the coated seeds, showing that the utilized materials did not absorb water. It also became clear that the drying made inside the coater during the coating was efficient, providing the removal of the water applied through the glue on the formation of the coating layers, avoiding absorption by the seed.

The coating of seeds with or without micronutrients was harmful to the normal seedlings formation and to the germination speed, being this effect progressive as the combinations of B and Zn were enhanced (Figures 4 and 5). A similar result was found by Pessoa et al. (2000), who observed an increase of heterogeneity and a delay in germination, and still a low initial development of plants when tested increasing doses of B in corn seeds. Yagi et al. (2006) also verified that the application of zinc sulfate in sorghum seeds in a dose of 28.56 g.kg of seeds resulted in a minor percentage of germination. However, Tavares et al. (2013) and Pletsch et al. (2014) using in the recoating of seeds doses of commercial products that possessed 780 $g.L^{-1}$ of Zn in wheat seeds and 780 $g.L^{-1}$ of Zn in canola seeds, respectively, observed that the treatments provided benefits for the germination and initial establishment of plants. Nonetheless, Ohse (2000), in irrigated rice seeds, observed that the combination of 0.67 $g.kg^{-1}$ of zinc sulfate and 0.065 $g.kg^{-1}$ of boric acid did not affect the germination, although it increased the number of abnormal seedlings, not being indicated by the author due to the diminishment caused in the seed strength.

However, it is a belief that negative results for germination are connected to the methodology used to execute the test recommended for non-coated seeds, when the seeds are disposed in the germination box,

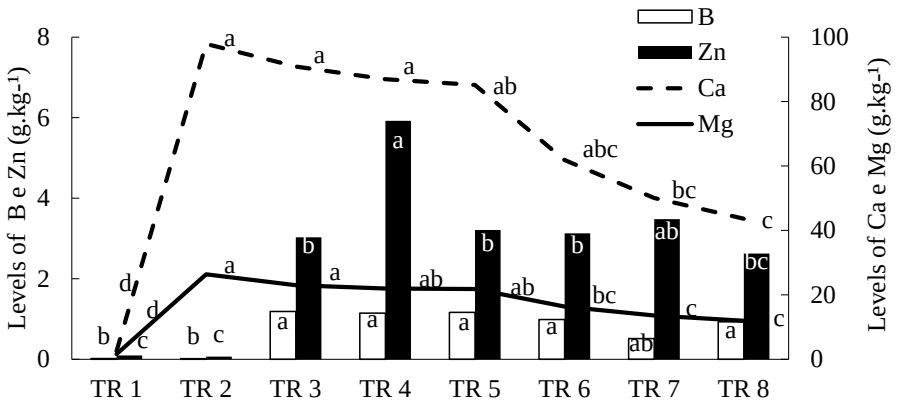

Figure 2. Levels of Boron, Zinc, Calcium and Magnesium present in seeds after coating. Treatments ($g.kg^{-1}$ of seeds): TR1: Uncoated; TR2: Without micronutrients; TR3: 0.8 H_3BO_3 + 0.8 $ZnSO_4$; TR4: 1.0 H_3BO_3 + 0.9 $ZnSO_4$; TR5: 1.2 H_3BO_3 + 1.0 $ZnSO_4$; TR6: 1.4 H_3BO_3 + 1.1 $ZnSO_4$; TR7: 1.6 H_3BO_3 + 1.2 $ZnSO_4$; TR8: 1.8 H_3BO_3 + 1.3 $ZnSO_4$.

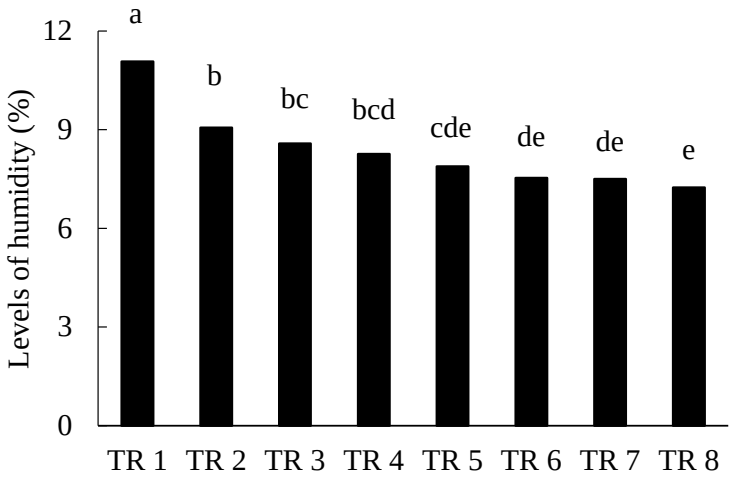

Figure 3. Levels of humidity in seeds after coating. Treatments ($kg.kg^{-1}$ of seeds): TR1: Uncoated; TR2: Without micronutrients; TR3: 0.8 H_3BO_3 + 0.8 $ZnSO_4$; TR4: 1,0 H_3BO_3 + 0.9 $ZnSO_4$; TR5: 1.2 H_3BO_3 + 1.0 $ZnSO_4$; TR6: 1.4 H_3BO_3 + 1.1 $ZnSO_4$; TR7: 1.6 H_3BO_3 + 1.2 $ZnSO_4$; TR8: 1.8 H_3BO_3 + 1.3 $ZnSO_4$.

which benefits the salt concentration present in the recoating after being put in a soluble state, altering the hydric potential of the substrate, creating an inhospitable condition for the seeds, on the higher doses of B + Zn (Figure 4) (Xavier, 2015).

As a consequence of substrate hydric potential alteration, there is a significant number in TR3 (41%), TR4 (39%) and TR5 (37.5%) of seeds which did not conclude the germination process, seeds that soaked but did not complete the third step of germination with the radicle development (Marcos Filho, 2015).

In addition to these conditions influencing negatively the germination, recoated seeds soak more slowly due to the necessity of rupture of more than one physical barrier to initiate the germination process (Derré et al., 2013), influencing directly the IVG (Figure 5).

This work's seed treatment goal was to transform them into seeds rich in B and Zn nutrients, to subsequently be made available for plants. However, the combination of 1.6 kg of $H_3BO_3.kg^{-1}$ of seeds + 1.2 kg of $ZnSO_4\,kg^{-1}$ of seeds (TR7) and 1.8 kg of $H_3BO_3\ kg^{-1}$ of seeds + 1.3 kg of $ZnSO_4\ kg^{-1}$ (TR8) was toxic for seeds, providing a bigger percentage of dead seeds in the germination box (Figure 4).

As apposed from the lab results, when seeds were sowed in sand and disposed in a greenhouse, there was an increase in the percentage of emerged seedlings compared to the percentage of germination (Figure 4). It is possible to relate this fact to the condition to which the seeds were submitted, the greenhouse expresses in a

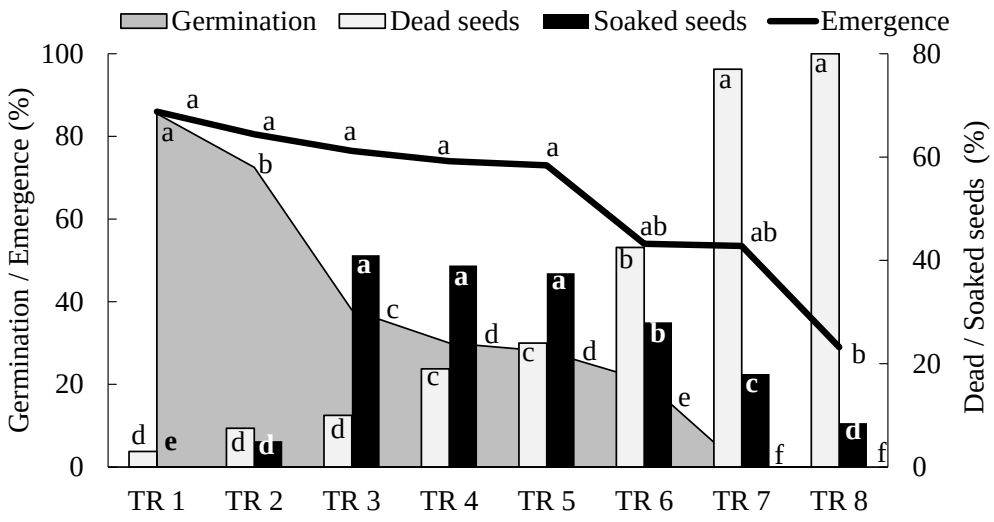

Figure 4. Germination, emergence, dead and soaked seeds rates. Treatments (Kg.Kg^{-1} of seeds): TR1: Uncoated; TR2: Without micronutrients; TR3: 0.8 H_3BO_3 + 0.8 $ZnSO_4$; TR4: 1.0 H_3BO_3 + 0.9 $ZnSO_4$; TR5: 1.2 H_3BO_3 + 1.0 $ZnSO_4$; TR6: 1.4 H_3BO_3 + 1.1 $ZnSO_4$; TR7: 1.6 H_3BO_3 + 1.2 $ZnSO_4$; TR8: 1.8 H_3BO_3 + 1.3 $ZnSO_4$.

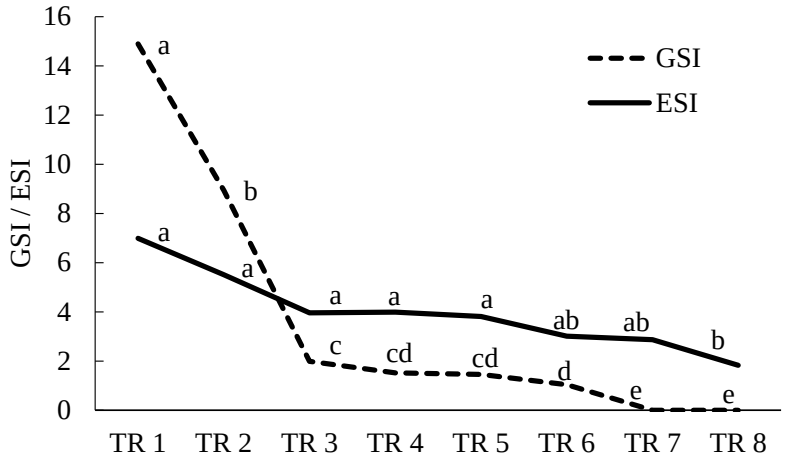

Figure 5. Germination Speed Index (GSI) and Emergence Speed Index (ESI). Treatments (Kg.Kg^{-1} of seeds): TR1: Uncoated; TR2: Without micronutrients; TR3: 0.8 H_3BO_3 + 0.8 $ZnSO_4$; TR4: 1.0 H_3BO_3 + 0.9 $ZnSO_4$; TR5: 1.2 H_3BO_3 + 1.0 $ZnSO_4$; TR6: 1.4 H_3BO_3 + 1.1 $ZnSO_4$; TR7: 1.6 H_3BO_3 + 1.2 $ZnSO_4$; TR8: 1.8 H_3BO_3 + 1.3 $ZnSO_4$.

more realistic way the field conditions, perforated trays that permit the leaching of salt and humidity excess, strengthened by the necessity of more intense irrigation, as reported by Abreu et al. (2001) and also because it is a test with longer duration (90 days). However, the effects of high doses of fertilizers are also felt in the greenhouse in the highest dose of B and Zn added to the recoating (TR8), when a significant diminishing of E and ESI was noticed (Figures 4 and 5).

Based on the evaluation done on plants, the combination of 1.8 kg of H_3BO_3 and 1.3 kg of $ZnSO_4$ kg-¹ of seeds (TR8) has shown itself harmful to the production of dry mass, this being the only treatment which differed from the control treatment (TR1) (Table 1). Thus, it is noticed that with the exception of the highest dose of B and Zn in the coating, there is no harm in the coating with B and Zn to the growth of plants related to control (TR1). These results agree with Araújo and Silva (2012), whose work with cotton trees highlights the direct connection between B and the plant development; and Funguetto et al. (2010) who highlight the Zn participation in different metabolic routes which provide growth, therefore

Table 1. Shoot Dry Mass (SDM) and Height (PHT) of perennial soybean plants (*Neonotonia wightii* cv. Common) 90 days after sowing in greenhouse sand.

Treatment (kg.kg^{-1} of seeds)(1)	SDM (g/pl)	PHT (cm)
Uncoated	0.13^{b}*	1.20^{ab}
Without micronutrients	0.12^{ab}	1.21^{ab}
0.8 H_3BO_3 + 0.8 $ZnSO_4$ (1)	0.11^{ab}	1.23^{a}
1.0 H_3BO_3 + 0.9 $ZnSO_4$	0.12^{ab}	1.27^{ab}
1.2 H_3BO_3 + 1.0 $ZnSO_4$.	0.13^{ab}	1.09^{ab}
1.4 H_3BO_3 + 1.1 $ZnSO_4$.	0.11^{ab}	1.09^{ab}
1.6 H_3BO_3 + 1.2 $ZnSO_4$	0.10^{bc}	1.04^{b}
1.8 H_3BO_3 + 1.3 $ZnSO_4$	0.08^{c}	1.03^{b}
Average	0.16	1.14
CV (%)	36.62	7 .22

*Average followed by the same letter do not differ statistically among themselves by Tukey test ($p<0.05$).

Table 2. Root Length (RL), Root Dry Mass (RDM), Ramification Number (RN) and Total Root Size (TRS) of perennial soybean plants (*Neonotonia wightii* cv. Common) 90 days after sowing in the greenhouse sand.

Treatment (kg.kg^{-1} of seeds) (1)	RL (cm)	RDM (g/pl)	RN	TRS (cm)
Uncoated	10.97^{a}*	0.36^{ab}	74.41^{a}	89.43^{a}
Without micronutrients	11.29^{a}	0.38^{a}	81.08^{a}	97.16^{a}
0.8 H_3BO_3 + 0.8 $ZnSO_4$ (1)	10.86^{a}	0.27^{ab}	75.88^{a}	88.07^{a}
1.0 H_3BO_3 + 0.9 $ZnSO_4$	10.77^{a}	0.29^{ab}	76.25^{a}	89.64^{a}
1.2 H_3BO_3 + 1.0 $ZnSO_4$.	10.61^{a}	0.29^{ab}	73.79^{a}	91.11^{a}
1.4 H_3BO_3 + 1.1 $ZnSO_4$.	10.43^{a}	0.28^{ab}	78.03^{a}	94.41^{a}
1.6 H_3BO_3 + 1.2 $ZnSO_4$	10.29^{a}	0.26^{b}	67.00^{a}	82.51^{a}
1.8 H_3BO_3 + 1.3 $ZnSO_4$	9.96^{a}	0.26^{b}	75.54^{a}	97.86^{a}
Average	10.65	0.30	75.25	91.27
CV (%)	6.77	16.63	13.5	13.67

* Average followed by the same letter do not differ statistically among themselves by Tukey test (0,05%).

influencing the plant's active photosynthetic area. Nevertheless, Albuquerque et al. (2010) report the negative influence of Zn to the plants growth when it is found in high amounts in the environment.

Regarding the roots production, which are important for growth parameters due to its importance in the absorption of nutrients and water, there was no significant effect of treatments for variables, root length, root dry mass, number of ramifications, total root size (Table 2). Also, Ohse et al. (2000), who tested in irrigated rice seeds the maximum dose of 0.67 g of Zn. kg-¹ of seeds and did not observe significant increments on the production of root dry mass compared to the control.

Meanwhile Yagi et al. (2006) and Prado et al. (2008) tested in sorghum seeds doses of zinc sulfate of 114.4 and of 28.56 g of Zn. kg^{-1} of seeds, respectively, and observed a significant decrease on the production of root dry mass. The toxicity symptoms of Zn in plants include the inhibition of radicular lengthening (Marschner, 1995), which was not observed in a significant way on the Table 2.

Perennial soybean plants developed in the greenhouse have shown an additional accumulation of B and Zn micronutrients when originated from coated seeds, accumulated in the aerial part, as well as in the roots. According to Marschner (1995), Zn accumulation in roots may have been caused by the accumulation of nutrients on its proximities, which is more common when the transport of nutrient happens by diffusion and it occurs when its supply rate is higher than its absorption, coherent with the situation found in this work (Figure 6).

The treatments which were prominent by the accumulation of B on the aerial part were the same treatments with bigger accumulation of Zn, also on the aerial part: TR4, TR7 and TR8, which presented a gain compared to the control treatment (TR1), of 66.55;

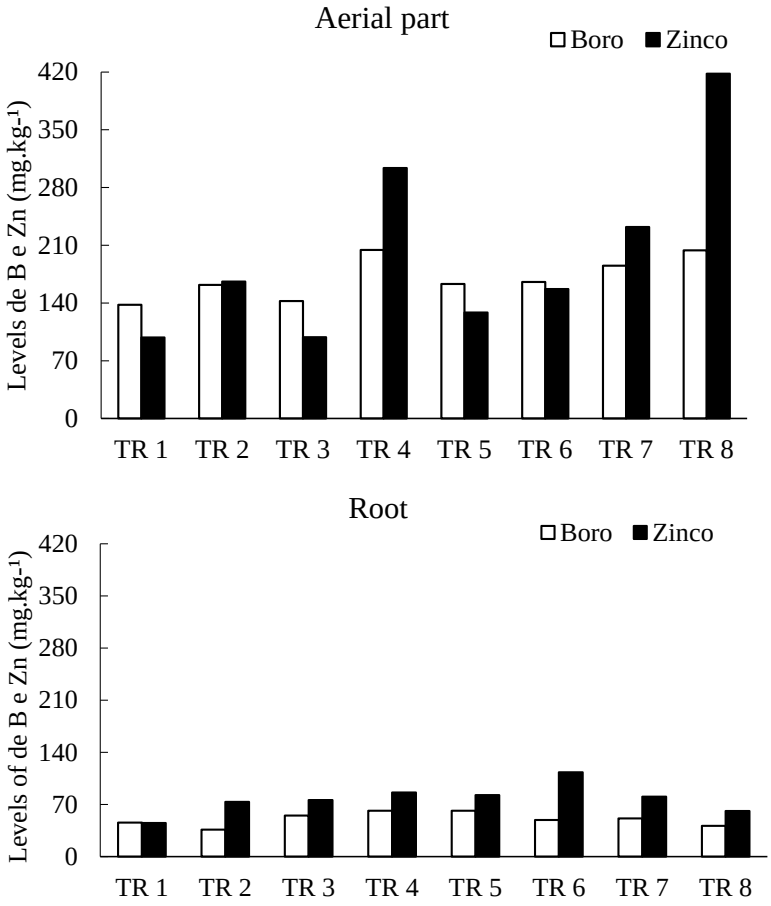

Figure 6. Levels of boron and zinc in the aerial part and root in perennial soybean plants (*Neonotonia wightii* cv. Common) 90 days after sowing seeds in the greenhouse sand. Treatments ($Kg.Kg^{-1}$ of seeds): TR1: Uncoated; TR2: Without micronutrients; TR3: 0.8 H_3BO_3 + 0.8 $ZnSO_4$; TR4: 1.0 H_3BO_3 + 0.9 $ZnSO_4$; TR5: 1.2 H_3BO_3 + 1.0 $ZnSO_4$; TR6: 1.4 H_3BO_3 + 1.1 $ZnSO_4$; TR7: 1.6 H_3BO_3 + 1.2 $ZnSO_4$; TR8: 1.8 H_3BO_3 + 1.3 $ZnSO_4$.

47.33; 66.00 mg of B per kg of the plant dry mass and 205.43; 133.91; 319.8 mg of Zn per kg of the plant dry mass, respectively (Figure 6). On these same treatments, different from the others, it is always observed a higher concentration of Zn than of B; and the maximum absorption of Zn, regarding TR8, gets to be two times bigger than the absorption of B in the same treatment, confirming that the nutritional interactions interfere on the plant mineral composition, one element being able to stimulate or inhibit the absorption of another element (Araújo and Silva, 2012).

Thus, on the results of other treatments, when absorption of B absorption was higher, the capacity of absorption of Zn was decreased. Ohse (2000), in his work, noticed a possible antagonistic effect between Zn and B, as well as between B and Cu; however, Hosseini et al. (2007) reported a significant interaction between B and Zn on corn plants growth, with a synergetic effect among these nutrients. Lefebre et al. (2002) report that the Zn response in function of the concentration of B depends on the analyzed organ, because when analyzing tobacco plants the effect on leaves was the opposite of what was found on the root; in other words, the decrease of Zn levels in function of the increase of concentrations of B. Ziaeyan and Rajaie (2009) also observed the diminishing of Zn concentration in corn leaves with an increase of B concentration. The different responses can occur based on the organs and species analyzed.

On the basis of the levels of B and Zn on the aerial part of the plants, the minimum level was 137.85 and 98.2 $mg.kg^{-1}$ and the maximum was of 204.40 and 418 $mg.kg^{-1}$, respectively (Figure 6). In Yamada's work (2004), the adequate level of micronutrients in the foliar analysis was listed for some crops in the *cerrado* region (Brazilian savanna). Among them, it is the perennial soybean that is considered an adequate amount of 30-50 $mg.kg^{-1}$ of B and 20-50 $mg.kg^{-1}$ of Zn. However, Fageria (2000a, b), regarding beans and soybean plants, which are from the same family, observed as ideal levels for these plants an amount of 10 - 55 $mg.kg^{-1}$ of B and 21 - 35 $mg.kg^{-1}$ of Zn.

There are few data with toxicity values for micronutrients, but Fageria (1992) reported values referring to the toxicity of zinc in the aerial part of annual crops, with higher levels than 400 mg kg^{-1} of dry mass. The outcomes of the nutritional analysis performed in this work were superior to those reported by these authors, including the control treatment (TR1). Still, it is not possible to compare these results directly, due to the different environment conditions in which the plants were developed and due to the evaluation criteria used by each author to perform the analysis; Besides, *fabaceae* species differ in nutritional efficiency, being identified variations on the absorption efficiency, translocation and utilization of macro and micronutrients related to the species and/or cultivars (Vieira, 2013).

Conclusions

1) The coating with a dosage of 0.8 kg of H_3BO_3 + 0.8 kg of $ZnSO_4$ kg^{-1} seed (TR3) provides greater weight and diameter of the seeds. All treatments increase the minimum diameter and decrease the humidity of seeds, but they are not capable of changing their irregularity of contour.
2) The combination of 1.8 kg of H_3BO_3 + 1.3 kg of $ZnSO_4$ kg^{-1} seed (TR8) decreases shoot dry mass production, whereas other treatments do not affect plant growth variables.
3) Perennial soybean plants absorb and accumulate micronutrients added to the seed coating.

Conflict of Interests

The authors have not declared any conflict of interests.

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Agrobacterium-mediated transformation of *Jatropha curcas* leaf explants with a fungal *chitinase* gene

Mariana Crotti Franco[1], Kleber Alves Gomes[1], Marcel Mamede de Carvalho Filho[1], Ricardo Harakava[3], Nicolas Carels[4], Walter José Siqueira[1], Rodrigo Rocha Latado[2] and Daniela de Argollo Marques[1]*

[1]Centro de P&D de Recursos Genéticos Vegetais, Instituto Agronômico, Caixa Postal 28, Campinas, SP, 13075-630, Brazil.
[2]Centro APTA Citros Sylvio Moreira, Instituto Agronômico, Caixa Postal 04, Cordeirópolis, SP, 13490-970, Brazil.
[3]Instituto Biológico, Secretaria de Agricultura e Abastecimento, Laboratório de Bioquímica Fitopatológica, Av. Conselheiro Rodrigues Alves, 1252, Vila Mariana, 04014002 - São Paulo, SP – Brazil.
[4]Fundação Oswaldo Cruz, Laboratório de Modelagem de Sistemas Biológicos, CDTS, Av. Brasil, 4365, Manguinhos, 21040-900 - Rio de Janeiro, RJ – Brazil.

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***Jatropha curcas* L. oil has been shown to be suitable for the production of biodiesel. However, this species has not been domesticated yet. Genetic breeding through conventional methods is time consuming and costly, hence, genetic transformation could contribute positively to the improvement of interesting traits. Although *in vitro* regeneration and stable genetic transformation has been pursued for several years, variation in transformation efficiency remains strongly genotype-dependent and indicates that protocols optimization is still needed. Thus, this study was carried out to introduce a *chitinase* gene from the *Trichoderma viride* fungus into the genome of a *J. curcas* superior genotype by inoculating leaf explants with *Agrobacterium tumefaciens* EHA 105 strain in axenic conditions. Some key parameters such as pre-culture period and antibiotic doses were optimized with 500 mg.L^{-1} cefotaxime and 100 mg.L^{-1} kanamycin concentrations being suitable for *A. tumefaciens* inhibition and explant selection, respectively. The best transformation efficiency (50%) was obtained when leaf explants were incubated on a culture medium promoting shoot regeneration at 15 days before the induction of the transformation process. Plants where *chitinase* gene amplicons could be detected were assessed for transgene copy number and expression levels by quantitative real-time polymerase chain reaction (PCR). One, two and three copies of the introduced gene were confirmed in nine transgenic lines with two of them that were assessed for gene expression and showed quantitative variation for this variable. These results bring valuable information for further gene insertions in breeding programs of *J. curcas* for fungal disease resistance.**

Key words: *Agrobacterium tumefaciens*, pre-culture time, kanamycin, quantitative real-time polymerase chain reaction (PCR), transgene copy number.

INTRODUCTION

Considering the importance of renewable energy sources to sustain the global energy matrix, biofuels are among the available alternative to mitigate the current energy crises and climate changes (Divakara et al., 2010; Varshney and Johnson, 2010). In Brazil, nowadays, the blend of 7% biodiesel into fossil oil (B7) is already mandatory; it is scheduled to reach 10%, until 2019 and even 20%, until 2030 (Giersdorf, 2013). The progressive increase in commercial diesel blend on the market has resulted in positive environmental, economic and social impact. However, it will also require urgent oil sources diversification to sustain the growing demand for biodiesel.

Due to its high oil content and quality, *Jatropha curcas* L., has been proposed as a promising plant source to diversify biodiesel and biokerosene production in tropical climates (Carels, 2013; Sharma, 2011; Li et al., 2007). Despite its potential, *J. curcas,* also known as physic nut, is a perennial and semi-wild species, which still needs years of selective breeding until the release of a stable cultivar to the world. Plant domestication and genetic improvement process could be sped up by the integration of biotechnology approaches to the conventional breeding process for the introduction of agronomical desirable traits such as oil content and quality, synchronous fruit maturity, early flowering and disease or pest resistance. Although considered as a stress resistant species (Openshaw, 2000; Debnath and Bisen, 2008; Sabandar et al., 2013), physic nut has proven to be highly susceptible to a wide array of pest and disease when it is raised as a monoculture, which may limit its seed and oil yields (Kumar et al., 2013; Sujatha, 2013; Argollo Marques et al., 2013).

The domestication process through selective breeding is a purifying process that leads to the reduction of genetic variability by capturing both additive and non-additive effects in elite clones via selection and clonal propagation (Carels, 2013). Despite some variations in agro-morphological and chemical properties reported in different studies (Wani et al., 2006; Kaushik et al., 2007; Rao et al., 2008; Argollo Marques et al., 2013), only a limited number of traits has been observed in the *J. curcas* genotypes evaluated so far. The major part of these traits involves genes engaged in mechanisms of resistance to biotic and abiotic stresses (Argollo Marques et al., 2013).

The genetic base broadening can be obtained by interspecific hybridization and/or biotechnological approaches. The introgression of stress resistance genes as well as other favorable genes found in wild congeneric species into *J. curcas* may be a promising breeding strategy (Sujatha et al., 2013; Argollo Marques et al., 2013), however, it is time consuming, expensive and limited to the closest wild species (Guidolin, 2003). Furthermore, undesirable genes are inevitably co-inherited and need cycles of backcrossing to be eliminated. In turn, the genetic engineering strategy has many advantages such as the shortening of the number of selective breeding steps and the ability to introduce genes for traits that may not be even available within congeneric species (Visarada et al., 2009; Herr and Carlson, 2013; Fu et al., 2015). However, to be successful, the genetic transformation process depends from the availability of an efficient and reproducible *in vitro* regeneration system. To date, plant regeneration protocols have been developed using various types of explants (Wei et al., 2004; Jha et al., 2007; Deore and Johnson, 2008; Kumar and Reddy, 2010; Kumar et al., 2010; Sharma et al., 2011; Toppo et al., 2012; Franco et al., 2014). Based on these regeneration systems, stable transformation procedures have been developed employing both *Agrobacterium tumefaciens* (Li et al., 2008; Kumar et al., 2011; Pan et al., 2010; Misra et al., 2012; Fu et al., 2015; Nanasato et al., 2015) or particle bombardment (Joshi et al., 2011; Purkayastha et al., 2010).

Due to little transgene re-arrangement, low copy number and preferential integration into transcriptionally active regions of chromosomes, *Agrobacterium*-mediated transformation is the most widely used method to generate transgenic *J. curcas* (Kumar et al., 2013; Fu et al., 2015). Li et al. (2008) first reported *J. curcas* genetic transformation via *A. tumefaciens* infection of cotyledon disc using the phosphinothricin herbicide as selective agent, however, the transformation efficiency was low (13%). Recently, Nanasato et al. (2015) improved the rate of cotyledon transformation efficiency to 23.3% using vacuum infiltration for the step of bacterial inoculation and co-culture on filter-paper wicks instead of agar for the step of latency before shoot regeneration. Fu et al. (2015) further increased the rate of transformation efficiency to 56% by optimizing kanamycin concentration in the selective medium and the duration of the selection process. Despite their higher potential of *in vitro* shoot regeneration, juvenile explants such as cotyledons and hypocotyls may results in a higher rate of unexpected somatic variation (Bhatia et al., 2005). Due to the smaller rate of somatic variation in regenerated shoots obtained

*Corresponding author. E-mail: d-argollo@iac.sp.gov.br.

from sporofitic explants such as stem and leaves, these tissues should be preferred for transformation (Kumar et al., 2010; Misra et al., 2012). Zong et al. (2010) using leaf explants, kanamycin for selection medium and a 2 days pre-culture period, increased the rate of transformation efficiency to 23.91%. Kumar et al. (2010) using hygromycin as another selective agent with leaf explant and a pre-culture period of 4 days achieved an even greater efficiency of 29%. Despite these efforts, large variations among different *Jatropha* genotypes in terms of their morphological responses to phytohormones is still observed *in vitro* and the rate of transformation efficiency of differentiated tissues still varies between 4 and 56% (Basa and Sujatha, 2009; Fu et al., 2015). Thus, the optimization of the regeneration and transformation procedures need to be conducted according to their genetic profile, which makes sense at least for a small number of high-yielding elite genotypes that have been recently selected by conventional breeding programs (Basa and Sujatha, 2009).

Currently, efficient *in vitro* regeneration protocols were developed using foliar explants for cloning elite genotypes selected under the breeding programs of Instituto Agronômico of Campinas (IAC) in Brazil (Franco et al., 2014). High regeneration rates (~40 shoots per explant) and efficient rooting via micrografting (85%) were obtained for the L4P49 genotype. The micro-propagated shoots were considered genetically stable after assessing their ploidy by flow cytometry and their DNA polymorphism by marker-tartrate-resistant acid phosphatase (TRAP), indicating preservation of clonal stability and phenotype uniformity. The subsequent adaptation of this regeneration protocol to an effective and reproducible *Agrobacterium*-mediated transformation protocol with optimization of some key transformation parameters such as pre-culture period and antibiotics concentration was the purpose of the present report. Then, the combined transformation and regeneration protocol was applied to transfer a fungal *chitinase* gene into the genome of a *J. curcas* elite plant to confer fungal disease resistance.

MATERIALS AND METHODS

Plant

Young apical and fully developed leaves were collected from plants of L4P49, a high fruit and oil yielding accession of *J. curcas* selected as an elite genotype by IAC. The leaves were surface sterilized for 15 min with 2.5% sodium hypochlorite (NaClO) solution and rinsed three times with sterile distilled water. Foliar segments (5 × 5 mm) were excised and cultured on a shoot regeneration medium (RM), composed by the salts and vitamins of Murashige and Skoog (1962) and denoted here as MS, which were supplemented with myo-inositol (100 $mg.L^{-1}$), cysteine (10 $mg.L^{-1}$), glutathione (25 $mg.L^{-1}$), sucrose (30 $g.L^{-1}$), hydrolyzed casein (0.5 $g.L^{-1}$), copper sulfate (6 $mg.L^{-1}$), indole-3-butyric acid (IBA, 0.2 $mg.L^{-1}$), thidiazuron (TDZ, 1 $mg.L^{-1}$), and phytagel (2.4 $g.L^{-1}$).

Regenerated shoots were elongated on MS medium supplemented with 6-benzylaminopurine (BAP, 0.3 $mg.L^{-1}$) and IBA (0.1 $mg.L^{-1}$). The pH of all medium was adjusted to 5.8, prior to sterilization (121°C for 20 min). The elongated shoots of 1.5 cm length after 30 days old cultures were used as a source of foliar explants for testing kanamycin resistance for further.

All cultures were maintained at 25 ± 2°C under a 16/8 h (day/night) photoperiod with light provided by cool white fluorescent lamps at an irradiance of 35 to 40 µmol m^{-2} s^{-1}.

Agrobacterium strain and binary vector

A cDNA of the *ech42 chitinase* gene was amplified by quantitative amplification of reversed transcripts by polymerase chain reaction (qRT-PCR) from *Trichoderma viride* grown on media supplemented with colloidal chitin. The *ech42 chitinase* gene was initially cloned in pGEM-T Easy (Promega) and completely sequenced. Subsequently, it was sub-cloned in pCAMBIA 1201 using the NcoI and BstEII restriction sites, substituting the *uidA* (GUS) gene, and transferred to pCAMBIA 2201 using the EcoRI and BstEII sites.

The *ech42* gene is under the control of a CaMV 35S promoter in Pcambia 2201 vector and the resistance to kanamycin for transgenic plant selection is conferred by a *neomycin phosphotransferase II* gene (*nptII*). The pCAMBIA 2201 vector (Figure 1) was introduced by electroporation into the disarmed *A. tumefaciens* EHA105 strain.

A. tumefaciens strain containing a binary vector pCAMBIA 2201 were inoculated in solid LB (Miller, 1972) medium supplemented with kanamycin (100 $mg.L^{-1}$) and rifampicin (100 $mg.L^{-1}$) under 28°C for 72 h. A single colony of the transformed bacteria was isolated and inoculated in liquid LB medium supplemented with kanamycin (100 $mg.L^{-1}$). Bacterial cultures were grown at 200 rpm and 28°C for 16 h. The bacterial suspension was centrifuged at 3,000 rpm for 15 min and the cell pellets were re-suspended in sterile distilled water before further use.

Determination of phytotoxic level of antibiotics

The *A. tumefaciens* EHA105 strain was inoculated in LB medium containing cefotaxime at 0, 300, 400 and 500 $mg.L^{-1}$ and maintained in biochemical oxygen demand (BOD) at 28°C. After five days, the bacterial growth was evaluated in order to determine the minimum dose necessary for total growth inhibition.

The phytotoxic level of kanamycin was determined in order to find the optimal dose that should be used in selective medium. Two types of untransformed foliar explants were cultured separately on RM including an inhibitory dose of cefotaxime previously identified as 500 $mg.L^{-1}$ and kanamycin at 0, 25, 50, 75 and 100 $mg.L^{-1}$. The first type of foliar explants consisted of leaf sections from shoots grown *in vitro* (called *in vitro* leaf explants) and the second was constituted by leaf segment taken from *in vivo* cuttings, grown in a greenhouse (called *in vivo* leaf explants). All cultures were maintained at 25±2°C and 16/8 h (day/night) photoperiod. Both antibiotics were filter-sterilized (Millipore ® 0.22 µm pore size) and added to media after autoclaving. The control medium was RM without antibiotic (cefotaxime and kanamycin). To assess the threshold of kanamycin toxicity, an experimental scheme of five plates per treatment and six explants per plate were used. Three repetitions of the test of kanamycin toxicity assessment were performed independently. The assessment of kanamycin toxicity was obtained through the relative number (%) of bleached explants after six weeks of subculture. The rate of shoot regeneration was calculated as the average number of regenerated shoots per explant.

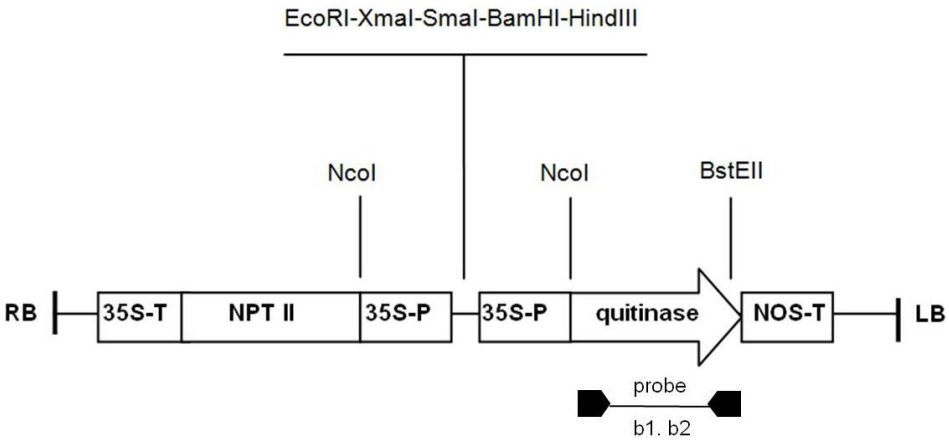

Figure 1. Expression cassette of the vector (pCAMBIA 2201 – *ech 42*). RB: right border of T-DNA; LB: left border of T-DNA; 35S-P: CaMV 35S promoter; 35S-T: CaMV 35S terminator; NOS T: nopaline synthase terminator.

Genetic transformation and effect of pre-culture time

Leaf explants were pre-cultures on RM during 0, 2, 4, 6, 8, 12 and 15 days prior to *Agrobacterium* inoculation in order to find the optimal conditions for the infection process. Explant inoculations were carried out by immersion in a bacterial suspension (OD_{600}=0.8) for 20 min. Then, explants were blotted dry on sterile paper and cultured on solid MS medium (7 $g.L^{-1}$ agar) without antibiotic during three days under total darkness at 24 ± 1°C. Then, explants were transferred to a selective medium, which was RM supplemented with cefotaxime (500 $mg.L^{-1}$) and kanamycin (100 $mg.L^{-1}$), to induce callus and shoot regeneration. Explants were kept for 4 weeks under 16 h photoperiod (35 to 40 µmol m^{-2} s^{-1} irradiance) at 25 ± 1°C and transferred to fresh media every 15 days until shoot regeneration. Shoots that survive on selective medium (with kanamycin) were transferred to solid MS (7 $g.L^{-1}$ agar) with cysteine (10 $mg.L^{-1}$), reduced glutathione (25 $mg.L^{-1}$), hydrolyzed casein (500 $mg.L^{-1}$), copper sulfate (6 $mg.L^{-1}$), adenine sulfate (50 $mg.L^{-1}$), sucrose (30 $g.L^{-1}$), agar (7 $g.L^{-1}$), BAP (0.3 $mg.L^{-1}$), IBA (0.1 $mg.L^{-1}$), and cefotaxime (500 $mg.L^{-1}$). Each treatment included 36 leaf explants.

After six weeks, the rate (%) of kanamycin-resistant shoots that overcame 1.5 cm was calculated relative to the total number of inoculated leaf explants. The transformation efficiency was estimated on the basis of one kanamycin-resistant shoot per explant as the ratio between the number of shoots with *ech42* amplicons (PCR^+) relative to the total number of inoculated explants.

Confirmation of transgenic shoots by PCR

Transformation was confirmed by PCR amplification using total genomic DNA extracted according to Doyle and Doyle (1990). DNA concentration was measured using a Nano Drop spectrophotometer (NanoVue plus-GE). Specific primers for the *ech42* gene (F: 5' CACTTCACCATGTTGGGCTTCCTC 3' and R: 5' GATCTCTAGTTGAGACCGCTTCGG 3') were obtained using Primer3 (Rozen and Skaletsky, 2000) and expected to produce an amplicon of 1.3 Kb. The amplification reaction was conducted on a Bio Rad T100™ thermocycler in a final volume of 25 µl with 50 ng of each DNA sample in 4.0 µl buffer reaction, 1.2 µl $MgCl_2$ (25 mM), 0.4 µl dNTP mix (2.5 mM of each), 0.5 µl of each primer F and R (10 µM), 0.25 µl of Taq DNA polymerase (Thermo Fisher Scientific). The amplification started at 95°C for 4 min, followed by 30 cycles of 30 s at 95°C, 30 s at 62°C and 2 min at 72°C, followed by a final extension of 7 min at 72°C. PCR products were separated on 1% agarose gel electrophoresis (0.5X TBE, 110 V) and visualized on Alpha Imager HP (Cell Biosciences) using GelRed staining.

Estimation of transgene copy number by qRT-PCR

Nine putative transgenic primary shoots were analyzed by qRT-PCR using SYBR green to estimate the number of *ech42* copies inserted in the *J. curcas* genome. Standard curves were prepared for the *ech42* transgene and endogenous *JcKASIII* gene using different dilution of genomic DNA: 75.0, 37.5, 18.75, 9.37, and 4.68 ng. Triplicates of PCR amplifications were performed in a final volume of 20 µl containing: 1 µl DNA, 4 µl primer mix (forward and reverse - 200 nM each), 5 µl H_2O milli Q (Ambion) and 10 µl SYBR® Green master mix (Life Technologies, USA). qRT-PCR experiments were performed using *ech42* primers (F: 5'-AGAACGGTATCTGGGACTACAAGGT-3' and R: 5'-GTAGTACGCCTGTGC GACAGAGT-3'; amplicon size: 80 bp) as well as primers (F: 5' GCACTTGGCTGCAAAACAAAT 3' and *JcKASIII* R: 5' CGTCCAGTCAACATATCGAG 3'; amplicon size: 174 bp) for *JcKASIII*, which is present in a single copy in the *J. curcas* genome according to Jha et al. (2013).

Amplifications were performed using the following program: 55°C for 5 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min, followed by a dissociation step (95°C - 15 s, 60°C - 30 s, and 95°C - 15 s) and repeated twice independently with three replicates each. The estimation of *ech42* copy number was made according to Mason et al. (2002) by comparing its standard curves plotted using threshold cycle (Ct) values relative to those of *JcKASIII* in control and transgenic plants. The r1 coefficient (virtual calibrator) was calculated for *ech42* using data from all transgenic and control plants, as discussed by Mason et al. (2002).

Analysis of gene expression using qRT-PCR

For gene expression analyses, total RNA was extracted from two

Table 1. Effect of antibiotics (cefotaxime and kanamycin) on explants of *J. curcas* after six weeks of subculture.

Explant type/treatment	Bleaching (%)	Callus (%)	Shoots (%)	Number of shoots per explants[1]
In vitro				
Control	15.6	100.0	41.6	1.8^{cd}
T1 (C^2 +$K^3$0)	17.8	100.0	63.0	3.9^{bc}
T2 (C + K25)	81.5	94.9	89.5	9.1^{a}
T3 (C+K50)	96.1	77.0	72.6	7.1^{a}
T4 (C+K75)	98.9	51.3	48.5	4.6^{b}
T5 (C+K100)	94.6	16.3	13.1	0.6^{d}
In vivo				
Control	58.8	100.0	82.5	4.4^{AB}
T1 (C^2 +$K^3$0)	32.0	100.0	76.0	13.0^{A}
T2 (C + K25)	53.3	100.0	100.0	3.2^{B}
T3 (C+K50)	43.0	31.5	23.0	1.3^{B}
T4 (C+K75)	64.0	60.0	34.0	2.0^{B}
T5 (C+K100)	84.0	14.0	10.0	0.2^{B}

[1]Values with different letters for the same explant are significantly different ($P < 0.05$, Tukey's test), CV=52.47%. [2]C: Cefotaxime (500 mg.L^{-1}). [3]K: Kanamycin doses with 0 (K0); 25 (K25); 50 (K50); 75 (K75) and 100 mg.L^{-1} (K100).

independent PCR^+ shoots randomly selected and amplified in quadruplicates and one non-transgenic plant (negative control) using PureLink™ RNA mini kit (Ambion). The RNA was quantified with a NanoDrop spectrophotometer (NanoVue plus-GE). The first-strand cDNA was synthesized according to Super Script III First Strand synthesis (Invitrogen). qRT-PCR reactions were performed using: 4 µl cDNA (10 ng/µl), 10 µl SYBR Green, 4 µl forward and reverse primers mix (final concentration 200 nM each) and 2 µl Milli-Q water. A total of four primers pairs were tested: three for *ech42* (pr1, pr2 and pr3) and one for the internal control (*actin* gene) as described subsequently: pr1 – F: 5' TGCCTACGCCGATTATCAGAA 3' and R: 5' TGCTTCACACAGCCGTATGC 3' (amplicon size: 81 bp), pr2 – F: 5' AGAACGGTATCTGGGACTACAAGGT 3' and R: 5' GTAGTACGCCTGTGCGACAGAGT 3' (amplicon size: 80 bp), pr3 – F: 5' AACGCATACGGCTGTGTGAAG 3' and R: 5' GCCACCGATAGAGAGCATAACCT 3' (amplicon size: 81 bp), *actin* gene – F: 5' GAACTGGAATGGTGAAGGCT 3' and R: 5' ACATAGGCATCCTTCTGACC 3' (amplicon size: 124 bp).

qRT-PCR reactions were carried out in the same conditions as described earlier. The expression levels were determined using the ΔCt and $2^{\Delta\Delta Ct}$ methodology (Bookout, 2003).

Statistical analysis

Experimental data were subjected to Tukey's test ($p < 0.05$) todetermine the statistical significance of the differences among the means using the Sanest Software (Machado and Zonta, 1995).

RESULTS

Antibiotic effects on explants and shoots in *in vitro* culture

Sensitivity tests revealed that the *Agrobacterium* growth was completely inhibited by 500 mg.L^{-1} cefotaxime but lower concentrations did not prevent its overgrowth; consequently, this dose was used in posterior transformation experiments.

The effect of cefotaxime (500 mg.L^{-1}) and increasing kanamycin doses was assessed separately on leaf explants from *in vitro* and *in vivo* sources. Cefotaxime seems to stimulate shoots regeneration in both explant kinds. *In vitro* leaf explant showed higher percentage of shoots production (63%) and shoots per explant (3.9) compared to control (without cefotaxime) in which shoots production was 41.6% and shoots per explant was 1.8. Similarly, the number of shoots per *in vivo* explants increased from 4.4, in the control, to 13.0 upon cefotaxime addition (Table 1).

The phytotoxic level of kanamycin was 100 mg.L^{-1} in both type of explants with a rate of shoots production almost nil after 30 days of culture: 0.6 shoots per *in vitro* leaf explant and only 0.2 shoots per *in vivo* leaf explant. Explants started to bleach after 15 days subculture, resulting in the total loss of chlorophyll (bleaching) in approximately 95 and 84% of *in vitro* and *in vivo* leaf explants, respectively (Table 1). After 30 days on kanamycin medium, all shoots became necrotic and started to die. Based on these results, RM was supplemented with 100 mg.L^{-1} of kanamycin for effective selection of transformed shoots.

Genetic transformation and effect of pre-culture time

EHA strain of *A. tumefaciens* was efficient for infecting foliar explants of *J. curcas* superior genotype L4P49

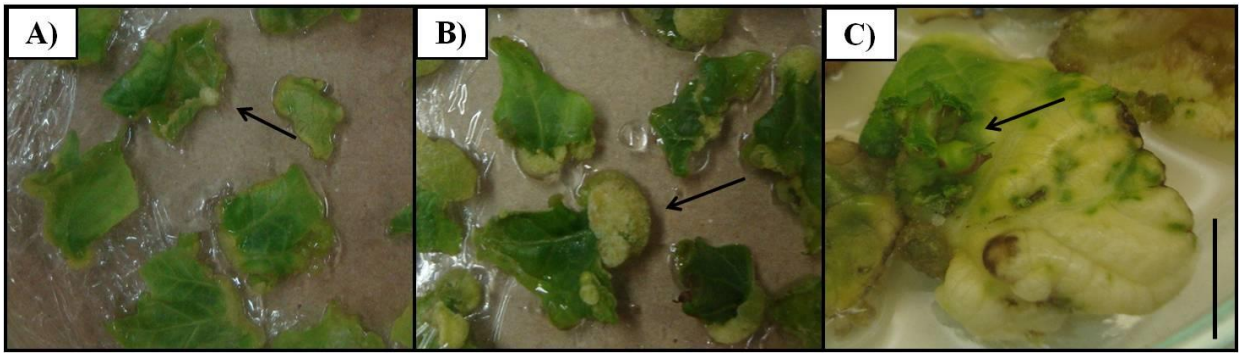

Figure 2. Aspect of explants precultured in regeneration medium (RM) during 6 (a) and 15 days (b). Arrows indicate the onset of callus formation. (c) Shoot regeneration observed after explant co-cultivation with *A. tumefaciens*. Putative transformed explants were kept on RM supplemented with 100 mg.L^{-1} of kanamycin. Bar=1 cm.

Table 2. Effect of pre-culture period on shoot regeneration and transformation efficiency.

Pre-culture period (days)	Inoculated explants	Kan^R shoots[(a)]	Regeneration rate[(b)]	PCR$^{(+)}$ shoots[(c)]	Transformation Efficiency (%)[(d)]
6	36	280	7.8	12	33.3
8	36	122	3.4	10	27.8
12	36	201	5.6	16	44.4
15	36	254	7.0	18	50.0

[(a)]Kan^R shoots (kanamycin-resistant shoots): Number of shoots that regenerated on selective medium containing kanamycin (100 mg.L^{-1}). [(b)]Regeneration rate: Kan^R shoots/total number of inoculated leaf explants. [(c)]PCR$^{(+)}$ shoots: Number of independent Kan^R shoots (one Kan^R shoot from each different explant) with positive result after PCR amplification. [(d)]Transformation efficiency (%): (number of PCR$^{(+)}$ shoots/total number of inoculated explants) × 100.

using bacterial suspension of 0.8 OD_{600}, three days co-culture period and suitable selective medium (RM medium supplemented with 500 mg.L^{-1} cefotaxime and 100 mg.L^{-1} kanamycin). Leaf explants directly infected with *A. tumefaciens* (without pre-culture) did not show any transformation and those pre-cultured on RM for two to four days before bacterial infection showed only low regeneration rate with few incompletely developed shoots unsuitable to the transformation efficiency assessment. Pre-culture time larger than six days allowed the onset of callus on the foliar explant edges (Figure 2). The explants pre-cultured on RM for 12 to 15 days prior to co-cultivation with *A. tumefaciens* gave the highest rate of callus formation resulting in larger rate of kanamycin-resistant shoot production on selective medium (Table 2). The largest transformation efficiency (50% of explants responded positively to transformation procedure) was observed when explants were pre-cultured on RM for 15 days with an average of seven kanamycin-resistant shoots per explant (Table 2).

Confirmation of transgenic shoots and estimation of transgene copy number

PCR analyses confirmed the presence of a 1.3 kb fragment equivalent to the expected amplicon size of the *ech42* gene into independent primary shoots grown on selective medium (Figure 3).

Standard curves of Figure 4 show the reaction efficiency and Ct values of each primer pairs for qRT-PCR assays as well as for calculation of copy number ratios. The evaluated amplification efficiency for the target gene (*ech42)* and for the endogenous reference gene (*JcKASIII*) was over 100% (Figure 4). The virtual calibrator (r_1 coefficient) calculated for *ech42* as described by Mason et al. (2002) was 0.9. Thus, the estimated *ech42* to *JcKASIII* ratios gave measures of the number of *ech42* copies effectively inserted in the *J. curcas* genome of each transformed plant (Table 3). Single, double and triple copies of the *ech42* gene were found in the transgenic lines evaluated.

Measures of gene expression by qRT-PCR

The experimental values of dissociation curves for *chitinase* and *JcKASIII* genes overlapped the adjusted regression lines, which demonstrate the reliability of our results. A single peak was observed in the dissociation curves, confirming the amplification of only one fragment

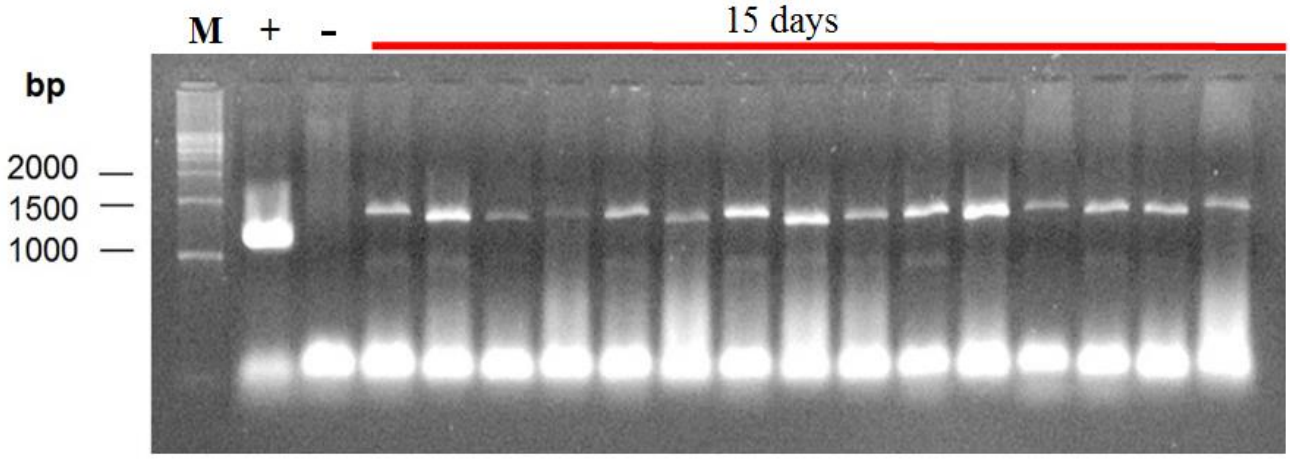

Figure 3. PCR analysis of 1000 and 1500 bp amplicons of the *ech 42* gene in transgenic plants. Lanes: *M* molecular size marker (1 Kb ladder), lane "+": positive control (plasmid DNA), lane "-": negative control (non-transgenic plant), red bar: Amplicons in putative transgenic plants from 6, 12 and 15 days pre-culture on RM.

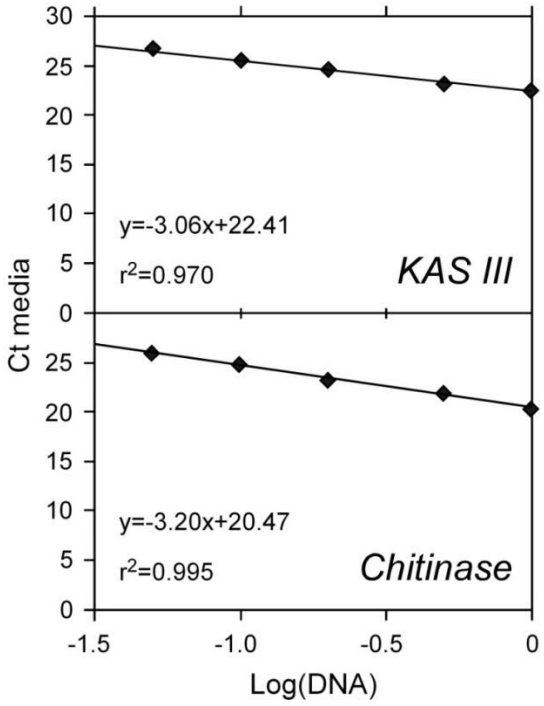

Figure 4. Plot of standard regression lines (Ct media) of *JcKASIII* (internal control) and *chitinase* genes (target) for quantities of genomic DNA corresponding to 75, 37.5, 18.75, 9.37, and 4.68 ng (Log(DNA)). Reaction efficiency percentages for *JcKASIII* and *chitinase* were 112.74% and 105.35%, respectively.

with all primers combinations analyzed for both primary shoots #12 and #23. In these two particular cases, it was found out that qRT-PCR allowed the differentiation of transgene expression levels by reference to the *actin* gene used as a control. Actually, the level of *ech42* expression observed in the #12 plant was 2 times larger than that of *actin* gene whereas it was 168 time larger than this internal control in the # 23 plant (Figure 5).

Table 3. Estimated and assumed copy number for *chitinase* gene in nine PCR$^+$ shoots.

Plant	Estimated copy number	Assumed copy number
1	0.86	1
2	1.04	1
3	1.64	2
4	0.83	1
5	0.90	1
6	0.28	0
7	0.24	0
8	0.76	1
9	2.76	3

DISCUSSION

Agrobacterium-mediated transformation requires previous determination of specific antibiotics at suitable concentration to completely inhibit bacterial growth without affecting shoot regeneration. Beta-lactam antibiotics, such as cefotaxime, are the most commonly used antibiotics in plant transformation protocols, since

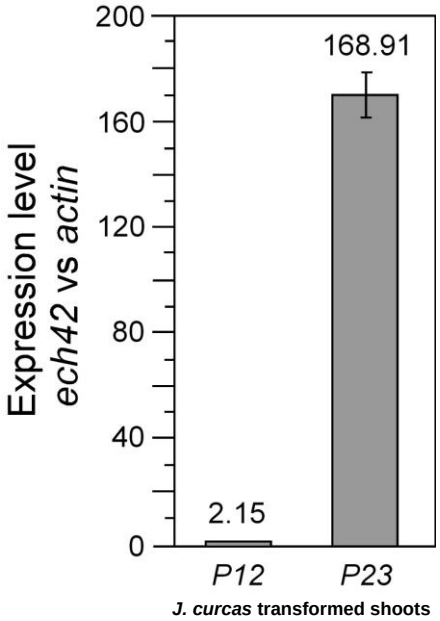

Figure 5. Expression levels of *ech42* in two *J. curcas* transformed shoots: Plant #12 and Plant #23 in relation to the *actin* gene of *J. curcas.*

they have a broad spectrum of activity against bacteria and a low toxicity to eukaryotes (Yu et al., 2001). Although inhibitory effect on shoot regeneration have already been previously reported (Tang et al., 2004; Mendes et al., 2009), stimulatory effects have also been reported for some species such as *Zea mays* (Danilova and Dolgikh, 2004) and *Citrus* (Oliveira et al., 2010). Stimulatory effect of antibiotics on plant morphogenesis may be explained by their chemical structure, which may mimic plant growth regulators (Nakano and Mii, 1993), or their degradation by-products, which may generate metabolites with plant growth regulator activity (Mathias and Mukasa, 1987).

Several antibiotics such as cefotaxime, augmentin, sporidex, and carbenicillin have been tested in *Jatropha* transformation procedure using different *Agrobacterium* strains (Kumar et al., 2010; Misra et al., 2012; Fu et al., 2015). Cefotaxime has been an excellent antimicrobial agent for suppressing *Agrobacterium,* especially EHA105 and LBA4404 strains (Kumar et al., 2010; Misra et al., 2012; Fu et al., 2015). Even if its inhibitory effect on shoot regeneration has been reported in some studies (Zong et al., 2010), cefotaxime, even at doses as high as 500 $mg.L^{-1}$, did not inhibit neither calluses induction nor shoots regeneration on cotyledon explants of *J. curcas* (Li et al., 2008). The complete elimination of *A. tumefaciens* EHA105 warrant transformed plant material free of bacterial contamination. In addition, cefotaxime at 500 $mg.L^{-1}$ was also stimulating the shoot regeneration on *in vitro* and *in vivo* leaf.

Considering bacterial concentration, preliminary results indicated that 0.8 OD_{600} was the most suitable density for transformation because it promoted the largest number of kanamycin-resistant shoots without having a negative impact on plant cells due to bacterial excess (data not shown). Bacterial inocula with larger OD_{600} resulted in a bacterial excess that inhibited callus induction and induced pH changes of the incubation media, which led to a corresponding reduction in transformation efficiency (Yong et al., 2006; Xu et al., 2009; Zong et al., 2010).

The *nptII* gene that confers kanamycin resistance was used here because of its recognized efficiency as a selectable marker of transformed cells in dicotyledonous plants including *J. curcas*, which have been found to be hypersensitive to kanamycin (Pan et al., 2010; Kajikawa et al., 2012). According to Tran and Mishra (2015), excessive doses of kanamycin may not only kill untransformed cells, but also inhibit the growth of transformed cells, thereby leading to lower transformation efficiency. Reduction of kanamycin concentration or/and delay selection strategies was necessary for transgenics of almond (Ramesh et al., 2006) and *J. curcas* (Fu et al., 2015). In other species such as rice (Tran and Mishra, 2015) and some trees (Stevens and Pijut, 2014), kanamycin selection is ineffective because of their natural cell resistance to this antibiotic.

In contrast to Purkayastha et al. (2010), Deng et al. (2005), Li et al. (2008) and Zong et al. (2010) who indicate low kanamycin concentration (<40 $mg.L^{-1}$) to select *J. curcas* transformed shoots, we did not find any noticeable kanamycin sensitivity of our *J. curcas* material at concentrations lower than 75 $mg.L^{-1}$ independently of explant kind (*in vitro* or *in vivo* leaf explants), which suggests that the *J. curcas* sensitivity to kanamycin is largely depending on its genotype.

Explant pre-culture has been reported to be necessary for transformation of several species with *A. tumefaciens* (Lawrence and Koundal, 2000; Barik et al., 2005; Xu et al., 2009). Kumar et al. (2010) reported that the transformation rate peaked in *J. curcas* (18.3%) when leaf explants were pre-cultured in regeneration medium for 4 days. Here, a positive effect of increasing the time of pre-culture was observed on RM until 15 days on kanamycin-resistant shoots production. The pre-culture of explants on media with phytohormones induces mesophyll cells to divide and form callus. Due to their particular dedifferentiation stage typical of highly dividing meristematic cells (Sangwan et al., 1992), these cells are competent for *Agrobacterium* transformation (Nagl et al., 1997; Guidolin, 2003; Ribas et al., 2011).

qRT-PCR was recently used to assess the copy number of transgenes due to its high sensitivity and also because of its requirement for only very small amounts of genomic DNA (Jha et al., 2011; Casu, 2012; Pinheiro et

al., 2014). Transgenic lines showed single, double and triple transgene insertions through qRT-PCR analysis and a difference of gene expression according to the internal control. Interestingly, no positive correlation has been found between copy number and expression of the trait associated to integrated gene(s) (Hobbs et al., 1990). Rather, it has been shown that a larger number of transgene copies resulted in a lower level of their expression (Beltrán et al., 2009; Hadi et al., 2012), while single or double copy integration tends to result in higher expression levels (Flavell, 1994; Pinheiro et al., 2014).

In conclusion, multiple kanamycin-resistant shoots (typically seven per explant) may be produced in 50% of explants using 15 days pre-culture on RM and selection with kanamycin (100 $mg.L^{-1}$). Based on our optimized procedure for the *J. curcas* accession considered, a *chitinase* gene was successfully introduced into an elite genotype selected for its high seed and oil yield by the IAC breeding program. Following the complete characterization of the gene insertion pattern as well as biological and field testing, transgenic genotypes will be backcrossed with other valuable genotypes to transfer them the fungal disease resistance trait. Furthermore, it was believed that we succeeded in identifying the key variables that may affect the transformation process according to the elite *J. curcas* genotype considered.

Conflict of Interests

The authors have not declared any conflict of interests.

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Germination, growth and physiological responses of *Senegalia senegal* (L.) Britton, *Vachellia seyal* (Delile) P. Hurter and *Prosopis juliflora* (Swartz) DC to salinity stress in greenhouse conditions

Dioumacor Fall[1,2,3]*, Niokhor Bakhoum[2,3], Fatoumata Fall[2,3,4], Fatou Diouf[2,3], Mamadou O. Ly[1], Mayécor Diouf[1,5], Djamel Gully[6], Valérie Hocher[6] and Diégane Diouf[2,3,4]

[1]Centre National de Recherches Forestières (CNRF)/Institut Sénégalais de Recherches Agricoles (ISRA), Route des Pères Maristes, BP 2312, Dakar, Sénégal.
[2]Laboratoire Commun de Microbiologie IRD/ISRA/UCAD (LCM), Centre de Recherche de Bel-Air, BP 1386, Dakar, Sénégal.
[3]Laboratoire Mixte International Adaptation des Plantes et Microorganismes Associés aux Stress Environnementaux (LAPSE), BP 1386, Dakar, Sénégal.
[4]Département de Biologie Végétale, Université Cheikh Anta Diop de Dakar, BP 5005, Dakar, Sénégal.
[5]Centre de Recherches Zootechniques (CRZ) / Institut Sénégalais de Recherches Agricoles (ISRA), BP 2330, Kolda, Sénégal.
[6]Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM)/Institut de Recherche pour le Développement (IRD) TA A-82/J, Campus International de Baillarguet, 34398 Montpellier, France.

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Salinity is among the most widespread environmental threats to global plant production, especially in arid and semi-arid climates. Thus, the selection of salt tolerant species is necessary for sustainable plant productivity. The purpose of this study was to measure and understand the salt tolerance of three multipurpose trees used in reforestation programs in many Sahelian countries (*Senegalia senegal*, Syn. *Acacia senegal*; *Vachellia seyal*, Syn. *A. seyal*, and *Prosopis juliflora*). The effect of salinity was evaluated at seed germination stage on Petri dishes containing water agar (0.9%, w/v) with seven concentrations of NaCl (0, 86, 171, 257, 342, 428, and 514 mM). Our results showed that all the species had a germination rate higher than 85% at 257 mM. However, it decreased at 342 mM with a reduction of 70 and 20%, respectively for *S. senegal* and *V. seyal*. For plants growth and physiological responses, seedlings were individually cultivated in plastic bags (25×12 cm) containing non-sterile soil and watered with four salt solutions (0, 86, 171 and 257 mM NaCl). Four months after the plants' cultivation, the results showed that for all species, the salinity reduced significantly the height, the collar diameter, the shoot and root dry biomass as well as the total chlorophyll, K^+ and K^+/Na^+ ratio. In the meantime, proline content, Cl^- and Na^+ accumulation in leaves were increased. It was also found that *S. senegal* and *V. seyal* tolerated high concentrations of NaCl (257 mM) and developed physiological and molecular mechanisms, such as salt tolerance genes (NHX1), which allow them to be considered as moderated salt tolerant species and seemed to be potential species for the restoration of salt-affected land as *P. juliflora*.

Key words: Multipurpose leguminous trees, abiotic stress, salt tolerance, Senegal.

INTRODUCTION

Soil salinization is an emerging environmental problem around the world and represents a major limiting factor for plants production and ecological environment, especially in arid and semi-arid regions (Zahran, 1999). Over 953 million ha of land are salt-affected throughout the world, covering about 8% of the world's land surface (Singh, 2009). In Senegal, 1 700 000 ha of the 3 800 000 ha of the agricultural lands are salt-affected (FAO-LADA, 2009). Numerous studies have shown that high NaCl concentrations in the growth medium of plants generate primary and secondary effects that negatively affect plant growth and development. Several physiological functions, including photosynthesis, mineral nutrition and carbohydrate metabolism have been shown to be affected by the high salinity (Chen et al., 2008). Primary effects are ionic toxicity and osmotic stress. Ionic toxicity occurs because of the high Na^+ and Cl^- concentrations in the cytoplasm of cells. The lowering of the water potential that causes turgor reduction and cellular water loss also induces osmotic stress. Secondary effects of NaCl stress include inhibition of K^+ uptake, membrane dysfunction and generation of reactive oxygen species (ROS) in the cells (Rout and Shaw, 2001; Ghoulam et al., 2002; Agarwal and Pandey, 2004; Upadhyay and Panda, 2005). Salinity modifies photosynthetic parameters, including osmotic and leaf water potential, transpiration rate, leaf temperature, and relative leaf water content (RWC); salt also affects. The components of photosynthesis such as enzymes, chlorophylls, and carotenoids are also affected by the salt. Changes in these parameters depend on the severity and duration of stress (Misra et al., 1997) and on plant species (Liu et al., 2011).

Senegalia senegal (L.) Britton*, Vachellia seyal* (Delile) P. Hurter and *Prosopis juliflora* (Swartz) DC are trees species widely found in arid and semi-arid zone of Senegal. *S. senegal* is a multipurpose perennial legume, widespread in the semi-arid zone of tropical Africa and the Middle East. *S. senegal* has huge potential in agroforestry systems, fuelwood production, forage, medicinal products and gum production (Von Maydell, 1986). In this respect, more attention is given to *S. senegal* which is among the selected forest species for the Great Green Wall, from Senegal to Djibouti (GMV, 2009). *V. seyal* contributes to soil fertility in agroforestry systems. It produces fodder and gum and is also an important source of rural energy because of its role in the production of both firewood and charcoal. In Senegal, *V. seyal* is widely distributed in the salt-affected coastal steppes. *P. juliflora* is used in agroforestry systems. This legume tree is an important component of the system, because it serves as a source of high quality animal feed. It can be sold for fuel and timber, as well as it improves soil physiochemical and biological properties, generating "fertility islands" or "resource islands" beneath its canopy (Reyes-Reyes et al*.,* 2002; Diedhiou et al., 2009; Dossa et al., 2010). It also has been described as salt-tolerant species (Basavaraja, 2007).

Adaptation of plants during germination and early seedling stages in saline environments is crucial for the establishment of species (Debez et al., 2004; Vicente et al., 2004). After seeds germination, seedlings are the most vulnerable in the life cycle of plants (Kolb and Barsch, 2010). Thus, for the successful establishment of plants in saline environments, seeds must remain viable at high salinity in an imposed secondary dormancy and germinate when salinity decreases (Vicente et al., 2004). Furthermore, seedlings will be able to grow in these conditions. One key mechanism of salinity tolerance is the ability to remove Na^+ ions from the cytosol and its sequestration into the vacuole to limit cell damage. The transport of Na^+ into vacuoles is thought to be mediated by vacuolar Na^+/H^+ antiporters of the NHX family, which are driven by the electrochemical gradient of protons (Gaxiola et al., 1999; Pardo et al., 2006). Several studies showed that NHX1 overexpression increased salinity tolerance in plants (Apse et al., 1999; Tian et al., 2006; Brini et al., 2007; Chen et al., 2015).

In Senegal, like in most arid and semi-arid regions, reforestation of salty lands has become a priority. *S. senegal*, *V. seyal* and *P. juliflora* are leguminous multipurpose trees selected in many reforestation programs. However, few studies have been done on the adaptation of these species to environmental conditions in relation to climate change such as drought and salinity. The objective of our study was to evaluate the effect of NaCl on *S. senegal*, *V. seyal* and *P. juliflora* germination, growth and some physiological and molecular traits in order to measure and understand their salt tolerance; with the aim to facilitate the on-going initiatives of Senegalese Forest Department to find suitable species for plantation in the salt affected areas in a context of climate change.

MATERIALS AND METHODS

Effect of NaCl on *S. senegal*, *V. seyal* and *P. juliflora* seeds germination

Seeds of *S. senegal* (Provenance Dahra-2013), *V. seyal*

*Corresponding author. E-mail: dioumacor.fall@isra.sn. Tel: +221 77 532 37 91. Fax: +221 33 849 33 02.

Table 1. Physical and chemical characteristics of soils collected in non-saline zone at 0 to 25 cm layer.

Soil parameter	Values
Clay	05.5%
Silt	11.5%
Sand	83.0%
Chemical characteristics	
pH H_2O	5.5
Electrical conductivity (at 25°C)	27 µS/cm
Salinity	0.00‰
Total nitrogen	0.05%
Total carbon	0.56%
Total phosphorus	52.00 mg/kg
Calcium (Ca)	0.78 méq%
Magnesium (Mg)	0.25 méq%
Sodium (Na)	0.09 méq%
Potassium (K)	0.15 méq%
Cation exchange capacity	2.99 méq%

(Provenance Ndiaffate-2013) and *P. juliflora* (Provenance Ndiaffate-2013) were provided by the National Centre for Forestry Research (CNRF) of the Senegalese Institute of Agricultural Research (ISRA). *P. juliflora* was chosen as a reference of wood species for salt tolerance. Seeds scarification and germination were performed as described by Fall et al. (2008). Seeds (n=20) were germinated on Petri dishes containing water agar (0.9% w/v) with the different concentrations of sodium chloride (NaCl). Seven concentrations of NaCl were tested: 0, 86, 171, 257, 342, 428 and 514 mM (Fall et al., 2009). The experiment was repeated three times (20 seeds × 3 = 60 seeds) for each salt treatment. Seeds were considered as germinated when emerging radical was visible. The number of germinated seeds was counted daily for 10 days. The germination rate was expressed in mean final percent germination, calculated from cumulative germinated seeds on the final day of assessment to that of the total number of seeds in the sample at different salinity levels.

Growth and physiological responses of *S. senegal*, *V. seyal* and *P. juliflora* seedlings under saline conditions

Growth conditions and salt treatment

Seeds were germinated as described on water agar. Seedlings were then individually cultivated in plastic bags (25×12 cm) containing 1.3 kg of non-sterile soil (Table 1) collected from Sadioga (Centre of the groundnut basin of Senegal, 16° 23' 18 W, 14° 03' 53 N). The experiment was carried out in greenhouse conditions at the Laboratoire Commun de Microbiologie IRD/ISRA/UCAD (LCM) of Dakar (Senegal) in Bel-Air (14°44'N, 17°30'W) under natural sunlight (35°C day, 27°C night, with 14 h photoperiod). The relative humidity was about 75%. Accordingly to the results on the species germination rate and the level of salinity of our study site (EC = 0.414 to 34.3 mS/cm), four concentrations of NaCl (0, 86, 171, and 257 mM) were tested. A randomized experimental design was used with four treatments and 10 replicates per treatment. Salt stress treatment was performed one month after transplantation. Seedlings were gradually exposed to NaCl in order to minimize any salinity shock. NaCl concentrations were increased by 43 mM per day until reaching the required final concentration. The salinity of the leachate from representative pots was monitored regularly with a salinometer (Digit 100 ATC Salinity pocket refractometer, CETI, Optical Instruments, Belgium) to ascertain actual NaCl concentrations within the rooting medium.

Plants growth measurement

Three months after salt stress application, plants growth was evaluated by measuring plant height, collar diameter, shoot (leaves + stems) and root dry biomass.

Physiological traits measurements

Relative water content: Relative water content (RWC) estimation was done by incubating stem fragment (5 cm) in 15 ml distilled water for 24 h and calculated according to Yamasaki and Dillenburg (1999) after 96 h at 80°C in a stove.

Leaf water potential: Water potential is defined as the potential energy per unit mass of water with reference to pure water at zero potential (atmospheric pressure and 20°C), (Campbell, 1977). Leaf water potential (LWP) was measured using a Scholander pressure chamber (Scholander et al., 1965).

Total chlorophyll content: The total chlorophyll content was evaluated from 100 mg of fresh leaves according to Arnon (1949) method. The total chlorophyll content was calculated as follow: C = [20.2 (A645) + 8.02 (A663)] × V/M; where, V and M are the extraction volume (L) and weight (mg) of crushed leaves, respectively.

Proline content: Free proline content was determined by spectrophotometry from 100 mg leaf samples according to Monnevaux and Nemmar (1986). The proline concentration on a fresh-matter basis was obtained from a calibration graph prepared with a series of standard proline solutions.

Concentration of Na and K in roots and leaves: Concentrations of Na and K were determined by atomic absorption spectrophotometer after HNO_3-H_2O_2 digestion. Chloride was extracted by contact with boiling deionized water and colorimetric assay was done using the method of mercuric thiocyanate and ferric nitrate.

Salt tolerance index of *S.senegal, V. seyal* and *P. juliflora* at germination and growth in greenhouse conditions: Salt tolerance index (STI) was calculated as the ratio of the parameters salt stressed plants versus those of control plants (Cano et al., 1998).

$$STI\ (\%) = (MV \text{ at } C_x / MV \text{ at } C_0) \times 100$$

Where, MV is the measured variable, C_0 is the control, and C_x is a given concentration of salt.

Identification of a vacuolar antiport Na^+/H^+ (NHX1) in *S. senegal, V. seyal* and *P. juliflora*

Total RNA extraction

The seedlings of *S. senegal*, *V. seyal* and *P. juliflora* were grown on

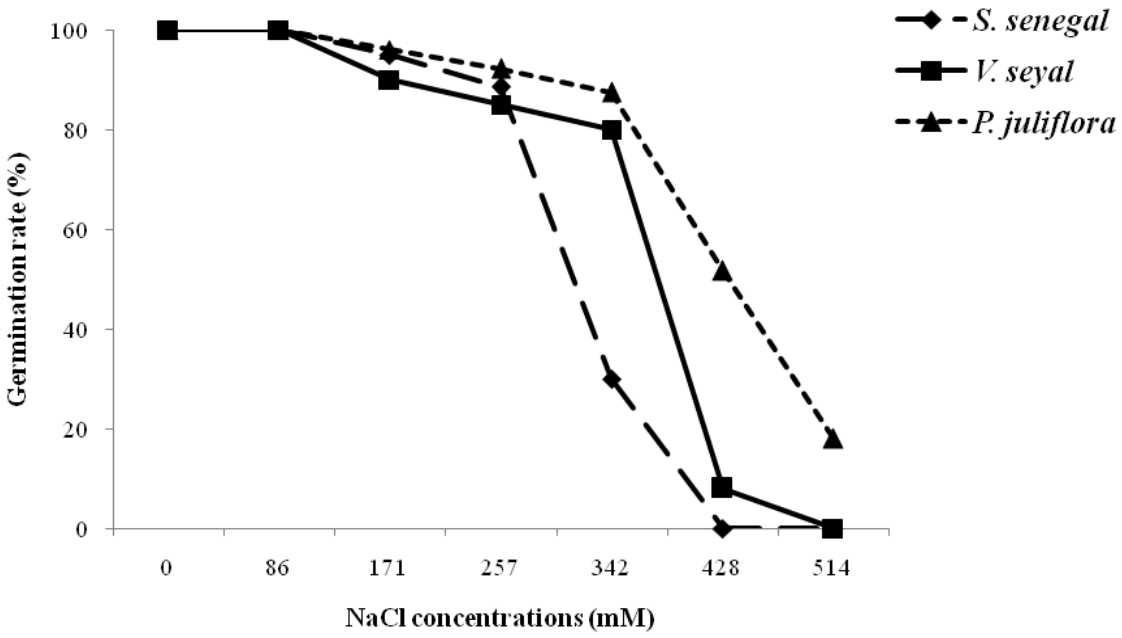

Figure 1. NaCl effect on final percent germination (%) of *S. senegal, V. seyal* and *P. juliflora*. The mean final percent germination was calculated from cumulative germinated seeds in water-agar (0.9%, w/v) after 10 days on the indicated NaCl concentrations (0, 85, 171, 256, 342, 428, and 514 mM) to that of the total number of seeds in the sample. Each value represented the mean of three replications (20 seeds × 3 = 60 seeds).

a mix of peat and vermiculite (2/1) during three weeks. Total RNA was extracted from young leaves using the RNeasy total RNA isolation kit (Qiagen). About 100 mg of fresh leaves were excised and ground using liquid nitrogen to fine powder. One milliliter of RTL buffer containing 10 mg of polyvinylpyrrolidone (PVPP) and 10 µl de Mercaptoethanol was added to the powder. The mix was incubated at 56°C for 2 min in a water bath. The lysate (650 µl) was transferred to a QIAshredder column and centrifuged at room temperature (25°C) at 18000 rpm for 2 min. The supernatant was collected in a fresh Eppendorf tube and 0.5 volumes of alcohol (96%) were added and mixed very well. The mixture was transferred on a spin column and centrifuged at 10 000 rpm for 45 s. Total RNA were washed with 700 µl of RW1 buffer by centrifugation at 10 000 rpm for 20 s and with 500 µl of RPE buffer at 10 000 rpm for 20 s. Total RNA was eluted with 30 µl of RNase-free water. To remove contaminating DNA, RNAs were treated with RNase-free DNase. The integrity of total RNA was estimated by a bioanalyzer before reverse transcription. One microgram of RNA was reverse-transcribed using Supercript II reverse transcriptase. The reverse transcription (RT) reactions were performed at 42°C for 50 min and using oligo-dT by following the instruction of the manufacturer.

NHX1 gene amplification

Since any specific primers were described in the literature to amplify NHX1 gene for the three species, degenerated primers were used. Thus, the complete sequences of NHX1 gene in several salt tolerant and non-tolerant species were searched in Genbank. After alignment of the obtained sequences, primers were designed in the highly conserved regions. Thus, one forward primer (NHX1F_5'-TTYAATGCHGGSTTTCARG-3') and 3 reverse primers (NHX1R1_5'-GABGTDGCATCATTHACAACWCC-3'; NHX1R2_5'-ACCTCDCGATCWGTNGART GC-3' and NHX1R3_5'-TGRGACRTVACAATMCCAC-3') were designed and theoretical sizes for three combinations were estimated. Two microliters of cDNAs were used as templates for PCR amplification with degenerated primers. Three couples of primers were tested (NHX1F/NHX1R1, NHX1F/NHX1R2 and NHX1F/NHX1R3). Samples were denatured for 3 min at 94°C and then run for 30 cycles of 30 s each at 94 and 50°C, 45 s at 72°C with a final extension of 7 min at 72°C. The PCR products were separated by 1% agarose gel electrophoresis (Figure 2).

Data statistical analysis

A one-way analysis of variance (ANOVA) analysis was performed for all data sets using XLSTAT™ for Windows statistical data analysis package (version 2009, Addinsoft, Paris, France). Student-Newman-Keuls´s post-hoc test was employed to determine if significant ($P \leq 0.05$) differences occurred in parameters measured between salinity treatments, and also the STI of species.

RESULTS

Effect of NaCl on *S. senegal, V. seyal* and *P. juliflora* seeds germination

The effect of NaCl on *S. senegal*, *V. seyal* and *P. juliflora* seeds germination, evaluated by the percentage of germinated seeds after 10 days, is as shown in Figure 1.

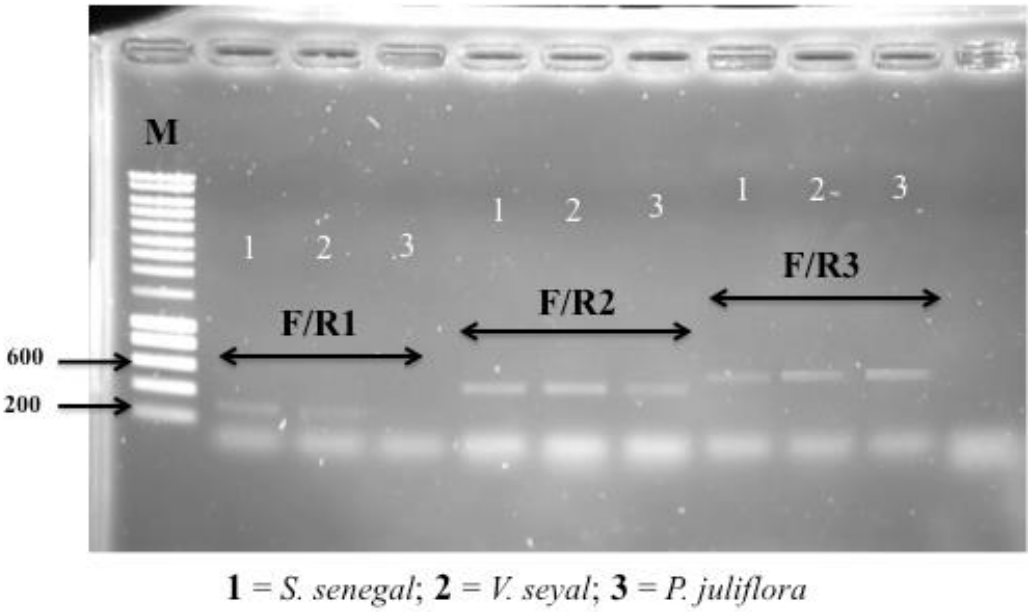

Figure 2. PCR profiles obtained after amplification of NHX1 gene with three couples of primers in leaves of *S. senegal, V. seyal* and *P. juliflora*. F = forward primer: NHX1F_5'-TTYAATGCHGGSTTTCARG-3'. R1 = Reverse primer 1: NHX1R1_5'-GABGTDGCATCATTHACAACWCC-3'. R2 = Reverse primer 2: NHX1R2_5'-ACCTCDCGATCWGTNGARTGC-3'. R3= Reverse primer 3: NHX1R3_5'-TGRGACRTVACAATMCCAC-3'. M = Molecular weight marker (200 pb).

The results show that for all species, the germination rate decreased with an increase of the NaCl concentration. Nevertheless, the negative effect of NaCl varied according to species. Below 257 mM, the germination rate of species was nearly equal to 100%. However, from this concentration, a difference in salinity tolerance was observed between species. For example, at 428 mM of NaCl, a reduction was observed in the germination rate of 100 (no germination), 92 and 48%, respectively for *S. senegal*, *V. seyal* and *P. juliflora*. At 514 mM NaCl, no germination was observed in *V. seyal* while a germination rate of 18% was observed for *P. juliflora*.

Effect of NaCl on *S. senegal, V. seyal* and *P. juliflora* seedlings growth

Salinity reduced height, shoot and root dry biomass of *S. senegal, V. seyal* and *P. juliflora* seedlings as shown in Table 2. However, the collar diameter seemed to increase with NaCl concentration. The low concentrations of NaCl (86 mM) seemed to increase the growth of *P. juliflora*. No significant negative effect of salinity was observed on seedlings height of the three species and on *P. juliflora* shoots dry biomass (SDB). In contrast, a significant negative effect was noted on SDB of *S. senegal* and *V. seyal* seedlings. For *S. senegal*, the average SDB was 0.95 g plant^{-1} at control and this value gradually decreased throughout the increasing salt concentrations, and reached to 0.28 g plant^{1} at 257 mM NaCl. In *V. seyal*, the SDB was 1.16 gplant^{-1} at 0 mM NaCl against 0.28 g plant^{-1} at 257 mM NaCl. At 257 mM, when compared to the control, the species had approximately the same percentages reduction in height with 24, 21 and 21%, respectively for *P. juliflora, S. senegal* and *V. seyal*. The percentages reduction in shoots dry weights were 76, 71 and 37%, respectively for *V. seyal, S. senegal*, and *P. juliflora* (Table 2). RDB at 257 mM NaCl decreased by 71, 57 and 51%, respectively for *P. juliflora, S. senegal* and *V. seyal* when compared with the seedlings control.

Physiological responses of *S. senegal, V. seyal* and *P. juliflora* seedlings under saline conditions

Chlorophyll, proline, leaves Cl^-, Na^+ and K^+ contents were not evaluated in *S. senegal* at 257 mM NaCl because seedlings had no leaves. Table 3 showed total chlorophyll and proline contents, RWC and LWP of *S. senegal, V. seyal* and *P. juliflora* seedlings. The chlorophyll content decreased in *S. senegal* and *V. seyal* with the increase in NaCl concentration. However, the chlorophyll content seemed to be increased in *P. juliflora*.

Table 2. Effect of NaCl concentrations (0, 86, 171 and 257 mM NaCl) on collar diameter, height, shoot and root biomass of *S. senegal, V. seyal* and *P. juliflora* seedlings grown during four months in greenhouse conditions on non-sterile sandy soil.

Species	NaCl (mM)	Col. dia. (mm plant^{-1})	Height (cm plant^{-1})	SDW (g plant^{-1})	RDW (g plant^{-1})
S. senegal	0	4.25±0.43^{a}	19.97±1.60^{a}	0.95±0.06^{b}	2.41±0.30^{b}
	86	4.42±0.29^{a}	18.57±1.29^{a}	0.92±0.03^{b}	2.03±0.14^{ab}
	171	4.89±0.45^{a}	16.43±0.75^{a}	0.75±0.24^{b}	1.69±0.48^{ab}
	257	4.98±0.13^{a}	15.83±3.33^{a}	0.28±0.03^{a}	1.04±0.64^{a}
V. seyal	0	2.60±0.36^{a}	26.90±5.91^{a}	1.16±0.03^{c}	1.62±0.04^{b}
	86	2.33±0.49^{a}	24.13±4.23^{a}	0.62±0.12^{b}	1.46±0.13^{b}
	171	2.34±0.52^{a}	20.47±4.68^{a}	0.55±0.15^{b}	0.94±0.11^{a}
	257	2.77±0.42^{a}	21.27±1.54^{a}	0.28±0.03^{a}	0.79±0.14^{a}
P. juliflora	0	2.18±0.17^{a}	25.87±1.40^{a}	0.67±0.07^{a}	0.49±0.12^{b}
	86	2.51±0.52^{a}	27.80±5.30^{a}	0.73±0.27^{a}	0.35±0.18^{ab}
	171	2.35±0.25^{a}	23.13±2.67^{a}	0.42±0.10^{a}	0.26±0.07^{ab}
	257	2.32±0.06^{a}	19.73±2.25^{a}	0.42±0.08^{a}	0.14±0.02^{a}

For each species, values within a column sharing same letter comparing NaCl treatments are not significantly different at *P<0.05* (Student-Newman-Keuls test). Each value represented the mean of three replications. Col. dia.: Collar diameter; SDW: shoot dry weight; RDW: root dry weight.

Table 3. Total chlorophyll (a + b) and proline contents, relative water content (RWC) and leaf water potential (LWP) of *S. senegal, V. seyal* and *P. juliflora* seedlings grown under greenhouse on non-sterile sandy soil and exposed during four months to four salinity levels (0, 86, 171 and 257 mM NaCl).

Species	NaCl (mM)	Chlorophyll a+b (mg g FW^{-1})	Proline (µg g FW^{-1})	RWC (%)	LWP (MPa)
S. senegal	0	2.17±0.13^{c}	1.08±0.02^{a}	83.1±1.8^{a}	-1.03±0.15^{b}
	86	1.41±0.05^{b}	1.09±0.01^{a}	84.1±2.4^{a}	-1.13±0.14^{b}
	171	0.75±0.03^{a}	2.11±0.14^{b}	89.1±6.2^{ab}	-1.24±0.10^{ab}
	257	nd	nd	90.8±3.6^{b}	-1.30±0.05^{a}
V. seyal	0	2.31±0.13^{c}	0.11±0.01^{a}	86.2±1.1^{a}	-1.17±0.04^{b}
	86	2.78±0.15d	0.12±0.04^{ab}	85.9±4.3^{a}	-1.20±0.10^{b}
	171	2.10±0.07^{b}	0.19±0.03^{b}	78.4±0.4^{a}	-1.18±0.08^{b}
	257	1.84±0.03^{a}	0.55±0.02^{c}	75.6±11.9^{a}	-1.43±0.10^{a}
P. juliflora	0	1.79±0.04^{a}	1.01±0.01^{a}	90.7±1.4^{a}	-0.67±0.10^{b}
	86	2.52±0.02^{c}	1.02±0.01^{a}	82.6±7.7^{a}	-1.00±0.11^{a}
	171	1.92±0.05^{b}	3.11±0.01^{b}	87.1±1.5^{a}	-1.15±0.12^{a}
	257	1.99±0.03^{b}	5.00±0.05^{c}	85.1±2.3^{a}	-1.17±0.05^{a}

For each species, values within a column sharing same letter comparing NaCl treatments are not significantly different at *P<0.05* (Student-Newman-Keuls test). Each value represented the mean of three replications. nd: Not determined (no leaves).

The reduction rate in chlorophyll content at 171 mM NaCl was 65 and 9%, respectively in *S. senegal* and *V. seyal* when compared with controls, while an increase of 11% was observed in *P. juliflora* at the same concentration of NaCl. In contrast to chlorophyll, free proline content increased with the NaCl concentration. This increase of proline content was more pronounced, respectively in *P. juliflora, S. senegal* and *V. seyal* for NaCl concentrations tested, with respectively 208, 95 and 73%. No significant difference (excepted *S. Senegal* at 257 mM) was observed in RWC of seedlings grown under salinity stress compared to those grown in non-saline soil.

Table 4. Mineral elements accumulation (g kg^{-1} dry weight) in roots and leaves of *S. senegal, V. seyal* and *P. juliflora* seedlings grown under greenhouse on non-sterile sandy soil and exposed during four months to four salinity levels (0, 86, 171 and 257 mM NaCl).

Species	NaCl (mM)	Cl^-		Na^+		K^+		K^+/Na^+	
		Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves
S. senegal	0	$^{*A}11.0\pm3.1^{a**}$	$^{A}12.4\pm2.5^{a}$	$^{A}1.6\pm0.7^{a}$	$^{A}0.4\pm0.2^{a}$	$^{A}9.3\pm2.8^{a}$	$^{A}27.4\pm1.9^{b}$	$^{D}5.8\pm1.7^{a}$	$^{C}68.5\pm4.6^{b}$
	86	$^{AB}17.0\pm3.6^{a}$	$^{B}26.2\pm2.8^{b}$	$^{B}4.2\pm1.4^{a}$	$^{B}6.9\pm1.1^{b}$	$^{A}7.7\pm1.6^{a}$	$^{A}26.9\pm2.8^{b}$	$^{C}1.9\pm0.2^{a}$	$^{B}3.9\pm0.2^{b}$
	171	$^{B}18.0\pm1.5^{a}$	$^{C}32.7\pm1.1^{b}$	$^{BC}7.0\pm1.8^{a}$	$^{C}12.6\pm3.3^{b}$	$^{A}7.2\pm0.7^{a}$	$^{A}24.3\pm3.5^{b}$	$^{B}1.0\pm0.1^{a}$	$^{A}1.9\pm0.1^{b}$
	257	$^{B}22.2\pm3.6$	nd	$^{C}11.2\pm4.4$	nd	$^{A}7.1\pm0.2$	nd	$^{A}0.6\pm0.12$	nd
V. seyal	0	$^{A}14.6\pm0.6^{b}$	$^{A}8.8\pm1.2^{a}$	$^{A}5.8\pm0.9^{b}$	$^{A}1.4\pm1.0^{a}$	$^{A}11.9\pm3.2^{a}$	$^{C}24.5\pm2.5^{b}$	$^{B}2.1\pm1.0^{a}$	$^{C}17.5\pm2.9^{b}$
	86	$^{B}29.1\pm4.9^{a}$	$^{B}25.8\pm4.1^{a}$	$^{A}7.1\pm0.7^{a}$	$^{B}12.8\pm1.5^{b}$	$^{A}10.0\pm1.3^{a}$	$^{A}17.2\pm2.6^{b}$	$^{A}1.4\pm0.4^{a}$	$^{B}1.3\pm0.1^{a}$
	171	$^{B}24.9\pm4.2^{a}$	$^{B}33.3\pm2.5^{b}$	$^{A}7.3\pm1.5^{a}$	$^{B}16.7\pm2.2^{b}$	$^{A}9.5\pm1.9^{a}$	$^{A}16.7\pm2.5^{b}$	$^{A}1.3\pm0.2^{a}$	$^{AB}1.0\pm0.2^{a}$
	257	$^{B}24.2\pm2.2^{a}$	$^{B}32.2\pm3.0^{b}$	$^{A}8.2\pm2.8^{a}$	$^{C}23.4\pm0.9^{b}$	$^{A}8.7\pm2.3^{a}$	$^{A}15.9\pm1.1^{b}$	$^{A}1.1\pm0.1^{b}$	$^{A}0.7\pm0.2^{a}$
P. juliflora	0	$^{A}14.6\pm2.6^{a}$	$^{A}17.3\pm1.9^{a}$	$^{A}4.1\pm0.1^{b}$	$^{A}2.1\pm1.5^{a}$	$^{A}14.3\pm0.3^{a}$	$^{A}28.9\pm2.4^{b}$	$^{C}3.5\pm0.1^{a}$	$^{C}13.8\pm1.3^{b}$
	86	$^{B}29.1\pm4.9^{b}$	$^{B}21.1\pm1.7^{a}$	$^{B}15.2\pm 2.9^{b}$	$^{B}9.7\pm1.0^{a}$	$^{A}11.6\pm0.6^{a}$	$^{B}17.7\pm1.6^{b}$	$^{B}0.8\pm0.2^{a}$	$^{B}1.8\pm0.3^{b}$
	171	$^{C}36.8\pm2.6^{b}$	$^{AB}18.3\pm2.4^{a}$	$^{C}20.0\pm1.9^{b}$	$^{B}9.9\pm1.2^{a}$	$^{A}10.2\pm1.6^{a}$	$^{B}17.5\pm4.1^{b}$	$^{AB}0.5\pm0.1^{a}$	$^{A}1.8\pm0.25^{b}$
	257	$^{C}40.9\pm2.1^{b}$	$^{AB}18.9\pm0.7^{a}$	$^{C}21.6\pm4.2^{b}$	$^{B}12.0\pm1.1^{a}$	$^{A}9.4\pm1.6^{a}$	$^{B}17.2\pm0.6^{b}$	$^{A}0.4\pm0.1^{a}$	$^{A}1.4\pm0.1^{b}$

*****For each species, values within a column sharing same upper case letter comparing NaCl treatments are not significantly different at *P<0.05* (Student-Newman-Keuls test). ******For each element, values within a line sharing same lower case letter comparing the repartition of element between roots and leaves are not significantly different at *P<0.05* (Student-Newman-Keuls test). Each value represented the mean of three replications after pooling seedlings. nd: Not determined (no leaves).

However, RWC seemed to increase in *S. senegal* and decrease in *V. seyal* and *P. juliflora* seedlings when increasing NaCl concentration. LWP was more negative under salinity stress for all species. Significant difference was noted between controlled and stressed plants for all species (Table 3). At 171 mM NaCl, LWP was negatively increased by 75, 26 and 22%, respectively in *P. juliflora, S. senegal* and *V. seyal.* Our results indicated that Cl^- and Na^+ content in leaves and roots increased with salinity (Table 4). The accumulation of Cl^- and Na^+ was higher in leaves than roots in *S. senegal* and *V. seyal*, while it is higher in roots than leaves in *P. juliflora*. However, the accumulation of K^+ was higher in leaves than roots for all species (Table 4). At 171 mM NaCl, leaf Cl^- content was increased by 6, 164 and 278%, respectively in *P. juliflora, S. senegal* and *V. seyal*. However, leaf Na^+ content was increased by 471, 1093 and 3050%, respectively in *P. juliflora, V. seyal* and *S. senegal.* The K^+ content and the K^+/Na^+ ratio were reduced in roots and leaves of *S. senegal, V. seyal* and *P. juliflora*. The K^+ content was significant higher in leaves than roots for all species.

Salt tolerance index of *S. senegal, V. seyal* and *P. juliflora* at germination and growth

Results show that salt tolerance index (STI) decreased with increasing NaCl concentration at germination and growth (Table 5). For germination, no significant difference was noted between STI of species for NaCl concentrations less than or equal to 257 mM NaCl with 89, 85 and 92%, respectively in *S. senegal, V. seyal* and *P. juliflora*. Nevertheless, when the NaCl concentration increased, a difference in STI occurred. For *S. senegal*, STI was 30% at 342 mM and became 0% from 428 mM of NaCl, while *V. seyal* and *P. juliflora* had an STI of 80 and 88%, respectively at 342 mM. At 514 mM, the STI of *V. seyal* became 0%; however, *P. juliflora* had a STI of 18% (Table 5). For growth (shoot dry biomass), STI of *S. senegal* was higher than *V. seyal* for all NaCl concentrations. At 86 mM NaCl, no significant difference was observed between *S. senegal* and *P. juliflora* which had STI significantly higher than *V. seyal*. At 257 mM NaCl, STI was 24, 29 and 63%, respectively for *V. seyal, S.*

Table 5. Salt tolerance index (%) of *S. senegal, V. seyal* and *P. juliflora* at germination and growth after four months under greenhouse.

Parameter	Species	NaCl concentrations (mM)						
		0	86	171	257	342	428	514
Germination	*S. senegal*	100±0^{a}	100±0^{a}	95±5^{a}	89±4^{a}	30±4^{a}	0±0^{a}	0±0^{a}
	V. seyal	100±0^{a}	100±0^{a}	90±3^{a}	85±3^{a}	80±5^{b}	8±1^{b}	0±0^{a}
	P. juliflora	100±0^{a}	100±0^{a}	96±4^{a}	92±4^{a}	88±4^{b}	52±3^{c}	18±2^{b}
Growth (shoot biomass)	*S. senegal*	100±0^{a}	97±4^{b}	79±5^{c}	29±4^{a}	nd	nd	nd
	V. seyal	100±0^{a}	53±3^{a}	47±5^{a}	24±3^{a}	nd	nd	nd
	P. juliflora	100±0^{a}	109±9^{b}	63±6^{b}	63±5^{b}	nd	nd	nd

For each column, values with same letter comparing the salt tolerance index of species are not significantly different at $P<0.05$ (Student-Newman-Keuls test). nd: Not determined.

senegal and *P. juliflora*.

Identification of NHX1 gene in *S. senegal, V. seyal* and *P. juliflora*

Results showed that the three primers generated amplicons for all species except for *P. juliflora* with NHX1F/NHX1R1 couple. NHX1F/NHX1R1, NHX1F/ NHX1R2 and NHX1F/NHX1R couples produced good amplicons with the expected size with an approximately size of 300, 500 and 600 pb, respectively.

Any difference in terms of amplicon size was obtained among species for the same primer.

DISCUSSION

Our results show that for all species, the percentage of germination decreased as the degree of salinity increased. Reduction in germination by increasing salinity levels has been described by numerous studies (El-Tayeb, 2005; Abari et al., 2011; Tsegay and Gebreslassie, 2014; Sharma and Vimala, 2016). High concentration of NaCl in the salt solution increases its osmotic potential and the germination rate reduction could be attributed to the osmotic effect of NaCl, which limits the seed hydration (Tobe et al., 2000; El-Keblawy and Al-Rawai, 2005). It could also be due to the toxic effect of NaCl on seed embryo or endosperm cell membranes (Bliss et al., 1986; Khajeh-Hosseini et al., 2003). The inhibition of germination can be due to high absorption of Na and Cl ions during seed germination (Taiz and Zeiger, 2002). On the basis of their salt tolerance at germination, the species can be arranged as follows: *P. juliflora* > *V. seyal* > *S. senegal*.

It was found that the seedlings growth (height, shoot and root dry weight) was affected by salinity and that the salt effect depends on salinity level and species. Results for height, shoot and root dry weight in response to increasing salinity level suggested that there was maximum reduction in shoot dry weight for *V. seyal* and *S. Senegal*, while the highest reduction of root dry weight was observed in *P. juliflora*. According to Alam et al. (2004), it is possible that the decrease in plant growth in saline condition was due to several reasons. One possibility is that salinity reduced photosynthesis, which in turn limited the supply of carbohydrates needed for growth (da Silva et al., 2011). A second possibility is that salinity reduced shoot and roots growth by reducing turgor in expanding tissues resulting from lowered water potential in root growth medium. Third, a disturbance in mineral supply, either an excess or deficiency, induced by changes in concentrations of specific ions in the growth medium, might have affected directly the growth (Lazof and Bernstein, 1998; Zhu, 2002). The decrease in shoot biomass and the yellow leaves might be due to disturbance in carbohydrates allocation in shoots. Our results for reduction of seedlings growth of all species with increase in NaCl concentration are in conformity with the finding of several authors (Abari et al., 2011; Saini et al., 2012; Sharma and Vimala, 2016). The low concentration of NaCl (86 mM) seemed to increase *P. juliflora* seedlings growth. This result corroborated those obtained by Viégas et al. (2004) on *P. juliflora* seedlings in hydroponic condition. The absence and the falling leaves at 257 mM NaCl, observed respectively in *S. senegal* and *V. seyal*, reduced the photosynthetic area which result in the reduction of plant growth and yield. On the basis of tolerance at growth (shoot biomass production), the species can be arranged as follows: *P. juliflora* > *S. senegal* > *V. seyal*. This ranking was confirmed by STI, which were 63, 29 and 24%, respectively in *P. juliflora, S. senegal* and *V. seyal* at 257 mM NaCl. The total chlorophyll (a + b) content decreased significantly with increasing NaCl concentration for all species. The decrease in chlorophyll content under salt stress is a commonly reported phenomenon. It is related

to its adverse effects on membrane stability (Ashraf and Bhatti, 2000), the weakening of protein-pigment-lipid complex (Ayala-Astorga and Alcaraz-Meléndez, 2010) and the increasing chlorophyllase (EC: 3.1.1.14) activity (Lakhdar et al., 2008). Also, Santos (2004) pointed out that the induced decrease in chlorophyll content in severely NaCl stressed leaves is mainly due to a decrease of 5-aminolinolic acid (ALA) synthesis, and therefore to limitations of chlorophyll synthesis. These observations corroborated with the results obtained by several authors (Hardikar and Pandey, 2008; Turan et al., 2009; Molazem et al., 2010; Heidari, 2012; Taibi et al., 2016). In contrast, the increase in NaCl concentration seemed to increase leaf total chlorophyll of *P. juliflora* suggesting that it was more tolerant in saline medium than *S. senegal* and *V. seyal* whose chlorophyll content decreased.

Free proline content increased with NaCl concentration for all species. Salt tolerant plants are distinguished by their capacity to accumulate high concentrations of compatible osmotica such as glycine betaine, proline, in cytoplasm to balance the osmotic pressure of ions in the vacuoles. Increasing leaf proline content under salinity stress might be caused by induction or activation of proline synthesis from glutamate or decrease in its utilization in protein synthesis or enhancement in protein turnover. The high accumulation of proline indicated that *S. senegal* (95%) and *V. seyal* (73%) seemed to have the capacity to tolerate salinity as *P. juliflora* (208%). Similar results were observed in many plant species such as *Acacia auriculiformis* (Diouf et al., 2005), date palm (Sané et al., 2005), rice (Shereen et al., 2007), maize (Cha-um and Kirdmanee, 2009), walnut (Akça and Samsunlu, 2012), and potato (Jaarsma et al., 2013).

Relative water content (RWC) and leaf water potential (LWP) are the basic parameters of plant water status. Water status is the main factor affecting the plants growth and development. Even if no significant difference was observed, the decrease of RWC in *V. seyal* and *P. juliflora* seedlings grown under salinity stress, compared to those grown in non-saline soil, indicated that salinity resulted in dehydration at cellular level and dehydration symptoms were greater in higher NaCl concentration treatment because of the increase in cellular water loss. This is a common reaction to salinity similar to those reported for other species such as pepper plants (Navarro et al., 2003), *Avicennia germinans* (L.) (Suárez and Medina, 2008). Therefore, plants should have osmotic adjustment inside the cell, since turgor maintenance is required for cell expansion and the biochemical, physiological and developmental processes (Flowers, 2004). Even so, the RWC seemed to increase in *S. senegal* with NaCl concentrations. This exception can be explained by the reduction and/or the absence of leaves, which reduced the transpiration area along the water retention in cells. Water potential in *S. senegal, V. seyal* and *P. juliflora* became increasingly more negative with the corresponding increase in media salinity, indicating that these species osmotically adjust in response to increases in salinity. Water potential was more negatively increased by 75, 26 and 22%, respectively in *P. juliflora, S. senegal* and *V. seyal*, suggesting that *P. juliflora* adjust better its osmotic pressure followed by *S. senegal* and *V. seyal*.

According to Greenway and Munns (1980), NaCl, the predominant form of salt in most saline soils, enhances Na^+ and Cl^- contents and consequently affects the uptake of other mineral elements. Our results showed that Cl^- and Na^+ accumulation increased in roots and leaves when increasing NaCl concentration while K^+ accumulation and K^+/Na^+ ratio decreased, but more Na^+ was taken up than Cl^-, indicating that *S. senegal*, *V. seyal* and *P. juliflora* are an ion accumulators. The strong Na^+ accumulation in leaves could be responsible for their loss as observed in *S. senegal* and *V. seyal*. Previous studies have shown that salinity increases Na^+ and Cl^- and decreased K^+ and K^+/Na^+ in plant leaves (Saghir et al., 2002; Hosseini and Thengane, 2007; Taffouo et al., 2010; Silini et al., 2016) found. This implies a competition between Na^+ and K^+ absorption in plants, resulting in a Na^+/K^+ antagonism (Mori et al., 2011). The reduction in K^+ uptake caused by Na^+ is likely to be the result of the competitive intracellular influx of both ions (Tripathi and Müller, 2015). It is well established that many K transport systems have significant affinity for Na^+ (Rodriguez-Navarro and Rubio, 2006).

Our preliminary results on molecular studies show the presence of salt tolerant gene NHX1 in *S. senegal, V. seyal* and *P. juliflora*. To our knowledge, this is the first study of the tonoplast associated Na^+/H^+ antiporter in these species. This result indicated that these species have the ability to sequester the Na^+ in the vacuole which in turn, will enhance their adaptation to salinity. The overexpression of NHX1 in saline conditions will increase their tolerance to salinity as shown by several studies (Gouiaa et al., 2012; Baltierra et al., 2013; Hasegawa, 2013; Panahi et al., 2013; Hu and Wu, 2014; Chen et al., 2015).

Conclusion

The results show that salt stress decreased germination rate, growth, total chlorophylls content, relative water content, leaf water potential, K^+ accumulation and K^+/Na^+ ratio in *S. senegal*, *V. seyal* and *P. juliflora*. Salt stress increased proline, Cl^- and Na^+ accumulation in all species. Nevertheless, results showed that species maintained a good germination rate and an overall growth at high level of salinity. In summary, the results show that *S. senegal, V. seyal* and *P. juliflora* accumulated Na^+ to achieve a negative water potential gradient and also accumulate

proline as an osmoprotectant or to achieve osmotic balance in the cytoplasm. According to our results, *S. senegal* and *V. seyal* should be considered as species that might be used to phytoremediate degraded saline lands as *P. juliflora*. However, molecular studies will be continued to evaluate the expression salt tolerant gene (NHX1) in saline conditions and field trials will be conducted to confirm their ability.

Conflict of interests

The authors have not declared any conflict of interests.

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Somatic embryogenesis from zygotic embryos and thin cell layers (TCLs) of Brazilian oil palm (*Elaeis guineensis* × *Elaeis oleifera*)

Bonetti, K. A. P.[1], Nesi, J.[1], Quisen, R. C.[2] and Quoirin, M.[1]*

[1]Department of Botany, Sector of Biological Sciences, Federal University of Parana, 81531-090 Jardim das Américas, Curitiba, PR, Brazil.
[2]EMBRAPA- Amazônia Ocidental, Manaus, Brazil.

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Oil palm hybrid BRS Manicoré is important for plantations in the north of Brazil, as it is resistant to fatal yellowing and is compact. Seed germination is slow and reduced, so somatic embryogenesis is a promising alternative for its propagation. Two kinds of starting explants were used: Zygotic embryos (ZE) and thin cell layers (TCL) from the base of seedling aerial parts. Two culture medium formulae were compared (Y3 and modified MS), and several growth regulators (2,4-D, Picloram, BA, 2iP), putrescine and activated charcoal (AC) were used during the different stages. Somatic embryos (SE) were obtained from ZE-derived embryogenic masses cultured on Y3 medium with or without 2,4-D (9 µM) + 1000 µM putrescine and 40% were converted into seedlings in the Y3 medium supplemented with 2 g.L^{-1} AC and without growth regulators, under light. SE developed in 49 to 53% TCL-derived calli and 50% of them were converted into seedlings in the same conditions described for ZE calli. The yield of both processes was compared, showing the superiority of TCL explants for SE production.

Key words: Y3 culture medium, clonal propagation, putrescine, thin cell layer.

INTRODUCTION

Among palms cultivated for oil production, oil palm (*Elaeis guineensis*) is the most productive, with yields higher than 25 tons of bunches per ha per year (SUFRAMA, 2003). Two types of oils are extracted from the fruits: Oil palm removed from the pulp and oil palm kernel obtained from the endosperm. They are used in the manufacturing of cosmetics, the oleochemical industry, food and animal feed, and in the preparation of commercial foods (SUFRAMA, 2003). The oil palm industry suffers from pests and diseases and slow seed germination (Pádua et al., 2013). In order to solve some limitations, such as disease sensitivity, the interspecific hybrid BRS Manicoré was created in 2010 by the breeding program of EMBRAPA (Brazilian Agricultural Research Corporation), resulting from crossing *E. guineensis* (African) with *Elaeis oleifera* (Amazonian)

*Corresponding author. E-mail: mquoirin@ufpr.br

Table 1. Media used in the first experiment of somatic embryogenesis of hybrid oil palm BRS Manicoré.

1st experiment	Salts and organic compounds	PGR*	Concentration (µM)	AC** (g.L-1)
Callus induction	mMS or	2,4-D	0 – 500	2
Medium	Y3	Picloram	0 – 500	2
1st subculture	mMS or Y3	2,4-D	100	2
		2-iP	7.9	2
		2,4-D	100	2
		BA	7.9	2
		Picloram	100	2
		2-iP	7.9	2
		Picloram	100	2
		BA	7.9	2
2d subculture	Y3	2,4-D	0, 9 or 27	0.5
		Putrescine	1000	
Maturation and conversion medium	Y3	-	-	2

*PGR: Plant Growth Regulator; ** AC: activated charcoal; mMS: Murashige and Skoog medium (1962) with 500 mg.L-1 cysteine; Y3: Eeuwens medium (1976).

(Collares, 2011). It is resistant to fatal yellowing and the stipe has a reduced vertical growth (Conceição and Muller, 2000; Cunha and Lopes, 2010). The fruits of this cultivar have a high oil and unsaturated fatty acid content, which enables high quality biodiesel to be produced from its oil (Collares, 2011).

The conventional method of multiplication of palm tree hybrid is by seeds, but their germination rate is low (30%) (Angelo et al., 2007), due to the abortion of the embryo inside the seed (Alves et al., 2011). The process is also very slow and irregular, and various degrees of dormancy may exist. These aspects constitute a bottleneck for seedling production.

Somatic embryogenesis is a process whereby somatic cells differentiate into somatic embryos (SE) (George et al., 2008). It has innumerable applications for plant vegetative propagation and is a promising alternative for the production of oil palm saplings on a large scale.

In the case of *E. guineensis*, several types of explants were already used to initiate somatic embryogenesis process: Mature or immature zygotic embryos (Balzon et al., 2013; Silva et al., 2014), cell suspensions from immature leaf segments (De Touchet et al., 1991), leaf segments (Pádua et al., 2013) and immature male inflorescence (Jayanthi et al., 2015).

Within micropropagation techniques, thin cell layer (TCL) has the advantage of using explants of reduced size which have a greater contact with the medium than other kinds of explant (Tran Thanh Van, 1980). This technique has been used successfully for peach palm (*Bactris gasipaes*) (Steinmacher et al., 2007), *Acrocomia aculeate* (Padilha et al., 2015) and oil palm (*E. guineensis*) (Scherwinski-Pereira et al., 2010). This study was developed to address the need for multiplication and production of hybrid oil palm BRS Manicoré, aimed at more rapid production on a scale that meets national demand. A somatic embryogenesis protocol was established from zygotic embryos and TCL using the shoots of *in vitro* grown seedlings.

MATERIALS AND METHODS

Somatic embryogenesis from mature zygotic embryos

Mature fruits were collected 150 days after pollination from mother plants obtained by controlled crossing of selected plants of the hybrid BRS Manicoré (*E. guineensis* x *E. oleifera*), provided by EMBRAPA - Western Amazon, Brazil. Several lots of seeds (every lot comes from one tree) were used, differing among the experiments. The endocarp was removed and almonds washed with mild soap and tap water and immersed in 70% ethanol for 5 min, then in a 10% (v/v) solution of sodium hypochlorite (NaClO) containing 1% Tween-20 for 20 min and rinsed four times with sterile distilled water. The zygotic embryos were isolated and disinfected with commercial NaClO at 2% (v/v) for 5 min, washed in sterile distilled water and inoculated on Petri dishes containing 20 mL of culture medium.

Two experiments were performed; comprising several stages until SEs were converted into seedlings. The composition of media used in the first experiment is indicated in Table 1. Seed lots were: CS428, 736, 1477 and 1681. In a second experiment, the first medium was Y3 without 2,4-D or supplemented with 250, 500 or 750 µM 2,4-D. Seed lots were: CS 47 and 1951. The calli were subcultured on the same media indicated in Table 1. In both experiments, sucrose (30 $g.L^{-1}$) and agar (Vetec®) (6 $g.L^{-1}$) were added to all media.

The growth of calli was observed every 30 days and the number

of embryogenic and non-embryogenic calli was recorded after 150 days. After 30 days of first subculture, calli with globular embryos and non-embryogenic calli were counted. Those containing globular and torpedo embryos were transferred to Y3 medium without growth regulators for maturation and conversion into seedlings. After one to three weeks the presence of root and leaf was recorded.

The cultures were maintained in the dark at 25 ± 2°C during the day and 21 ± 2°C by night. During the maturation and conversion stage, they were transferred under fluorescent light (white light) with an irradiance of 40 µmol. $m^{-2}.s^{-1}$ and a photoperiod of 16 h.

The experimental design was completely randomized with 10 repetitions (Petri dishes) and five embryos/dish. For the conversion of SE, 616 replications were used with one embryo per tube (15 cm high × 2,5 cm diameter). The variables evaluated were: percentage of formation and consistency of callus, formation of embryogenic callus and SEs. The data were subjected to ANOVA variance analysis, homogeneity by Bartlett´s test and comparison of means by Tukey´s test at 5% probability.

Somatic embryogenesis from leaf TCLs

After 30 to 50 days of *in vitro* culture, seedlings 2 to 6 cm in length were used as a source of explants. The root primordium and cotyledon petiole were removed, leaving the leaf primordia. The primordium was sectioned transversely from the apex to the base into segments approximately 1 mm thick (TCL) which were used as explants. Two experiments were conducted: The first one consisted of three treatments: Y3 medium supplemented or not with 2,4-D (500 or 800 µM) and the second one tested four concentrations of 2.4 -D (250, 500, 800 and 1000 µM) in Y3 medium and a control without 2,4-D. The media were supplemented with 2 $g.L^{-1}$ AC.

After 90 days, the calli obtained in all treatments of both experiments were sub-cultured in Y3 medium containing 100 µM putrescine and 0.5 $g.L^{-1}$ AC. Three treatments were compared (0, 50 and 100 µM 2,4-D). The SEs formed on these calli were transferred to Y3 medium supplemented with 2 $g.L^{-1}$ AC and without growth regulators for embryo maturation and conversion. All media contained 30 $g.L^{-1}$ sucrose and 2.5 $g.L^{-1}$ Gelzan. The pH of all culture media was adjusted to 5.8 with NaOH or 0.1 M HCl and the media were autoclaved at 120°C for 20 min. Activated charcoal was added along with Gelzan, after pH adjustment.

The cultures were kept in the dark at 25 ± 2°C during the day and 21 ± 2°C by night for four weeks and then transferred under fluorescent light (white light) with an irradiance of 40 µmol. $m^{-2}.s^{-1}$ and a photoperiod of 16 h.

The experimental design was completely randomized with 10 repetitions (Petri dishes) and six explants per dish. For the conversion of SEs, 250 replications were used (tubes) with one embryo per tube. The variables evaluated were: Consistency of callus (friable or compact), rate of callus formation, callus type and formation of SEs. The data were submitted to the same statistical analysis as for experiments using mature zygotic embryos.

RESULTS

Callus and somatic embryos formation from zygotic embryos

After inoculation of fifteen-day-old zygotic embryos on mMS and Y3 culture media, callus formation was observed in embryos cultured in media containing 2,4-D or Picloram (Figure 1A and B). After 30 days of culture, 76% of the explants formed calli on Y3 medium supplemented with 2,4-D (Table 2), whereas in the media without 2,4-D or Picloram, ZE germinated without callus development (data not shown). After 90 days the formation of calli was observed on the explants that did not previously respond. Friable calli developed in all media and embryogenic calli appeared (Table 2 and

Figure 1C, D, E and F). The mMS medium with 2,4-D and Y3 with Picloram were the most suitable for the formation of friable calli observed in 62.11 and 59.29% of the explants respectively, after 90 days, differing from the other means (Table 2). Considering these results, we chose Y3 as basal medium for further experiments.

When 2,4-D was added to Y3 culture medium at concentrations of 250 and 500 µM, there was a higher percentage of explants forming calli (49 and 62% respectively) than at 750 µM (31%) after 30 days of culture (Table 3). After 90 days, friable calli developed on the media containing 250 and 500 µM (58.7 and 72.1%, respectively) (Table 3).

Calli cultivated for 150 days on mMS or Y3 medium containing 2,4-D or Picloram and transferred to Y3 medium containing only auxin or auxin and cytokinin (BA or 2-iP) showed embryogenic calli (Figure 1G, H and I) irrespective of the type of auxin. The addition of 2iP to 2,4-D or Picloram promoted the formation of embryogenic calli by 34 and 143% respectively, and the combination of BA and 2,4-D or Picloram by 40 and 210% (Table 4).

SEs or pre-globular SE were formed on calli grown for two months on multiplication medium (Y3 with Picloram, 2,4-D + BA or 2iP) (Table 4) and transferred into Y3 medium with or without 2,4-D (9 or 27 µM), and with 1000 µM putrescine (Figure 1G and H). SE formation occurred in all these culture media, regardless of the presence and concentration of auxin (Figure 1G, H and I), but was higher in the media without 2,4-D or with 9 µM (37 and 43% respectively) (Table 5). Each callus formed an average of 25 to 30 embryos after 60 days.

The SEs in the torpedo stage (Figure 1J), transferred to the conversion medium (Y3 without regulators) under light conditions appeared white after one to two weeks (Figure 1J). After two weeks they turned green and the cotyledon petiole started to elongate, as well as the radicle and plumule (Figure 1 K). Some of the embryos were considered abnormal, as they were green but did not emit a root or cotyledon and others had only a root. 40% of SEs were converted into plants,

The process of somatic embryogenesis from zygotic embryos (ZE) was divided into four stages until the conversion of somatic embryos into plants (Table 6).

Callus and somatic embryos formation from TCLs

After 30 days shoot TCLs inoculated in Y3 culture medium supplemented with 500 µM 2,4-D exhibited formation of friable calli around the explant (Figure 2A), reaching 39.45% after 60 days (Table 7). The explants cultivated in the medium containing 800 µM 2,4-D showed slow

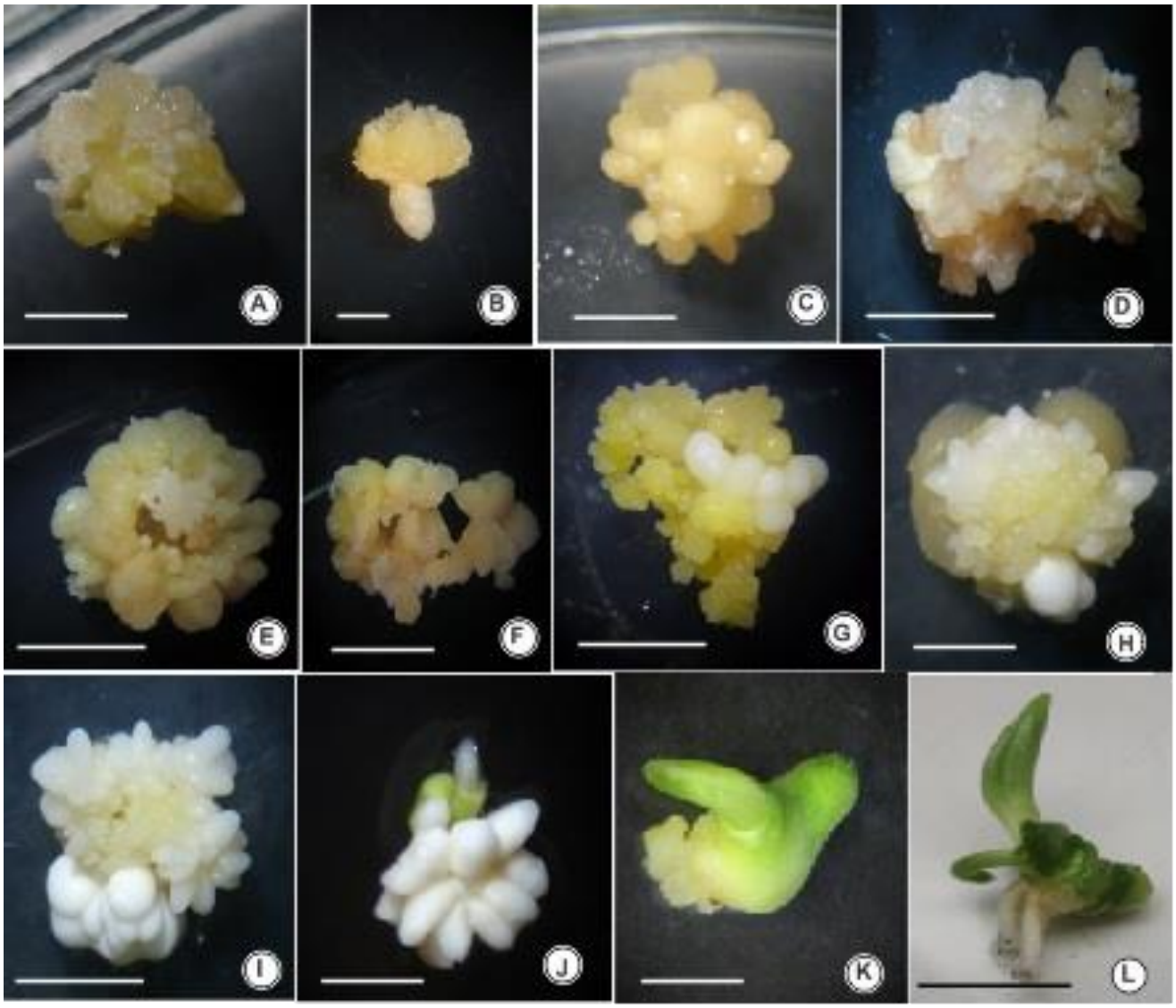

Figure 1. Stages of somatic embryogenesis from zygotic embryos of the hybrid oil palm BRS Manicoré (*Elaeis guineensis* × *E. oleifera*) cultured on Y3 medium. (A) Callus on medium containing 500 µM 2,4-D after one month. (B) with 500 µM Picloram after one month. (C) Friable callus on medium with 2,4-D (100 µM) and BA (7.9 µM). (D) Friable callus on medium with 2,4-D (100 µM) + 2iP (7.9 µM). (E) Embryogenic callus in medium + 2,4-D (100 µM) + BA (7.9 µM). (F) with 2,4-D (100 µM) + 2iP (7.9 µM). (G) Embryogenic callus with embryos in torpedo and globular stages in medium with 9 µM 2,4-D + 1000 µM putrescine after 7 months. (H) Somatic embryos in globular and scutellar stages in medium with 1000 µM putrescine. (I) Somatic embryos in growth regulator-free medium after 8 months. (J) In torpedo stage in growth regulator-free medium after 9 months. K) Converted embryo after two weeks in growth regulator-free medium under light. (L) After three weeks. Bars: A, C, F, H, I, J and K = 2 cm. B = 1 cm. D, E, G and L = 3 cm. Source: The author (2014).

development and a transparent gelatinous mass (Figure 2B and C) and only 17.7% of them formed calli (Table 7). After 60 days of culture, the calli were friable (Figure 2D). After 90 days the culture medium supplemented with 500 µM 2,4-D gave the highest percentage of yellow (embryogenic) and translucent (non-embryogenic) calli (47.7 and 32.7%) differing from medium supplemented with 800 µM 2,4-D (12.2 and 14.7% respectively) (Table 7).

The concentration of 2,4-D affected the development of the callus as 30% of the TCLs cultured in the presence of 250 and 500 µM 2,4-D formed calli while no callus was formed or was formed in low percentages at concentrations above 500 µM (2 and 4% respectively for 800 and 1000 µM) (data not shown).

After transfer of calli to Y3 culture media with or without 2,4-D (50 or 100 µM) and putrescine, embryogenic calli containing somatic pro-embryos developed independently of the presence of 2,4-D (49 and 53% respectively) (data not shown) (Figure 2E and F) with 5 to 20 embryos at

Table 2. Formation of callus from zygotic embryos of the hybrid oil palm BRS Manicoré after 30 days in two culture media supplemented with 500 µM auxin and consistency of calli after 90 days.

Culture media	30 days		90 days	
	Formation of callus (% of explants)	Non-responding explants (%)	Friable calli (%)	Compact calli (%)
MS+2,4-D	52.83[a]	47.17[a]	62.11[a]	15.80[b]
MS+Picloram	65.65[a]	34.35[a]	43.00[b]	39.00[a]
Y3+2,4-D	76.32[a]	23.68[a]	44.59[b]	36.40[a]
Y3+Picloram	60.90[a]	39.10[a]	59.29[a]	36.00[a]
LSD	32.6	38.62	7.66	6.82

Means followed by the same letter are not statistically different by Tukey´s test at 5% probability. Callus formation after 90 days: data not shown. LSD = Least significant difference.

Table 3. Formation of callus from zygotic embryos of hybrid oil palm BRS Manicoré in Y3 culture medium supplemented with three concentrations of 2,4-D after 30 days.

Concentration of 2,4-D (µM)	30 days	90 days	
	Formation of callus (% of explants)	Friable callus (%)	Compact callus (%)
Control	0.25[b]	----	----
250	49.10[a]	58.7[a]	41.4[b]
500	62.27[a]	72.1[a]	27.8[b]
750	31.30[ab]	23.7[b]	76.2[a]
LSD	47.22	34.23	33.89

Values are mean values of four seed lots. Means followed by the same letter are not statistically different by Tukey's test at 5% probability. LSD = Least significant difference.

Table 4. Multiplication of callus formed from zygotic embryos of oil palm hybrid BRS Manicoré after 60 days in Y3 culture medium supplemented with 100 µM auxin and 7.9 µM cytokinin.

Plant growth regulators	Embryogenic callus		Non-embryogenic callus	
	(n/N)*	(%)	(n/N)*	(%)
2,4–D	12/31	38.75[b]	19/31	59.87[bc]
PICLORAM	5/31	16.12[c]	26/31	83.875[a]
2,4–D + 2iP	15/29	51.65[a]	14/29	48.30[d]
PICLORAM + 2iP	25/64	39.06[b]	39/64	60.93[b]
2,4–D + BA	20/37	54.06[a]	17/37	45.88[d]
PICLORAM+ BA	28/56	50.00[a]	28/56	50.00[cd]
LSD	9.89		10.44	
CV%	5.99		4.55	

Means followed by the same letter are not statistically different by Tukey's test at 5% probability. Data not shown for calli without development. Values are averages of two replications. *n = Number of calli, embryogenic or not; N = total number of calli; CV = coefficient of variation; and LSD = least significant difference.

globular and torpedo stage per callus (Figure 2G). Somatic embryos at the torpedo stage were separately converted into plantlets (Figure 2H, I and J).

Approximately 230 SEs were converted into plants on conversion medium (growth regulator-free medium) under light. After two weeks they started to turn green and developed cotyledonary petioles and primary roots (Figure 2I and J). After 45 days, 50 plants out of a total of 230 (21.7%) had root and shoot parts.

The process of somatic embryogenesis from TCLs was divided into three steps until the conversion of SEs into plants (Table 6).

Table 5. Somatic embryo formation from zygotic embryo-derived calli of palm tree hybrid BRS Manicoré after 60 days in Y3 culture medium supplemented with 2,4-D and 1000 µM putrescine.

2,4-D (µM)	Callus with embryos*		Embryogenic callus***		Friable callus	
	(n/N)**	(%)	(n/N)*	(%)	(n/N)*	(%)
0	34/91	37.36[a]	53/91	58.24[a]	4/91	4.39[a]
9	53/121	43.80[a]	56/121	46.28[a]	14/121	11.57[a]
27	19/101	18.81[b]	58/101	57.48[a]	24/101	23.76[a]
LSD		79.74		69.59		39.33
CV%		119.76		65.56		149.71

Means followed by the same letter are not statistically different by Tukey´s test at 5% probability. Values are averages of two replications *In scutellar and torpedo stages. **n = Number of calli with embryos; N = total number of calli; *** with globular embryos; CV = Coefficient of variation; and LSD = least significant difference.

Table 6. Stages of somatic embryogenesis and media composition for palm tree hybrid BRS Manicoré. Basal medium is Y3 (Eeuwens, 1976).

Time (months)	From zygotic embryos	Time (months)	From leaf TCLs
1st - 3d	**Callus induction** 250 µM 2,4-D, 2 g.L^{-1} AC, 30 g.L^{-1} sucrose, 6 g.L^{-1} agar	1st - 3d	**Callus induction** 250 µM 2,4-D, 2 g.L^{-1} AC, 30 g.L^{-1} sucrose, 2.5 g.L^{-1} Gelzan
4th - 5th	**Callus multiplication** 100 µM 2,4-D or Picloram, 7,9 µM BA, 2 g.L^{-1} AC, 30 g.L^{-1} sucrose, 6 g.L^{-1} agar	4th - 6th	**Embryogenic callus and SE formation** 100 µM put, 0,5 g.L^{-1} AC, 30 g.L^{-1} sucrose, 2.5 g.L^{-1} Gelzan
6th - 8th	**Conservation of embryogenic calli and SE** formation** 1000 µM put*, 0.5 g.L^{-1} AC, 30 g.L^{-1} sucrose, 6 g.L^{-1} agar	7th - 8th	**Conservation, SE maturation and conversion** 2 g.L^{-1} AC, 30 g.L^{-1} sucrose, 2.5 g.L^{-1} Gelzan
9th - 10th	**Maturation and SE conversion** 2 gL^{-1} AC, 30 g.L^{-1} sucrose, 6 g.L^{-1} agar.		

*Putrescine, **somatic embryo.

Table 7. Formation of callus from shoot TCLs of hybrid oil palm BRS Manicoré cultivated in Y3 culture medium supplemented with 2,4-D after 60 and 90 days.

	60 days		90 days		
2,4-D Concentration (µM)	Callus Formation (%)	Non-responding explants (%)	Yellow Callus (%)	Translucent Callus (%)	Non-responding explants (%)
500	39.45 a	59.65 a	47.73 a	32.76 a	19.50 b
800	17.70 a	82.30 a	12.20 b	14.73 a	73.06 a
LSD	31.38	47.6	32.73	54.57	59.08
CV%	63.49	30.6	31.38	99.0	56.26

Means followed by the same letter are not statistically different by Tukey´s test at 5% probability. Average of four repetitions. CV = coefficient of variation. LSD = Least significant difference.

DISCUSSION

Both auxins 2,4-D and Picloram were efficient in inducing somatic embryogenesis from zygotic embryos of hybrid oil palm (BRS Manicoré). By culturing zygotic embryos of Tenera oil palm in N6 medium (Chu et al., 1975) supplemented with 9 µM 2,4-D, Dicamba or Picloram, Thuzar et al. (2011) were able to obtain 32, 17 and

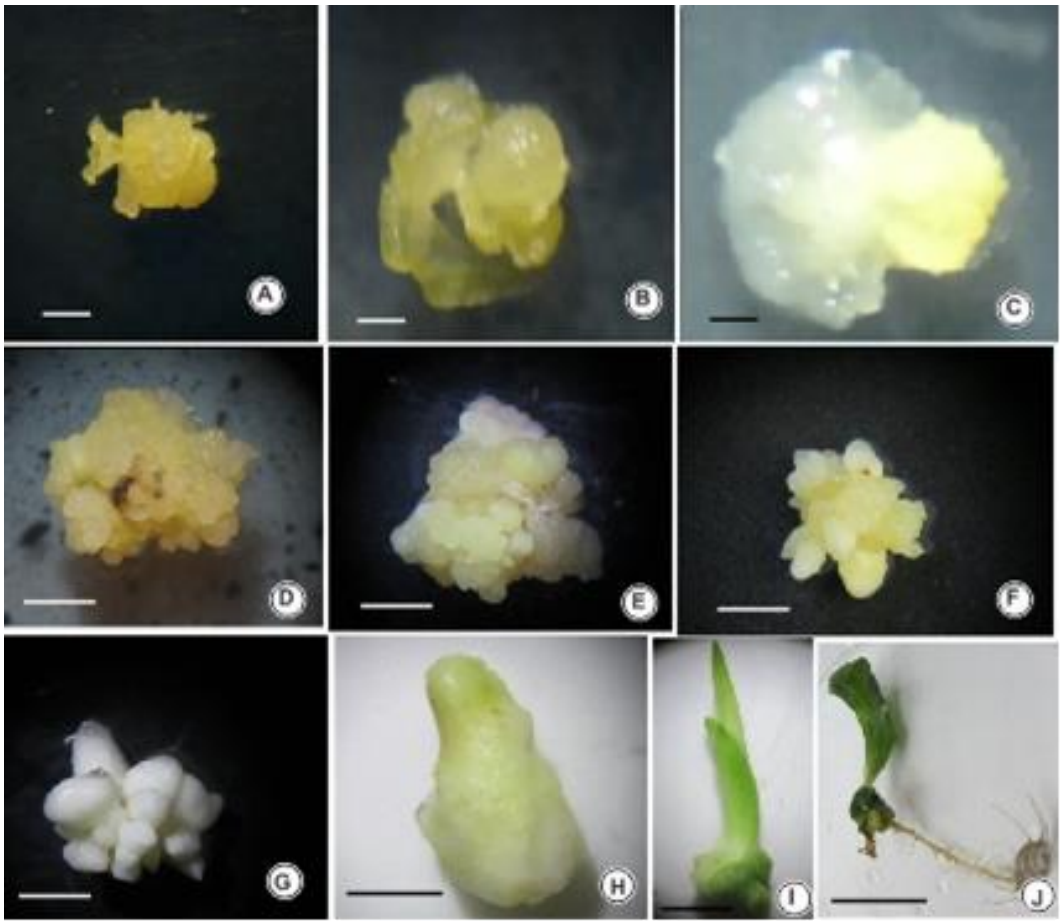

Figure 2. Stages of somatic embryogenesis from shoot TCLs of hybrid oil palm BRS Manicoré cultured on Y3 medium. (A) With 500 µM 2,4-D after one month. (B) and (C) With 800 uM 2,4-D after one month. (D) Embryogenic callus on medium with 100 uM 2,4-D + 100 uM putrescine after 5 months. (E) On medium with 50 uM 2,4-D + 100 uM putrescine after 5 months. (F) Globular embryos on growth regulator-free medium after 8 months. (G) Somatic embryo in growth regulator-free medium after 8 months and 10 days. (H) Somatic embryo individualized after 9 months. (I) Embryo converted into seedling after 14 days in growth regulator-free Y3 medium. (J) Seedling after 21 days. Bars: A, B and C = 1 cm. D, E, F and G = 1.5 cm. H, I and J = 2 cm. Source: the author (2014).

21.5% of embryogenic calli, respectively. These were smaller values than those obtained in the Y3 medium supplemented with 2,4-D or Picloram in the present study (76.3 and 60.9%).

For this hybrid, concentrations of 2,4-D or Picloram between 250 and 500 µM were suitable for initiation of somatic embryogenesis when the auxin was added to mMS or Y3 culture medium. Muniran et al. (2008) cultivated zygotic embryos of *E*. *guineensis* "Dura" and observed that Y3, MS and N6 media were effective for callus formation when supplemented with 11 µM 2,4-D, obtaining respectively 98, 80 and 82% of explants forming calli. It should be noted that although our hybrid cultivar needs a particular concentration and type of auxin, other cultivars or genotypes may require other concentrations. The concentrations of 250 or 500 µM used in this study were effective but for varieties SJ-165 and SJ-167 of *E. guineensis* × *E. oleifera* concentrations of 375 and 625 µM 2,4-D were necessary for callus formation in zygotic embryos cultured in half-strength MS culture medium (Alves et al., 2011). Similar responses were obtained by Silva et al. (2012) for different genotypes of *E. guineensis* in MS culture medium containing 450 µM Picloram. Those authors showed that two of those genotypes had high potential for the formation of embryogenic calli and differentiation into SEs.

The combination of low concentrations of auxin (2,4-D or Picloram) with a cytokinin (BA or 2iP) is necessary for the multiplication of the calli. In this study, 100 µM 2,4-D or Picloram, combined with 7.9 µM BA or 2iP, led to callus proliferation in 50 to 54% of explants. Similar results were obtained for *E. guineensis* genotypes BRS C2328: On MS medium containing 0.6 µM ANA and 12.3

µM 2iP, 48% of the calli were embryogenic and 52.5% of them produced SEs (Silva et al., 2012). Likewise, in *Acrocomia aculeata*, Padilha et al. (2015) observed friable calli in 53% of TCLs cultivated in Y3 culture medium supplemented with 75 µM Picloram and 12.5 µM 2iP. These results show the importance of auxin/cytokinin balance for embryogenic callus multiplication.

The project presented here depicts the formation of SEs on media without 2,4-D or with a low concentration of 2,4-D (9 µM) and in the presence of putrescine (1 µM), with an average of 25 to 30 embryos per callus. The effect of polyamines on growth, differentiation and senescence of tissues cultured *in vitro* has already been described by Kaur Sawhney et al. (1985), indicating a causal connection between polyamine titer and cellular activity and differentiation. The positive effect of putrescine on the SE process has been observed by Rajesh et al. (2003) who obtained somatic embryogenesis in palm tree calli cultivated in modified MS medium containing 1 µM putrescine and 0.045 µM 2,4-D. This effect was also verified for other species, such as *Araucaria angustifolia* (Silveira et al., 2006), *Ocotea catharinensis* (Santa-Catarina et al., 2007), *Citrus sinensis* (Wu et al., 2009) and *Saccharum officinarum* (Reis et al., 2015). Moreover, the latter authors showed that addition of putrescine to the culture medium induced proteomic changes in six classes of proteins previously associated with somatic embryogenesis process. In tissue culture of several species, addition of polyamines or their biosynthesis inhibitors to culture medium demonstrated their role in somatic embryogenesis regulation and the importance of the putrescine/spermidine ratio (Kakkar et al., 2000). However, their requirement for tissue culture may be not universal and varies according to the stage of embryo development (Kakkar et al., 2000; Bais and Ravishankar, 2002). In our case, putrescine applied alone positively affected somatic embryogenesis but spermidine was not tested. The internal level of these polyamines in the calli should be measured in order to understand the way they influence this process.

The formation of SEs in the absence of 2,4-D is very important as it avoids problems of somaclonal variation. This disturbance was defined as “a phenotypic variation, either genetic or epigenetic in origin, displayed among somaclones, namely plants derived from any form of cell culture involving the use of somatic plant cells” (Schaeffer, 1990). In Tenera oil palm, SEs were also obtained when the embryogenic calli were transferred to N6 maturation medium supplemented with 0.45 µM 2,4-D, 1.8 µM putrescine, 0.5 $g.L^{-1}$ hydrolyzed casein and 2 $g.L^{-1}$ AC (Thuzar et al., 2011).

For the conversion of somatic embryos into plants, plant growth regulator-free medium was efficient (Figure 1I and 2F-G). The plants produced shoots and roots and these were more developed than the roots of plants from *in vitro* germination of zygotic embryos. This may be because these somatic embryos are in contact with auxin from the beginning of callus induction and the roots develop after having absorbed a sufficient amount of auxin from the media of previous stages. Teixeira et al. (1994) also observed the regeneration and conversion of SEs in palm plants on MS medium devoid of growth regulators.

TCL technique using the base of the aerial part of seedlings grown *in vitro* is suitable for callus induction and obtaining SEs of the hybrid oil palm BRS Manicoré. This technique stood out from the works of Tran Thanh Van (1973) which considered that “the cells in TCL systems are literally reprogrammed in order to express all patterns of differentiation” (Tran Thanh Van, 2003). This system is considered efficient for the regeneration of embryos in different species, including palm trees (Samosir et al., 1998; Nhut et al., 2003; Steinmacher et al., 2007). The use of small explants is also advantageous as they have greater contact with the culture medium and can absorb the nutrients of the medium more easily (Fehér et al., 2003).

When cultivated in a medium containing 2,4-D (250 and 500 µM) TCLs formed calli in 33.1 and 31.8% of the explants but none when cultured in a 2,4-D-free medium. Similar results were obtained by Pádua et al. (2013) who observed callus induction in leaf explants of *E. guineensis* var. Tenera, grown in Y3 culture medium with Picloram or 2,4-D (9 µM) and no callus formation in auxin-free media. The pioneer work of Skoog and Miller (1965) showed that a proper balance between auxin and cytokinin in the medium stimulates callus formation in carrot tissues. However, internal balance of our explant is not known. In many cases, a strong auxin applied exogenously induces callogenesis without a need for cytokinin addition.

For the formation of embryogenic calli and SEs, calli need less exposure to auxin if it is combined with a cytokinin or putrescine. In this study, SEs were obtained in the culture medium with 2,4-D as well as in the medium without 2,4-D, both containing 100 µM putrescine. SE were also obtained in *Euterpe edulis*, using a combination of auxin and cytokinin (0.54 µM NAA and 9.8 µM 2-iP) (Guerra and Handro, 1998).

In this research, the process of somatic embryogenesis from TCL of hybrid oil palm BRS Manicoré was more effective than from ZE due to the fact that one plant provides five to eight explants to be used in the formation of callus. The cost of the technique is also approximately 20% lower because the process is reduced to three steps until SEs are converted into plants and the plant growth regulators (auxin, cytokinin and putrescine) are used in two phases only instead of three (Table 6). This means a smaller number of transfers to a new medium and lower labor costs. The calli derived from ZE provided one to five more embryos than those from TCL, since they remained two months in a culture medium containing cytokinin and auxin, whereas the TCLs were transferred directly to a

medium containing putrescine. However, TCL technique is still better, since an average of 30 to 50 SEs are formed from a single plant (five explants) whereas 3 to 4 ZE would be required to obtain the same number of ES.

In conclusion, protocols for somatic embryogenesis of hybrid *E. guineensis x E. oleifera* were described for the first time with two kinds of starting explants. In summary, for callus induction from ZE or TCLs, the use of 2,4-D at 250 µM is necessary. The combination of 100 µM auxin with a cytokinin is efficient for multiplication when calli are derived from ZE or with putrescine in TCL-formed calli. Embryogenic calli and SEs are formed in media without 2,4-D or with putrescine (100 or 1000 µM). SEs are converted into complete plantlets in Y3 culture medium devoid of plant growth regulators. Different auxin types, such as naphthaleneacetic acid, 2,4-D and Picloram, as well as concentrations should be adapted for each species or genotype/cultivar.

Conflicts of interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Genetic diversity of rice *(Oryza sativa)* germplasm from six countries using simple sequence repeats markers

Ibrahim Soe[1]*, Richard Akromah[2], Daniel Nyadanu[2], Maxwell Darko Asante[3] and Alex Tamu[1]

[1]Sierra Leone Agricultural Research Institute, Rokupr, Sierra Leone.
[2]Department of Crop and Soil Sciences, Faculty of Agriculture, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.
[3]CSIR-Crops Research Institute, Kumasi, Ghana.

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This experiment was carried out to determine the genotypic variation among rice *(Oryza sativa)* accessions using simple sequence repeats (SSR) markers. In the present study, a total of 12 SSR markers were used across 87 rice accessions from six countries. NTSYS-pc and PowerMarker software were used for data analysis. Six primers out of these 12 primers showed DNA amplification and polymorphism among the 87 rice accessions. The number of alleles detected by these six primers ranged from 2 to 9 with an average of 6.83 while polymorphism information content (PIC) ranged from 0.34 to 0.79 with an average of 0.55. The unweighted pair group method with arithmetic averages (UPGMA) cluster dendrogram generated based on the six SSR markers grouped the accessions into 4 clusters with 41% similarity coefficient. Accessions from these four clusters have late maturity, green basal leaf sheath colour, no awn and fewer tillers, respectively. This experiment has proven that even a small number of SSR markers are effective in assessing genetic diversity in rice. The genetic diversity revealed by the SSR markers in this study would be very important to select potentially good genotypes for future rice improvement programmes.

Key words: Dendrogram, genetic diversity, molecular markers, rice.

INTRODUCTION

Rice (*Oryza sativa* or *Oryza glaberrima*) is consumed by more than 50% of the world's population especially in developing countries. In terms of production levels, it is the third highest cereal after wheat and maize (FAOSTAT, 2012). By the year 2025, global demand for rice will be 880 million tonnes which is 70% more of the present world production (IRRI, 2010). The average growth rate of rice yield was 3.68% annually in the 1980s, but it decreased to 0.75% per year in the late 1990s (Nguyen and Ferrero, 2006). Besides, the plateauing of yields, other challenges that could limit increased rice production include biotic and abiotic stresses, declining productivity in intensive rice production systems, increasing cost of production, and increasing public

*Corresponding author. E-mail: soeibrahim@gmail.com.

concern for the protection of natural resources (Nguyen and Ferrero, 2006). In years to come, expanding the areas of rice cultivation will be limited because of land and water resource scarcity due to climate change, urbanization and population growth especially in Asia where more than 50% of the world rice is produced (Devi and Ponnarasi, 2009).

One of the major ways of addressing the issues affecting rice production and also increasing the average yield of this rice crop is through breeding for rice varieties that produce higher yields per unit land area, and also meeting the world's rice requirements will depend upon the development of high yielding genotypes that have resistance against biotic and abiotic stresses using conventional and biotechnological approaches (Paterson et al., 2005).

The effectiveness of any rice breeding programme depends on the utilization of different germplasm stock available in research organizations/institutes around the world (Susan et al., 2012). This will enable breeders to evaluate and select desirable varieties for breeding programmes.

There have been extensive efforts by rice breeders around the world to improve the quantity and quality of rice by crossing varieties. These efforts have produced many rice varieties. It is foreseen that more will follow as environmental conditions and consumers' desires are changing. However, for breeding efforts to be successful, the genetic resources available to plant breeders need to be assessed accurately using molecular markers (Meti et al., 2013). In contrast to morpho-agronomic traits, molecular markers can reveal differences existing among genotypes at the DNA level. Molecular markers have been proven to be very objective and independent of environmental conditions (Se-Jong et al., 2012). Therefore, the aim of this study was to evaluate the level of genetic diversity among 87 rice accessions from six countries using simple sequence repeats markers.

MATERIALS AND METHODS

Plant and DNA isolation

A total of 87 accessions of rice germplasm from Ghana (CRI and SARI), Thailand, Mali, Benin (Africarice), Cameroon and Philippines (IRRI) were collected and used in this study (Table 1). Genomic DNA was extracted from young fresh leaves of three weeks old plants of the 87 rice accessions using the cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle (1990). Samples were stored at 4°C until it was required for use.

Molecular markers and polymerase chain reactions

Twelve simple sequence repeats markers (SSR) covering 10 of the 12 chromosomes of rice were used to detect polymorphism among the rice accessions (Table 2). The SSRs markers were procured from Metabion International AG (Germany). Polymerase chain reaction (PCR) was carried out in Eppendorf mastercycler (Eppendorf, Hamburg, Germany) of 96-well plates. PCR kits (KAPA 3G Fast Ready Mix) procured from KAPA Biosystems (Pty) Ltd (South Africa) was used for the reaction. Total volume of 15 µl with final concentrations of 1 X KAPA Plant PCR Buffer + dNTPs and 1.5 mM $MgCl_2$, 0.3 µM forward and reversed primers, 0.1 X KAPA Plant PCR Enhancer, 1 U/µl KAPA 3G Plant DNA Polymerase and 10 µg/µl of crude DNA.

PCR amplification was subjected to initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 1 min and a final extension at 72°C for 7 min. The reactions (PCR products) were then held at 4°C until electrophoresis.

Gel electrophoresis

PCR products obtained were electrophoresed using 2% agarose gel stained with ethidium bromide solution. The tracking dye in the PCR premix (KAPA 3G) made visual tracking of the PCR products through the gel. Approximately, 10 µl of the amplified products and 5 µl 100 bp molecular ladder (Universal ladder) obtained from KAPA Biosystems (Pty) Ltd (South Africa) were electrophoresed at 120 V for 120 min using Galileo Bioscience (81 to 2325) tank. A control was loaded in the first well and the molecular ladder was loaded into well two and the DNA of the rice accessions were loaded in the adjacent wells. The gel was visualized by illumination on Benchtop UV transilluminator. The gels were photographed under UV light.

Scoring of DNA bands

Amplified polymorphic products from microsatellite analyses were scored qualitatively for absence (0) and presence (1) for each marker allele-genotype combination.

SSR data analysis

The number of alleles per locus, major allele frequency, gene diversity, heterozygosity and polymorphism information content (PIC) values were all calculated using PowerMarker version 3.25 (Liu and Muse, 2005). NTSYS-pc version 2.21q was used to construct unweighted pair group method with arithmetic averages (UPGMA) dendrograms to show the distance-based relationship among the rice accessions.

RESULTS

A total of 12 SSR loci were evaluated for their efficiency of polymorphism across 87 accessions of rice received from different countries. Among the 12 SSR primers used in this study, six yielded scorable amplification products (Table 3). Forty one alleles with a mean of 6.83 alleles per locus were obtained from these six SSR primers. The number of alleles per locus ranged from 2 [Xtxp 284 (1)] to 9 [Xtxp 10 (9)]. Major allele frequency ranged from 0.31 to 0.73 with an average of 0.54. The locus Xtxp 149 (1) was the most informative since it had the highest level of polymorphism with PIC value of 0.79 and gene diversity value of 0.81 (Table 3). Xtxp 149 (1) also had the highest heterozygosity of 0.91 followed by Xtxp 201 (2) 0.87. Xtxp 284 (1) with a scoring of 0.00 had the lowest heterozygosity.

Genetic relationship revealed by the six SSR primers

Table 1. Plant materials and their source.

S/N	Accession	Source	S/N	Accession	Source
1	WAB 2081-WAC B-TGR4-B	AfricaRice, Benin	47	BASMATI 123	CSIR-SARI, Ghana
2	WAB 2125-WAC B-1-TGR3-WAT B1	AfricaRice, Benin	48	CRI-30	CSIR-SARI, Ghana
3	IR 841 (CHECK)	AfricaRice, Benin	49	CRI-2	CSIR-SARI, Ghana
4	DKA-M2	AfricaRice, Benin	50	CRI-45	CSIR-SARI, Ghana
5	JASMINE 85	CSIR-SARI, Ghana	51	CRI-73	Cameroon
6	FAROX 508-3-10-F43-1-1	AfricaRice, Benin	52	CRI-48	Mali
7	FAROX 508-3-10-F44-2-1-1	AfricaRice, Benin	53	NERICA 1	Mali
8	WAB 2098-WAC2-1-TGR2-WAT B2	AfricaRice, Benin	54	AFRK-7	Mali
9	WAB 2056-2-FKR2-5-TGR1-B	AfricaRice, Benin	55	AFRK-8	Mali
10	WAB 2060-3-FKR1-WAC2-TGR4-B	AfricaRice, Benin	56	AFRK-5	Mali
11	TXD 88	AfricaRice, Benin	57	AFRK-13	Mali
12	WAB 2098-WAC3-1-TGR1-4	AfricaRice, Benin	58	NERICA 4	Mali
13	WAB 2076-WAC1-TGR1-B	AfricaRice, Benin	59	AFRK-6	Mali
14	WAB 2081-WAC2-2-TGR2-WAT B3	AfricaRice, Benin	60	AFRK-2	Mali
15	GBEWAA	CSIR-SARI, Ghana	61	AFRK-11	Mali
16	PERFUME IRRIGATED	Thailand	62	NERICA 14	Mali
17	WAS-122-13-WAS-10-WAR	AfricaRice, Benin	63	AFRK-9	Mali
18	LONG GRAIN ORDINARY 2	Thailand	64	AFRK-3	Mali
19	EXBAIKA	CSIR-SARI, Ghana	65	AFRK-1	Mali
20	WAS-163-B-5-3	AfricaRice, Benin	66	AFRK-10	Mali
21	FAROX 15	CSIR-SARI, Ghana	67	AFRK-5	Cameroon
22	PERFUME SHORT	Thailand	68	AFRK- 4	AfricaRice, Benin
23	KATANGA	CSIR-SARI, Ghana	69	IR 74963-2-6-2-5-1-3-3	IRRI, Philippines
24	TOX 3107	CSIR-SARI, Ghana	70	IR 81412-B-B-82-1	IRRI, Philippines
25	ANYOFULA	CSIR-SARI, Ghana	71	IR 55419-04	IRRI, Philippines
26	NABOGU	CSIR-SARI, Ghana	72	IR 79913-B-179-B-4	IRRI, Philippines
27	GR 21	CSIR-SARI, Ghana	73	APO	IRRI, Philippines
28	PHKA RUMDON	Cameroon	74	N22	IRRI, Philippines
29	MLI 20-4-1-1-1	Mali	75	IR 77298-14-1-2-10	IRRI, Philippines
30	DKA-M2	Mali	76	KALIAUS	IRRI, Philippines
31	SIK 353-A-10	Mali	77	UPL RI 7	IRRI, Philippines
32	DK 3	Mali	78	KALIA	IRRI, Philippines
33	MLI 6-1-2-3-2	Mali	79	IR 74371-46-1-1	IRRI, Philippines
34	MLI 25-1-2	Mali	80	IR 74371-54-1-1	IRRI, Philippines
35	DKA 4	Mali	81	IR 80411-49-1	IRRI, Philippines
36	DKA- M8	Mali	82	IR81023-B-116-1-2	IRRI, Philippines
37	SIK 350-A-150	Mali	83	WAY RAREM	IRRI, Philippines
38	DKA-M11	Mali	84	VANDANA	IRRI, Philippines
39	DKA 22	Mali	85	IR 77298-5-6-18	IRRI, Philippines
40	DKA-M9	Mali	86	IR 74371-70-1-1	IRRI, Philippines
41	DKA 1	Mali	87	UPL RI 5	IRRI, Philippines
42	DKA 21	Mali			
43	MLI 20-4-3-1	Mali			
44	SBT 70	Cameroon			
45	BASMATI 113	Thailand			
46	AGRA RICE	CSIR-CRI, Ghana			

using similarity coefficients based on UPGMA is shown in Figure 1. From the figure, the 87 rice accessions were clustered into four major groups at 41% similarity coefficient. Cluster I contained 10 accessions from Benin

Table 2. SSR Primers and their sequences used in DNA fingerprinting.

SSR locus and chromosome location	Primer sequence (5' To 3')	Type of SSRs
Xtxp 149 (1)	F=AGCCTTGCATGATGTTCC R=GCTATGCTTGGTGTGGG	$(CT)_{10}$
Xtxp 284 (1)	F=CCAGATTGGCTGATGCATACACACT R=AAGGGTAATTTATGCACTCCAAGGTAGGAC	$(AAG)_{19}$
Xtxp 201 (2)	F=GCGTTTATGGAAGCAAAAT R=CTCATAAGGCAGGACCAAC	$(GA)_{36}$
Xtxp 197 (2)	F=GCGTCAATTAATCCAAACAGCCTC R=GAGTTCCTATTCCCGTTCATGGTGAT	$(AC)_{10}$
Cba (3)	F=AAAGCTCGGCGTTAGAAATA R=CGTTTAACAACTCGTACCATC	$(TA)_{18}$
Xtxp 51 (4)	F=TCTCGGACTCAAGAGCAGAGG R=GGACAGCAGCGGCTTCAG	$(TG)_{11}$
Xtxp 274 (6)	F=GAAATTACAATGCTACCCCTAAAAGT R=ACTCTACTCCTTCCGTCCACAT	$(TTC)_{19}$
Xtxp 278 (7)	F=GGGTTTCAACTCTAGCCTACCGAACTTCCT R=ATGCCTCATCATGGTTCGTTTTGCTT	$(TTG)_{12}$
Xtxp 295 (7)	F=AAATCATGCATCCATGTTCGTCTTC R=CTCCCGCTACAAGAGTACATTCATAGCTTA	$(CT)_{19}$
Xtxp 258 (9)	F=CACCAAGTGTCGCGAACTGAA R=GCTTAGTGTGAGCGCTGACCAG	$(AAC)_{19}$
Xtxp 10 (9)	F=ATACTATCAAGAGGGGAGC R=AGTACTAGCCACACGTCAC	$(CT)_{14}$
Xtxp 217 (10)	F=GGCCTCGACTACGGAGTT R=TCGGCATATTGATTTGGTTT	$(GA)_{23}$

F, Forward primer; R, Reverse primer (Missihoun et al., 2015).

Table 3. Allele number, major allele frequency, gene diversity, heterozygosity and polymorphism information content (PIC) values generated from six SSR molecular markers.

Marker	Allele no.	Major allele frequency	Gene diversity	Heterozygosity	PIC
Xtxp 149 (1)	8.00	0.31	0.81	0.91	0.79
Xtxp 284 (1)	2.00	0.69	0.43	0.00	0.34
Xtxp 201 (2)	8.00	0.52	0.61	0.87	0.55
Xtxp 197 (2)	8.00	0.73	0.44	0.27	0.43
Xtxp 278 (7)	6.00	0.54	0.57	0.31	0.50
Xtxp 10 (9)	9.00	0.47	0.71	0.42	0.68
Mean	6.83	0.54	0.60	0.46	0.55

(Africa rice), Thailand, Mali and Philippines (IRRI) which are late maturing, cluster II six accessions from Ghana (CSIR-SARI), Thailand and Mali, they have green basal leaf sheath colour. Majority of the accessions (57) studied were grouped into cluster III, they are accessions with no awn and broad leaf width. This cluster contained accessions from all the six countries in which collections were done and cluster IV, contains 14 accessions from Benin (Africa rice), Ghana (CSIR-SARI and CRI), Mali, Thailand and Philippines (IRRI); they have short culm

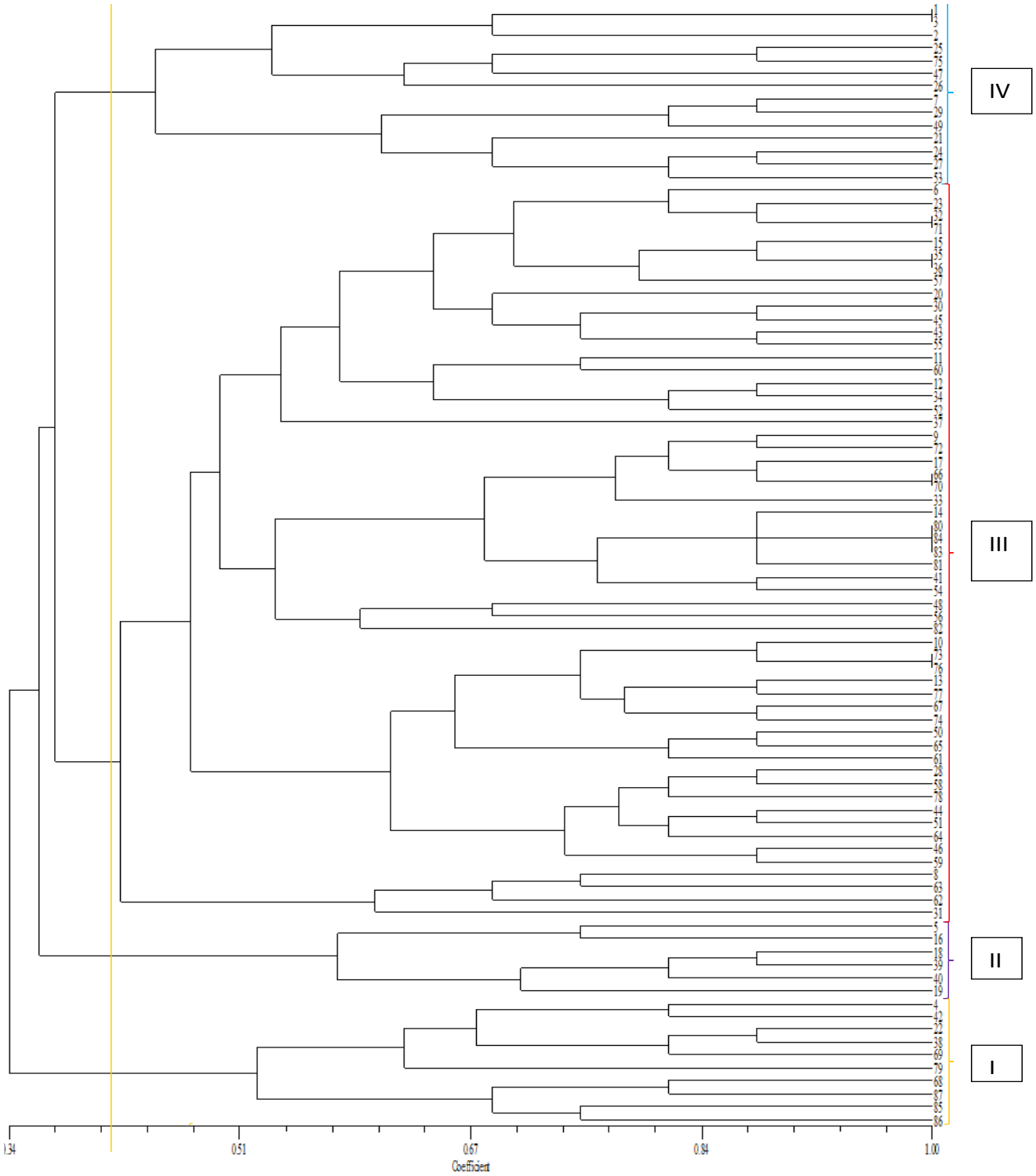

Figure 1. An UPGMA cluster dendrogram showing genetic relationships among 87 rice accessions based on six SSR markers. Number follows details in Table 1.

length and fewer tillers. Further, at 58% similarity coefficient, the four major clusters were divided into sub-clusters. Cluster I (IA and IB), cluster II (IIA and IIB), cluster III (IIIA, IIIB, IIIC, IIID, IIIE, IIIF and IIIG) and cluster IV (IVA, IVB and IVC). More than half of the accessions studied (49) from all six countries showed the

closest resemblance at a similarity coefficient of 88%. Accession [73(APO) and 76(KALIAUS) from Philippines (IRRI)], [66 (AFRK-10) from Benin (Africa Rice) and 70(IR 81412-B-B-82-1) from Philippines (IRRI)], [35(DKA 4) and 36(DKA- M8) from Mali], [32(DK 3) from Mali and 70 (IR 55419-04) from Philippines (IRRI)], [1(WAB 2081-WAC B-TGR4-B) and 3 (WAB 2125-WAC B-1-TGR3-WAT B1) from Benin (Africa Rice)] and finally [80 (IR 74371-54-1-1), 83(WAY RAREM) and 84(VANDANA) all three from Philippines (IRRI)] showed 100% similarity.

DISCUSSION

Success of rice improvement programmes depends on the amount of genetic variability and the degree to which the desirable traits are heritable (Ravi et al., 2003). Hence, assessment of genetic variability among genotypes becomes important in establishing relationships among different cultivars. Characterization using molecular markers is the alternative strategy to overcome the several limitations of morpho-agronomic traits characterization of genetic materials. Morphological characterization affected by environmental condition, requires a longer duration and may be more expensive. In the present investigation, twelve SSR markers were used to characterize and assess the genetic variability among 87 rice accessions collected from six countries. Only six out of these 12 microsatellite markers revealed genetic polymorphism and ensured unambiguous identification of the rice accessions. Small numbers of molecular markers can be used to assess genetic diversity as shown earlier in other studies. Ali et al. (2011) reported that a subset of 36 microsatellite markers gave nearly similar results as using 169 SSR markers for genetic diversity studies. These six SSR primers yielded a total of 41 alleles ranging from 2 [Xtxp 284 (1)] to 9 [Xtxp 10 (9)] with an average of 6.83 alleles per locus and were similar to those earlier reported by Ni et al. (2002). They used Indian quality rice germplasm and reported an average of 6.80 alleles per locus. The number of alleles detected in the present study is lower than those observed by Chakhonkaen et al. (2012), who reported a total of 127 alleles that ranged from 4 to 12 alleles using 19 InDel (Insertion-Deletion) markers to evaluate genetic diversity in 101 rice accessions. The average genetic diversity of 0.60 obtained was higher compared to 0.55 previously reported by Sajib et al. (2012), who used nine SSR markers to study genetic diversity among 12 aromatic landraces of rice. Polymorphism information content (PIC) is a measure of polymorphism among varieties for a marker locus used in linkage analysis (Sajib et al., 2012). It ranged from 0.34 to 0.79 with an average of 0.55 in this study. The PIC range and average observed in this study are similar to those reported earlier by Meti et al. (2013), they reported PIC range of 0 to 0.74 with an average of 0.58 using 12 SSR markers to estimate genetic diversity in 48 aromatic rice genotypes. Higher values of PIC might be the result of diverse genotypes and lower values may be the result of closely related genotypes (Prabakaran et al., 2010).

The dendrogram showed that there was genetic variation among the 87 rice accessions in relation to the SSR primers used. The similarity coefficient of these accessions ranged from 0.34 to 1.00, which is an indication of the genetic variation among the accessions based on the SSR primers. The variation observed among the accessions is an indication that SSR markers can reveal diversity existing between rice accessions. This is in agreement with earlier findings of Pervaiz et al. (2010); they reported that SSR markers are effective tools in discriminating rice genotypes. The accessions were grouped into four main clusters at a genetic similarity coefficient of 0.41. Also, at a similarity coefficient of 0.58, each of the four main clusters were divided into sub-clusters. Accession 73 (APO) and 76 (KALIAUS) both from Philippines (IRRI) showed 100% similarity revealing that no genetic variability exist between these two accessions based on the six SSR primers. The similarity could have risen from informal exchange of seeds (germplasm) among farmers, but given different names because of differences in dialect and ethnic groups. It is important to eliminate duplicates to enable effective management and conservation of germplasm.

Broadening the genetic base of rice in breeding programmes is urgently needed to enhance heterozygosity in crosses and create heterotic progenies. Overall, this study has explained the relevance of employing molecular markers to determine genetic distances and relationships in rice. Moderate level of genetic diversity was observed among the rice accessions.

Conflict of Interests

The authors have not declared any conflict of interests.

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The rolling circle amplification and next generation sequencing approaches reveal genome wide diversity of Kenyan cassava mosaic geminivirus

T. M. Kathurima[1, 2]*, E. M. Ateka[2], A. B. Nyende[2] and T. A. Holton[1]

[1]Biosciences East and Central Africa Hub, C/O ILRI, P. O. Box 30709-00100, Nairobi, Kenya.
[2]Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000-00200, Nairobi, Kenya.

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Rolling circle amplification is a simple approach of enriching populations of single-stranded DNA plant begomovirus genomes (genus, *Begomovirus*; family, Geminiviridae). This is an innovative approach that utilizes the robustness of the bacteriophage phi29 DNA polymerase used in circle amplification, together with deep sequencing using Illumina Miseq and bioinformatics to assess population diversity of begomoviruses in naturally infected cassava. The approach is suitable for detecting rare members in a population in begomoviral populations in situation where mixtures of isolates, strains, and multiple species occur. The main objectives were to increase the sensitivity of detection of next generation sequencing by enriching it using rolling circle amplification then determination of the diversity of the cassava mosaic geminivirus. This was done by total nucleic acids isolated from symptomatic, field cassava infected plants, then using rolling circle amplification to multiply the less abundant viral sequences. Enriched and non-enriched virus-libraries were subjected to deep sequencing using Illumina Miseq. Using bioinformatic CLC Genomics 5.5.1 software programs the quality assessment of reads and contig assembly of viral sequences. This was done through de novo and reference-guided assembly. The identity and diversities of the begomoviral sequences were compared with sequences in Sanger sequencing of viral components deposited in the NCBI Gene Bank. In this study we have demonstrated that RCA increases the chances of detecting the virus by approximately 10 to 1000 fold and wide genome diversity of cassava mosaic geminivirus in various cassava growing zones in Kenya were detected. In conclusion, this approach described herein is simple and will enhance the exploration of begomovirus diversities from cassava infected plants, irrespective of their viral abundance. This will make it possible for routine screening of field samples as the cost of deep sequencing NGS is decreasing and the advances of bioinformatic software development become enhanced. This is the first report of the RCA-Illumina-NGS approach to explore cassava infected with begomoviruses under field conditions and their diversities.

Key words: Illumina Miseq sequencing, geminivirus, ssDNA viruses, viral sequence enrichment, de novo genome assembly, rolling cycle amplification (RCA).

INTRODUCTION

Cassava is cultivated as a subsistence crop in developing countries across the world where its roots provide a source of dietary carbohydrates for over 700 million people. In East and Central Africa, cassava mosaic disease (CMD) is the most damaging plant virus disease as well as the world with an epidemic causing annual crop losses valued at between US$ 1.9 and 2.7 billion (Patil, 2009). Cassava mosaic disease is caused by a

complex of cassava mosaic geminiviruses (CMG's), family Geminiviridae, and genus *Begomovirus*. The viruses are spread by the whitefly *Bemisia tabaci* (Gennadius) (Brown, 2007). CMG's are of two types monopartite and bipartite genomes. The bipartite has genome components, called DNA-A and DNA-B, single-stranded DNA comprising 2.7 kb circular molecules while the monopartite lacks the DNA-B component and it is approximately 2.9 kb. DNA-A encodes proteins and regulatory elements that play the role of replication, encapsidation and control of gene expression while DNA-B encodes proteins enabling viral movement (Jeske, 2009). The rolling-circle mechanism just like the ssDNA-containing bacteriophages is the mechanism utilized by the genomic ssDNA to replicate utilizing double-stranded DNA (dsDNA) intermediates, in the nucleus of the host cell.

The DNA-A and DNA-B components share no much sequence similarity except for a short sequence regarded to as the 'common region' (CR) of approximately 200 nucleotides. The common region is the initiation replication site for rolling circle replication, and it is conserved among members of the family Geminiviridae (Harrison and Robinson, 2002). Both DNA components contain protein-coding regions in the viral strand and in the complementary strand. Six such genes seem to be universally present. On the complementary strand for the New World (NW) bipartite begomoviruses, contains one gene (*AV1*) on the viral sense strand and three genes (*AC1*, *AC2*, *AC3*) for the DNA-A component (Harrison and Robinson, 1999) and for the Old World (OW) bipartite begomoviruses an additional gene *AV2* in the viral sense strand and *C4* on the complementary strand (Hanley et al., 1999). The DNA-B component contains one gene each, *BV1* and *BC1*, on the sense and complementary strands of respectively (Sanderfoot et al., 1996).

In Africa, nine species of Begomovirus have so far been associated with cassava mosaic disease (Fargette et al., 1996; Bull et al., 2006; Tiendrébéogo et al., 2012). In Kenya, only four begomovirus species have previously been reported, namely ACMV, EACMV, and EACMZV (Stanley and Gay, 1983; Were et al., 2004a, 2004b).

The current experimental procedures used for detecting and determining the of genetic variability within begomoviral genomes utilize DNA by polymerase chain reaction (PCR) (Saiki et al., 1988) using specific or degenerate sequence primers, or use of random primers in rolling circle amplification (RCA) (phi29 DNA polymerase) (Dean et al., 2001), followed by cloning and sanger sequencing. Normally, the monopartite or bipartite DNA complete genome components of begomoviruses and their associated circular, ssDNA satellites are cloned from the products of RCA (Inoue-nagata et al., 2004) or virus-specific PCR (Briddon et al., 2000). The amplification of begomoviral genomic and associated components by PCR, followed by cloning and capillary DNA sequencing, are limited by the variant numbers produced at early steps of amplification specificity of the primers, and then by selection during the molecular cloning step. In another approach, begomoviral genome characterization has been achieved using a combination of restriction fragment length polymorphism (RFLP) and pyro-sequencing (Wyant et al., 2012).

Normally RCA produces high molecular weight products as dsDNA that are digested into unit-length components and cloned, with the inserts then verified by Sanger sequencing (Dean et al., 2001; Johne et al., 2009). This procedure is a time-consuming, and in addition, a few numbers of variants are represented among the resultant clones, based on the expectation that one or a few most frequent genotypes are the once represented in the starting material. In addition technical limitations can result in the inability to detect very low abundant begomoviral and/or associated DNA satellite molecules.

An innovative approach described here utilizes the robustness of the bacteriophage phi29 DNA polymerase used in RCA, together with deep sequencing using Illumina (Mardis, 2008; Bentley et al., 2008) and bioinformatics to assess population diversity of begomoviruses in naturally infected cassava. For virus discovery from field samples at the population level, traditional procedures such as RCA or PCR thereafter followed by cloning and Sanger DNA sequencing, are ineffective. The approach is suitable for detecting rare members in a population in begomoviral populations in situations where mixtures of isolates, strains, and multiple species occur. In the current study we demonstrated that circular single-stranded DNA-containing begomoviruses were enriched by RCA from total DNA extracts of naturally infected symptomatic cassava field plants. The enriched begomoviral genomes were subjected to Illumina Miseq sequencing. The short sequence reads obtained were assembled using bioinformatics tools, and were compared with genome sequences deposited in the genebank

MATERIALS AND METHODS

Cassava leaves identified with cassava mosaic disease were collected from Coast, Eastern, Western and Nyanza regions of Kenya where cassava growing is important. Twenty four samples

*Corresponding author. E-mail: kathurima07@gmail.com.

that detected positive for cassava mosaic geminiviruses with specific isolates primers on PCR were selected. Universal primers were used for detection of African cassava mosaic virus (ACMV) were JSP001 (5'-ATGTCGAAGCGACCAGGAGAT-3') ACMV for the forward primer and JSP002 (5'-TGTTTATTAATTGCCAATACT-3') ACMV for the reverse primer with an expected amplicon of 774bp. The detection of EACMV was done using EAB555 F/R primers whose sequences were EAB555/F (5'TACATCGGCCTTTGAGTCGCATGG-3') EACMV DNA-B and EAB555/R (5'CTTATTAACGCCTATATAAACACC-3') EACMV DNA-B with an expected amplicon of 556 bp fragment of EACMV DNA B component (Fondong et al., 1998). Of these four samples total DNA extract containing begomoviruses were enriched by RCA from and another four of the same samples were not enriched with RCA. The remaining 16 samples were not enriched but were chosen to represent major growing regions in Kenya.

The rolling circle amplification (TempliPhi)

The cassava geminivirus virus genome was amplified and isolated using the TempliPhi Kit (GE Healthcare, Buckinghamshire, United Kingdom) according to James et al. (2011). Two mixes were prepared. For master mix 1 (MM1), 5 µl of TempliPhi sample buffer was mixed with 1 µl of the isolated DNA and 1 µl of a 50 µM stock solution (4.16 pmol/ul of each primer) of TempliPhi degenerate primers. The mix was then heated at 95ºC for 3 min to denature the DNA followed by cooling to room temperature or 4°C. Master Mix2 was prepared by mixing 5 µl of TempliPhi reaction buffer and 0.2 µl of TempliPhi Enzyme Mix. Five micro liter of the TempliPhi premix (mix 2) was transferred to the cooled, denatured sample (MM1) then incubated at 30ºC for 18 h. After the incubation period, the enzyme (Phi29DNA polymerase) was heat-inactivated at 65ºC for 10 minutes. The samples were then cooled and stored at 4°C. Cleaning of the samples was done using Qiagen kit following the manufacturers' procedure. The amplified clean DNA was used directly in Illumina next generation sequencer.

Preparation of nextera libraries for Illumina MiSeq sequencing

A good quality DNA presented by optical density of 260/280 and 260/230 purity indices equal to or greater than 1.8 to 2.0 values from spectrophotometry were selected from 24 samples for next generation sequencing. The DNA libraries were prepared from total DNA extract concentration from ranging from 0.05 to 3.3 ng/µl using the Illumina nextera DNA Sample Preparation kit^{TM} according to the manufacturer's instructions (Illumina, San Diego, California). The first step involved DNA fragmentation with addition of adapter sequences to the ends to allow for amplification by PCR. Addition of indexes and enrichment was done. The final size and concentration of each library was estimated using a Bioanalyzer (Agilent, Santa Clara, CA, USA) and the Qubit (Invitrogen, Carlsbad, CA, USA), respectively. Library pools of 2 nM were prepared by mixing the libraries from each sample to achieve an equal molar concentration of each. Libraries were normalized, pooled and sequenced using a 2×300 -cycle PE V3 Illumina kit. Paired end reads were generated using the Illumina MiSeq System at the Biosciences Eastern and Central Africa - International Livestock research Institute (BecA-ILRI) Hub in Nairobi, Kenya.

Comparison of sensitivity between RCA enriched and non RCA enriched samples

Serial dilutions of 10^{-1} to 10^{-7} of the same positive batch of enriched and non-enriched total DNA extracted were made in duplicates and a negative control included. Polymerase chain reaction was carried out in a thermocycler in a reaction of 20 µl in AccuPower®Bioneer premix, 0.1 µM forward and reverse primer and 2.0 µl DNA template. The reaction profile was followed by 35 cycles of 94°C (30s), 52°C (30s), 72°C for 1 min and 72°C for final extension. PCR products were analyzed by electrophoresis in 1X TAE buffer on 2% agarose gel stained with gel red and image captured by a camera under UV light. The PCR assays were performed in technical triplicates.

Illumina Miseq Sequence short sequence assembly and analysis

Sequences from the 24 libraries were screened for quality where sequences with a value less than 25 were trimmed. The short reads were then subjected to reference/de novo assembly using CLC Genomics 5.5.1 software using default settings. The number of sequence reads from CMGs contigs extracted from non RCA and RCA were compared. The genome sequences assembled were also subjected to phylogenetic analysis together with those from genebank sequences by the neighbor-joining method, using MEGA6 software.

RESULTS

Of the 24 libraries sequenced 20 had concentrations ranging from 1.0 to 3.3 ng/ul produced good number of pair ended reads while 4 libraries had less than 0.06 ng/ul and had very low number of reads and consequently could not be assembled**.** The de novo assembly of the high-throughput Illumina 6 million (6M) reads was carried out using CLC Genomics 5.5.1 software default settings resulted in a large number of contigs that ranged from 1,456 to 42,181 with an average length of 254 to 388 nucleotides. The contigs were used to search in the NCBI-gene bank database in order to identify the most closely related begomovirus(es).

The search result showed that the contigs assembled from RCA containing begomovirus libraries consistently comprised the highest number of assembled reads, indicating that the RCA successfully enriched the begomoviruses DNA components looking at the number that assembled to arrive at a consensus genome (Figure 1). The ACMV_A was found in 4 libraries in samples collected from Western and Eastern regions, ACMV_B was found in 3 libraries in samples collected from western and eastern, EACMV_A in 10 libraries in Eastern, Western, Coast and Nyanza and EACMV_B was found in 6 libraries in Eastern, western, coast and Nyanza.

The complete DNA-A genome of CMG from *de novo* assembly resulted in 2754 to 2801 nucleotides, DNA-B genome 2737-2787nucleotides, ACMV_A genome 1835-2781 and DNA_B 2672-2718 nucleotides long.

The limits of detection for RCA enriched DNA derived from symptomatic cassava leaves by the PCR amplifications was observed to be up to the dilution factor of 10^{-7} for enriched RCA compared to 10^{-5} and 10^{-6} of non-enriched DNA contents (Table 1).

Table 1. Comparison of sensitivity between RCA enriched and non RCA enriched samples.

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Isolate Eastern_14 RCA enriched	+	+	+	+	+	+	+
Isolate Eastern _14 Non RCA enriched	+	+	+	+	+	-	-
Isolate Western_2 RCA enriched	+	+	+	+	+	+	+
Isolate Western_2 Non RCA enriched	+	+	+	+	-	-	-

+, Positive amplification; -, no amplification.

Table 2. Percent nucleotide sequence similarities of complete Kenyan EACMV DNA_A sequences.

Sequences	western_6	western_2	Eastern-14	Coast_42	Eastern_14	Coast_17	Nyanza_9	Nyanza_66	Western_40	Nyanza_11	Western_2	Coast_4	Eastern_16	Eastern_37	coast_4
K02029.TGMV	61	61	61	65	65	65	63	63	63	63	63	63	63	63	63
ACMV-UG_Nam	**99**	**96**	**96**	72	72	72	72	73	73	73	73	70	70	70	70
ACMV/Ug//Mld	**95**	**97**	**98**	72	72	72	73	73	74	74	73	70	70	70	70
EACMZV-K10	72	72	72	**98**	**98**	**98**	82	83	83	83	83	87	87	87	87
EACMZV-K212	72	72	72	**98**	**98**	**98**	82	83	83	83	83	87	87	87	87
Nam_CMD-MI54_12	73	73	73	83	83	83	**97**	**98**	**98**	**98**	**99**	92	92	92	92
gi|61191723_TZ10	73	74	74	82	82	82	**97**	**98**	**98**	**98**	**99**	92	92	92	92
EACMV-UGK66	73	74	74	83	83	83	**98**	**99**	**99**	**99**	**99**	93	93	93	93
EACMV-UGK127	73	74	74	82	83	83	**98**	**98**	**99**	**99**	**99**	92	92	92	92
EACMV-UGK115	73	74	74	82	83	83	**98**	**98**	**99**	**99**	**99**	92	92	92	92
EACMV-K6	70	70	70	86	86	86	92	92	92	92	93	**97**	**97**	**97**	**97**
EACMV-K312	70	70	70	86	86	86	91	92	92	92	92	**97**	**97**	**97**	**97**
EACMV-K313	70	70	70	86	86	86	91	92	92	92	92	**98**	**98**	**98**	**98**

Phylogenetic analysis of full length DNA_A of Kenyan CMGs

The thirteen complete genome DNA_A sequences were aligned and compared with NCBI sequences which grouped into two major clusters. Cluster one was EACMV containing sub cluster A, B and C which grouped according to three geographical regions. The sub cluster A contained isolates coast_42, eastern_14, coast_17, with 98% nucleotide sequence identity clustered with Zanzibar isolates (EACMVZV), sub cluster B isolates nyanza_66, nyanza_11, western_40 and western_2 with 97%-99% nucleotide identity clustered with Ugandan isolates (EACMV_UG) and sub cluster C isolates coast_4, eastern_16 and eastern_37 with 97 to 98% nucleotide identity clustered with Kenyan isolates (EACMVK) and cluster D was ACMV isolates eastern_6, western_2 and eastern_14 (Table 2) (Figure 2).

Phylogeny analysis of full length DNA_B of Kenyan CMGs

The nine complete DNA_B genome sequences obtained from *de novo* assembly were aligned and compared with NCBI sequences. They clustered into two major clusters which were ACMV and EACMV. Just like the EACMV_A, they clustered into four sub clusters with isolate cmd16; western_52 and nyanza_66 clustering with UG isolate eastern_8 clustered with KE isolate and isolate coast_42, eastern_17 and eastern_14 clustering with ZV isolates. Isolate cmd14 and cmd4 clustered with ACMV.

DISCUSSION

In this study we enriched circular single-stranded DNA-containing begomoviruses by RCA from total DNA extracts of naturally infected symptomatic cassava field plants. The enriched begomoviral genomes were subjected to Illumina Miseq sequencing. The short sequence reads obtained were assembled using bioinformatics tools, and were compared with genome sequences deposited in the genebank. We have demonstrated from the serial dilution assay that the viral components that were enriched using RCA procedure increased its detection limit by approximately 10 to 1,000 fold by RCA (Table 1), consequently observation from the de novo assembled

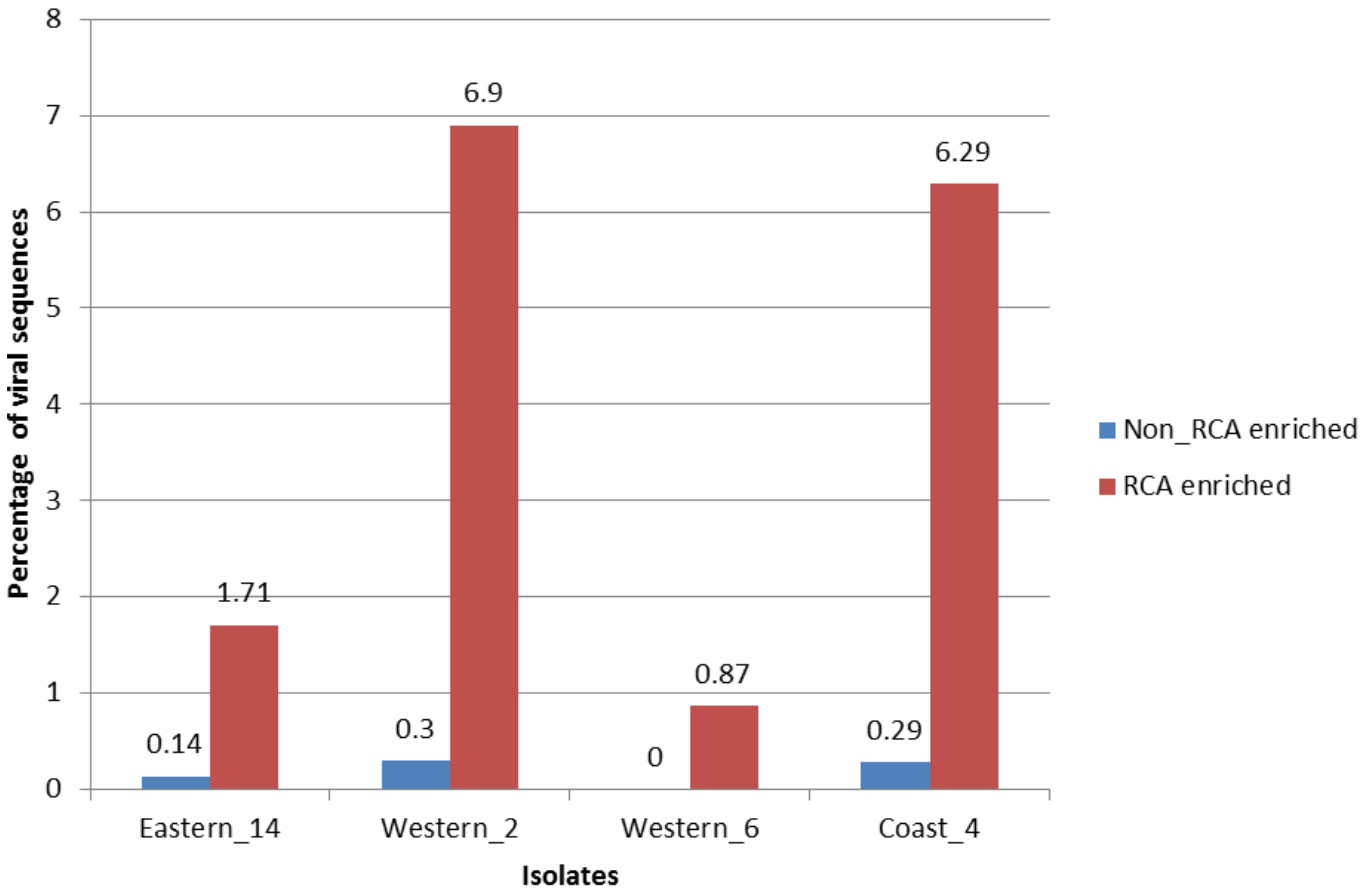

Figure 1. A graph showing percentage of viral de novo assembled sequences of various cassava geminivirus DNA components sequences obtained from total DNA extract enriched RCA and non-enriched with RCA.

reads also indicates generally a high number of sequences of CMGs were assembled from the total sequenced reads from the enriched isolates libraries than non-enriched viral components (Figure 1). Interestingly there was a library that did not record any geminivirus without enrichment except on enriched libraries. Then it is right to infer that RCA increases the chances of detecting ssDNA viruses in samples with very low virus titer. This procedure therefore steps up the sensitivity of detection by increasing the copy numbers of circular DNA. Contrary to the conventional sequencing that relies on primers to amplify a section of the genome resulting in limited information on similarities. It is possible to obtain a complete genome from this RCA and next generation sequencing approach because you need not to have prior knowledge of the viral sequences. Out of 24 libraries, we were able to assemble 25 complete genomes of Geminivirus from both DNA_A and DNA_B component infecting Kenyan cassava. To achieve such results with conventional Sanger method it will be tedious. The utility of metagenomics in detecting and determining the genome sequence and nucleotide similarities of cassava infecting begomoviruses has been used successfully demonstrated in this study (Table 3) which can be utilized in diagnostics and diversities studies of begomovirus in cassava.

The phylogenetic analysis of sequences obtained from the assembled reads from the isolates of this study and compared with genebank sequences grouped in distinct geographical distribution and others have overlapping patterns for both DNA components (Figures 2 and 3). The EACMV-UG isolates were detected in regions neighboring Uganda such Nyanza and Western regions and there was no evidence of EACMV-UG in eastern and coastal region. Previous study reports have been unable to identify EACMV-UG in coastal regions of either Kenya or Tanzania (Were et al., 2004a; Ndunguru et al., 2005). Both EACMV and EACMZV were identified in coastal and eastern region. Detection proportions result here showed that EACMV is more widespread than ACMV in the country it agrees well with (Mwatuni et al., 2015) report. Synergistic interaction between ACMV and EACMV were observed and this result could, lead to severe symptoms as reported by farmers and as observed in the CMD pandemic in Uganda (Bull et al., 2006).

The sequencing technologies cost is on downward

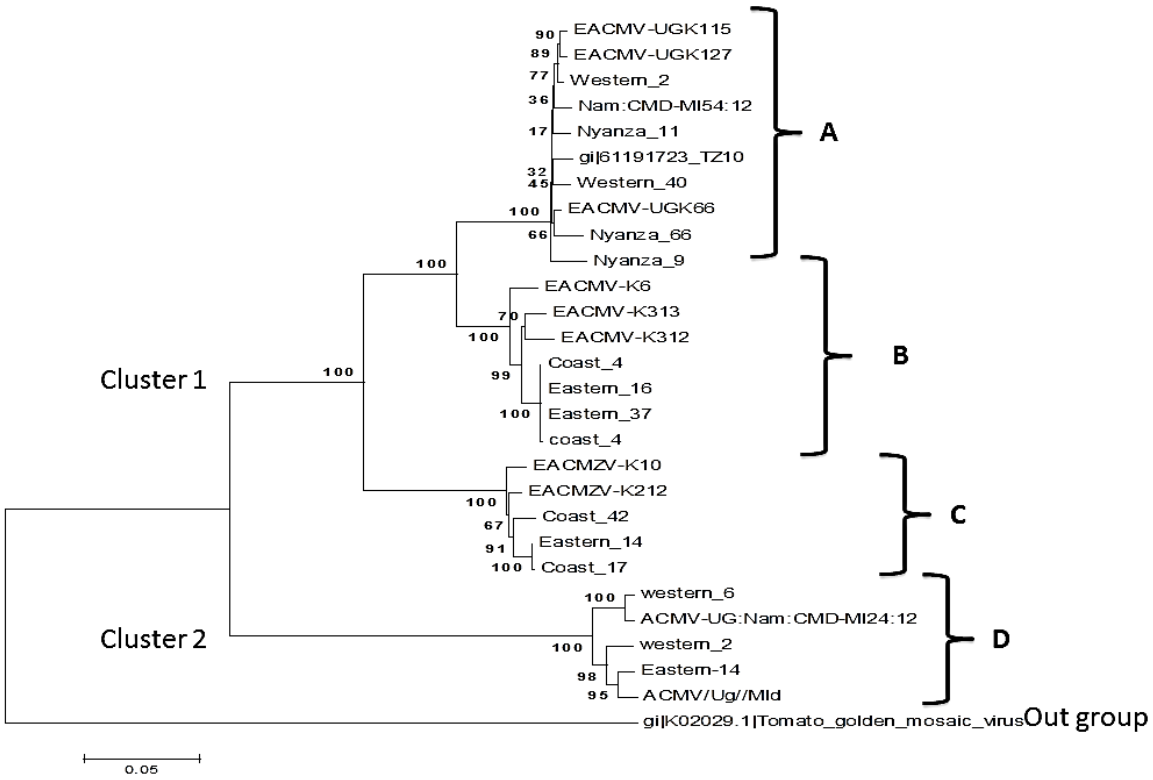

Figure 2. Neighbor-joining tree of the complete genome of DNA_A sequences obtained from de novo assembly of the resultant illumina Miseq deep sequencing. Numbers at nodes indicate percentage bootstrap values of 1000 replicates. Cluster 1 (EACMV) East Africa Cassava Mosaic Virus; sub_cluster (A) Ug isolate, (B) Ke isolate and (D) Zv isolate: Cluster 2 (D) (ACMV) Africa Cassava Mosaic Virus. The alignment was arbitrarily rooted with an out-group of (K02029) Tomato golden mosaic virus DNA_A component.

Table 3. Percentage nucleotide sequence similarities of EACMV DNA_B component and selected other begomoviruses from NCBI.

Virus isolate	Eastern-14	Western_6	Nyanza_66	Eastern_16	Western_52	coast_42	Eastern_17	Eastern_14	Eastern_8
gi|K02030.1_TGMV	46	44	44	44	45	45	44	44	44
ACMV-UG_Nam	**93**	**99**	50	50	50	50	50	50	50
EACMV/Ug3//Svr	51	50	**97**	**97**	**98**	89	89	89	90
EACMZV-K19_2_	51	50	88	89	89	**96**	**96**	**96**	90
EACMV-KE2K27	51	50	89	90	90	91	91	91	**96**
EACMV-UGK72	51	50	89	90	90	91	91	91	**96**

trend and with possibilities of multiplexing strategies. The exploration of begomovirus composition in populations can be done for as many as 96 samples per flow cell lane, infact decreasing the cost of deep sequencing. This allows large numbers of virus samples to be processed simultaneously. MiSeq libraries can be constructed using a high multiplexing barcode to lower the sequence throughput and reduce the period dedicated forcomputation and assembly (Smith et al., 2010) in order to considerably increase the number of samples per lane to make reasonable coverage for this short genome sizes and reduce the cost per sample.

Conclusion

From the results reported herein, we have demonstrated that enriching of the circular ssDNA begomoviral first by RCA, from total DNA extracts of symptomatic cassava by selectively boosting the viral concentration of the starting DNA template thereby increasing the sensitivity of detection. These results demonstrate a reliable approach

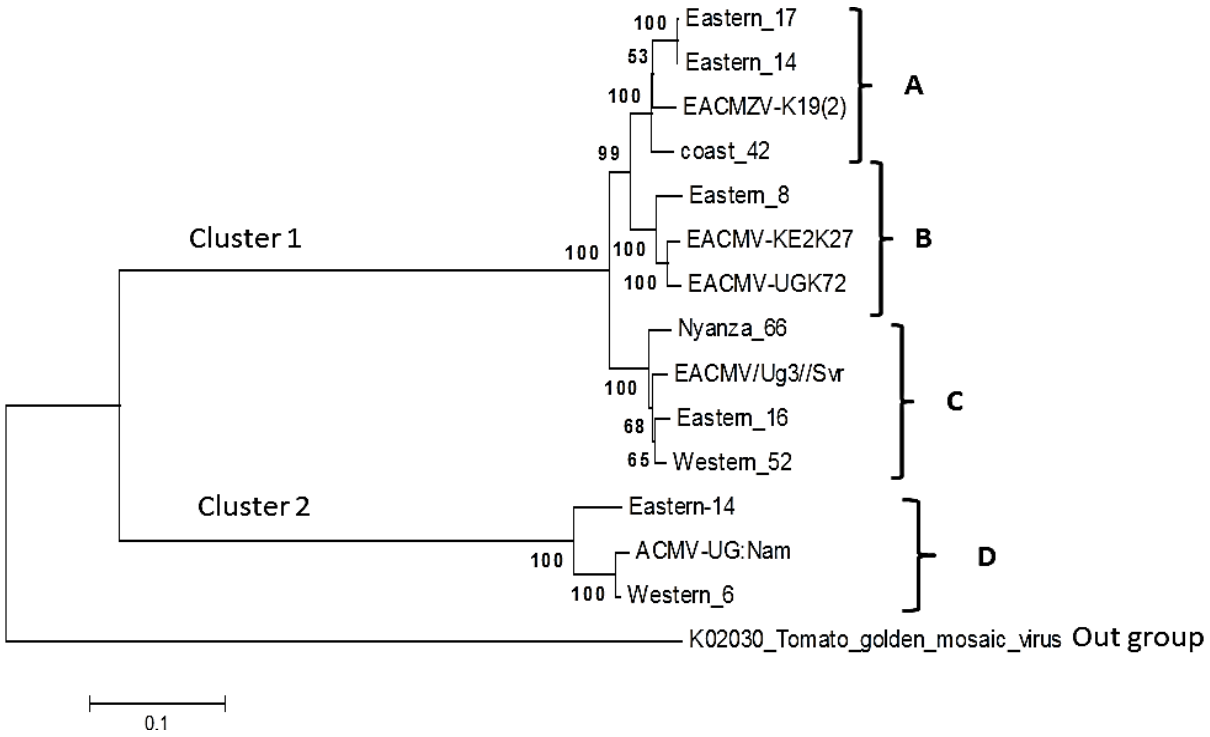

Figure 3. Neighbor-joining tree analysis of the complete genome of DNA_B component sequences obtained from de novo assembly of the resultant illumina deep sequencing. Numbers at nodes indicate percentage bootstrap values of 1000 replicates. Cluster 1(EACMV strain), sub cluster A, Uganda isolates, sub cluster B, Kenya isolate, sub cluster C, Zanzibar isolate and cluster 2 (ACMV strain) sub cluster D. The alignment was arbitrarily rooted with an out group of (K02030) Tomato golden mosaic virus DNA_B component.

for implementation of deep sequencing of begomovirus associated with of ssDNA that infections cassava plants and for exploring their diversities among viral genomes.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Influence of recirculation rate on the performance of a combined anaerobic-aerobic reactor applied to the removal of carbon and nitrogen from poultry slaughterhouse wastewater

Carla L. Lopes[1]*, Adriana N. de Lima Model[1], Jackeline T. Gotardo[1], Fernando H. Passig[2], Eloy L. de Mello[1], Benedito M. Gomes[1] and Simone D. Gomes[1]

[1]Center of Exact and Technological Sciences, State University of West Paraná (UNIOESTE), 2069 Universitária Street, 85819-210 Cascavel, PR, Brazil.
[2]Academic Department of Chemistry and Biology, Federal Technological University of Paraná – UTFPR, Deputado Heitor Alencar Furtado Street, 4900, Ecoville, 81280-340 - Curitiba, PR – Brazil.

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The objective of this study was to evaluate a combined anaerobic-aerobic upflow fixed-bed reactor with liquid phase recirculation for the removal of nitrogen and organic matter from poultry slaughterhouse wastewater. The reactor performance was evaluated with a hydraulic retention time (HRT) of 11 h and three different recirculation rates (R=0.5; 1 and 2). The highest nitrogen removal efficiency value was obtained with an HRT of 11 h (6.8 h in the anaerobic zone and 4.2 h in the aerobic zone) and a recirculation rate of 2. In this condition, the total nitrogen removal efficiency was 69%, with effluent concentrations of 6 mg NH_4^+ L^{-1} and 12 mg NO_3^- L^{-1}. For all tested conditions, there was good chemical oxygen demand (COD) removal, with efficiency above 95%. The effect of dilution and the favoring of mass transfer caused by the increase in the recirculation rate positively influenced reactor performance.

Key words: Anaerobic degradation, nitrification, denitrification, combined reactor.

INTRODUCTION

Effluents from animal processing industries, such as slaughterhouses, after anaerobic processing have been used in many biological nitrogen removal studies in view of their eutrophic potential and the risks to aquatic life and human health due to the presence of nitrogen. Such studies often use a sequencing batch reactor (SBR) and aim to obtain information regarding the operational parameters that influence biological nitrogen removal,

*Corresponding author. E-mail: limberger.carla@gmail.com. Tel: +55 (45)3220 3265.

such as the initial concentration of ammonia nitrogen, the carbon to nitrogen ratio, the air flow and the cycle time (Andrade et al., 2010; Kummer et al., 2011; Dallago et al., 2012; Mees et al., 2011; Lima, et al., 2014; Lopes, et al., 2015).

In an SBR, nitrification and denitrification occur in a single reactor by alternating aerobic and anoxic periods, with sequential operations over time, divided into four steps: input, reaction, sedimentation and liquid discharge (Metcalf and Eddy, 2003). Based on the fact that effluents from anaerobic reactors have high NH_4^+-N/COD ratios, it is necessary to add an external source of organic carbon to achieve full denitrification. Methanol, ethanol and acetic acid are commonly used in the nitrogen removal process, but the use of external sources as electron donors to promote denitrification has limitations related to costs (Cervantes et al., 2001; Barana et al., 2013). The use of industrial effluents as carbon sources for denitrification is also an important strategy (Bernet et al., 1996; Mees et al., 2013). This requirement for an organic carbon source enables the use of endogenous electron donors from the anaerobic digestion, such as volatile organic acids, methane, ammonia or reduced sulfur compounds (Foresti et al., 2006), which are made possible in continuous systems.

A reactor consisting of anaerobic and aerobic compartments, constantly fed and with recirculation of the nitrified effluent to the anaerobic compartment, can enable the use of endogenous electron donors for the reduction of the nitrate into nitrogen gas from industrial effluents (Del Pozo and Diez, 2005; Araujo and Zaiat, 2009; Chan et al., 2012; Kreutz et al., 2014) and domestic sewage (Fazolo et al., 2007; Abreu and Zaiat, 2008; Oliveira Netto and Zaiat, 2012). There are few studies on the application of combined systems in the treatment of poultry slaughterhouse wastewater. Del Pozo and Diez (2005) used an anaerobic-aerobic reactor on a pilot scale with polystyrene foam as the support medium, with internal recirculation, for the treatment of poultry slaughterhouse wastewater and obtained organic matter and nitrogen removal efficiencies of 93 and 67%, respectively. However, the high internal recirculation associated with the air-lift effect caused mixing between the anaerobic and aerobic zones. Thus, most of the organic matter was removed aerobically, and only 12 to 34% of the total nitrogen (TN) removal was due to denitrification, limited by the dissolved oxygen concentration in the anaerobic zone, which was above 0.5 mg L^{-1} due to the mixing regime. In addition, most of the nitrogen removed was used in the synthesis of heterotrophic bacteria.

Araujo and Zaiat (2009) presented a new combined anaerobic-aerobic reactor configuration for the treatment of wastewater from the industrial processing of lysine. They observed that the best reactor operating condition was obtained with a hydraulic retention time of 35 h (21 h in the anaerobic zone and 14 h in the aerobic zone) and a recirculation ratio (r) of 3.5. Under that condition, the removal efficiencies of COD, total Kjeldahl nitrogen (TKN) and TN were 97, 96 and 77%, respectively.

In this context, the configuration in the present study was based on the reactor presented by Araujo and Zaiat (2009) and was designed to promote the recirculation of liquid from the aerobic zone to an intermediate anaerobic-anoxic zone, aimed at using the gases produced in the reactor during anaerobic digestion, such as nitrate reducers during the denitrification step, thus eliminating the need for providing an external source of organic carbon.

Therefore, the objective of this study was to evaluate the influence of effluent recirculation on the performance of a continuous anaerobic-aerobic upflow fixed-bed reactor for the removal of organic matter and nitrogen from poultry slaughterhouse wastewater.

MATERIALS AND METHODS

Combined anaerobic-aerobic reactor

The combined anaerobic-aerobic upflow fixed-bed reactor was manufactured out of an acrylic tube with an internal diameter of 93 mm and a length of 1,000 mm (Figure 1), as proposed by Araujo and Zaiat (2009). The reactor consisted of an inlet chamber (0.543 L volume), a reaction bed, an aeration chamber (0.407 L volume) and an outlet chamber (0.679 L volume). The reaction bed was divided into three compartments: anaerobic compartment I (Vu = 0.752 L), anaerobic compartment II (Vu = 2.199 L) and an aerobic compartment (Vu = 1.071 L). The working volume of the reactor was 5.651 L. Peristaltic dosing pumps were used to feed the reactor under a continuous upflow regime and to recirculate the effluent from the aerobic compartment to anaerobic compartment II. The oxygen was supplied to the aerated zone via a Big Air A360 aquarium pump, and the air was dispensed through porous rocks. The dissolved oxygen concentration was maintained above 2.0 mg L^{-1}.

Biomass inoculation and immobilization

Expanded clay with mean particle sizes ranging from 5 to 15 mm was used for immobilization of the biomass in anaerobic compartment I to minimize clogging of the reaction bed due to the presence of suspended solids in the wastewater and also to induce the adhesion of acidogenic bacteria throughout this material (Ortega et al., 2001). However, this compartment was not previously inoculated.

In the anaerobic II and aerobic compartments, polyurethane foam cubic matrices were used, with 1 cm edges, an apparent density of 23 kg/m^3 and a porosity of approximately 95%. The foam cubic matrices were embedded into sturdy plastic rings to support the material. To accelerate system startup, the polyurethane foam supports in the anaerobic II and aerobic compartments were previously inoculated. For the inoculation of anaerobic compartment II, an anaerobic sludge with 24.65 g VSS L^{-1} was used, with a biomass to wastewater ratio of 1:3. The support material remained immersed in the sludge for 24 h before it was introduced into the reactor. The system was operated in the anaerobic zone for 60 days, when the system exhibited stability in the reduction of COD and the formation of ammonia nitrogen. After that, and with the nitrifying biomass properly inoculated, the modules that made up

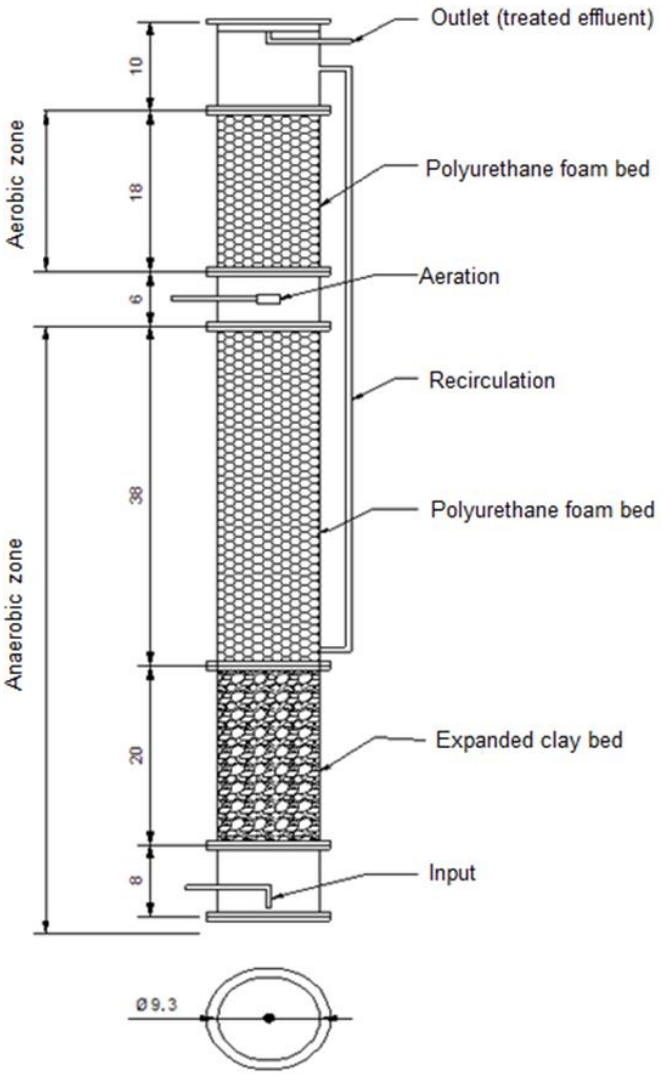

Figure 1. Diagram of the reactor. Araujo & Zaiat (2009).

the aerobic compartment were attached, and the system was operated under combined conditions for an additional 60 days.

Regarding the aerobic module, the support medium was inoculated with nitrifying sludge with 2.65 g VSS L^{-1} at a biomass to wastewater ratio of 1:3. This procedure was performed in a batch system in the laboratory with 24 h cycles over one week. When the support material exhibited significant nitrate formation, it was transferred to the reactor. Therefore, the adaptation stage of the biomass in the reactor lasted for 120 days and was divided into two steps; in the first 60 days, the reactor operated only with the anaerobic compartments, after which the aerobic compartment was then attached, and operations occurred under combined anaerobic-aerobic conditions. For both the anaerobic and aerobic adaptation stages, the hydraulic retention time (HRT) was 14 h with continuous input and no recirculation.

Substrate

The poultry slaughterhouse wastewater was sampled after the primary treatment (flotation system) to avoid problems caused by oil and grease deposition, such as obstruction, flotation, mass transfer problems and decreased methanogenic activity (Masse et al., 2003; Masse and Massé, 2005; Demirel et al., 2005; Saddoud and Sayadi, 2007).

After collection, the wastewater was stored into 2-L polyethylene terephthalate (PET) bottles and kept in a freezer until use. The wastewater characteristics are shown in Table 1.

Physico-chemical analyses

The system was monitored daily. The influent and effluent samples were characterized according to Standard Methods (APHA, 2005) for pH (potentiometric method 4500-H^+), total suspended solids (Method 2540-G), volatile suspended solids (Method 2540-E), TKN (5400-NT), total ammonia nitrogen (Method 4500-NH_4^+), and chemical oxygen demand (Method 5220-D); nitrate and nitrite were determined by flow injection analysis (Methods 4500-NO_3^- and 4500-NO_2^-). Alkalinity was determined by the method proposed by Ripley et al. (1986). The dissolved oxygen concentration was determined using an oximeter (model Orion 3 Star, Thermo Scientific, Beverly, MA, USA).

Experimental procedures

The reactor operation was divided into three stages (stages I, II and III) according to the recirculation conditions applied. The characteristics of each stage and the operating times are shown in Table 2. The HRT was 11 h, with 6.8 h in the anaerobic zone and 4.2 h in the aerobic zone. Thus, each stage was operated for at least 20 days after reaching a stationary state, defined by variations of less than 10% in three consecutive samples with respect to NH_4^+ removal, as proposed by Sahinkaya et al. (2011). The reactor was kept at a temperature of 30±0.1°C and controlled using a digital thermostat.

Data analysis

For data analysis, some equations were used, as described. The percentage of ammonia nitrogen was obtained with equation 1.

$$A(\%)= \left(1- \frac{TKN_a - NH_{4e}}{TKN_e}\right) *100 \quad (1)$$

Where, TKN_a: total influent Kjeldahl nitrogen; TKN_e: total effluent Kjeldahl nitrogen; NH_{4e}: effluent ammonia nitrogen. The denitrification efficiency through recirculation (E_{DN}) was calculated according to Equation 2.

$$E_{DN} = \frac{N_{nitr} - N_e}{N_{nitr}} \quad (2)$$

In Equation 2, N_{nitr} is the nitrified nitrogen concentration, and N_e is the sum of the concentrations of N-nitrite and N-nitrate present in the treated effluent. The removal efficiencies of TN, chemical oxygen demand (COD) and total suspended solids (TSS) were determined using equation 3:

$$S_e = \left(1- \frac{E}{100}\right) *S_i \quad (3)$$

Where, S_i: Influent substrate concentration (TN, COD or TSS); S_e: Effluent substrate concentration (TN, COD or TSS).

The upflow velocity was calculated according to Equation 4.

Table 1. Composition of the industrial wastewater used.

COD ($mg.L^{-1}$)	Total solids ($mg.L^{-1}$)	TKN ($mg.L^{-1}$)	NH_4^+-N ($mg.L^{-1}$)	NO_3^--N ($mg.L^{-1}$)	NO_2^--N ($mg.L^{-1}$)	Alkalinity ($mg.L^{-1}$)	pH
647±137	708±152	76±15	7.7±1.2	<0.05	<0.05	63±19	6.6±0.3

Note: Mean values and standard deviations of 16 analyzed samples.

Table 2. Operating time, upflow velocity (UFV), recirculation rate (Qr/Q), nitrogen loading rate (NLR) and organic loading rate (OLR) values for stages I, II and III, operated at a hydraulic retention time (HRT) of 11 hours and an inflow of 0.51 $L.h^{-1}$

Operating condition	Observation time (d)	UFV ($m.h^{-1}$)	Q_r/Q	NLR ($kg.N.m^{-3}.d^{-1}$)	OLR ($kg\ COD\ m^{-3}.d^{-1}$)
Stage I	22	0.112	0.5	0.134±0.010	1.137±0.112
Stage II	26	0.150	1	0.133±0.017	1.137±0.171
Stage III	25	0.225	2	0.157±0.007	1.329±0.133

$$UFV = \frac{Q_F + Q_R}{A} \quad (4)$$

Where, Q_F is the influent flow rate ($m^3\ h^{-1}$), Q_R is the recirculation flow rate ($m^3\ h^{-1}$) and A is the cross-sectional area of the reactor (m^2).

RESULTS AND DISCUSSION

Biomass startup and adjustment in the reactor

After 60 days of operation in the anaerobic zone, in which only the two anaerobic compartments were in operation, the ammonification efficiency reached 75.4%, and the removal efficiencies of VSS and COD were 86 and 88%, respectively. Rajakumar and Meenambal (2008) evaluated an anaerobic hydroxide sludge blanket reactor, consisting of a support medium of PVC rings at the top of the reactor, with 5.4 L of usable volume applied to the treatment of poultry slaughterhouse effluents. The authors observed that the reactor startup was completed after 120 days of operation at temperatures ranging from 29 to 35°C, with a COD removal efficiency of 80%.

Figure 2 shows the profiles of the parameters pH, total alkalinity and total volatile acids in the final effluent, along with the COD and NH_4^+-N profiles for the influents and effluents of the reactor, during the startup period. The pH ranged from 6.4 to 7.4 and increased during the process. There was an increase in alkalinity due to ammonification and a conversion of COD to volatile organic acids, which led to sufficient system buffering capacity. The same trend was observed by Rajakumar et al. (2012). The TVA/TA ratio at the end of the period was 0.14; in Rajakumar et al. (2012), this ratio remained between 0.13 and 0.19, indicating system stability. The anaerobic reactor startup strategy was considered efficient, as it performed in a way that allowed considering that the system was adapted, enabling the startup of the aerobic compartment to proceed to the nitrification stage. The conversion of organic nitrogen into ammonia nitrogen was observed at the beginning of the operation of the reactor in the anaerobic module. At the end of the combined stage (anaerobic-aerobic), the conversion reached a mean efficiency of 70%.

Nitrifying activity was observed starting in the second week after the incorporation of the aerobic compartment, indicating effluents with NH_4^+-N values < 20 mg L^{-1} (Figure 3) after the fifth week, which is compatible with the current environmental law for the discharge of effluents into water bodies (Brasil, 2011). The conversion efficiency of NH_4^+-N into NO_2^--N + NO_3^--N was 47.1±11.1%, and approximately 20% of the influent nitrogen was removed from the system by assimilation. The TKN, NH_4^+-N and NO_3^--N parameter results analyzed during this test phase are shown in Table 3. Figure 3 shows the COD and NH_4^+-N results for the biomass startup and adaptation periods in the reactor. The COD varied in the input loads due to different batches but remained stable regarding the output values, reaching a removal efficiency of 92% at the end of this adaptation period.

Another indication of the onset of nitrification is the significant consumption of alkalinity. The mean values of total alkalinity and pH were, respectively, 73.1 mg L^{-1} and 6.5 in the influent and 9.4 mg L^{-1} and 5.4 in the effluent. The profiles of these indicators are shown in Figure 4. With the onset of nitrification, the inorganic carbon began to be consumed by chemoautotrophic microorganisms, with a consequent decrease in alkalinity to effluent values close to zero, which became a limiting factor in the nitrification process. This change was also observed in Oliveira Netto and Zaiat (2012). Because the combined

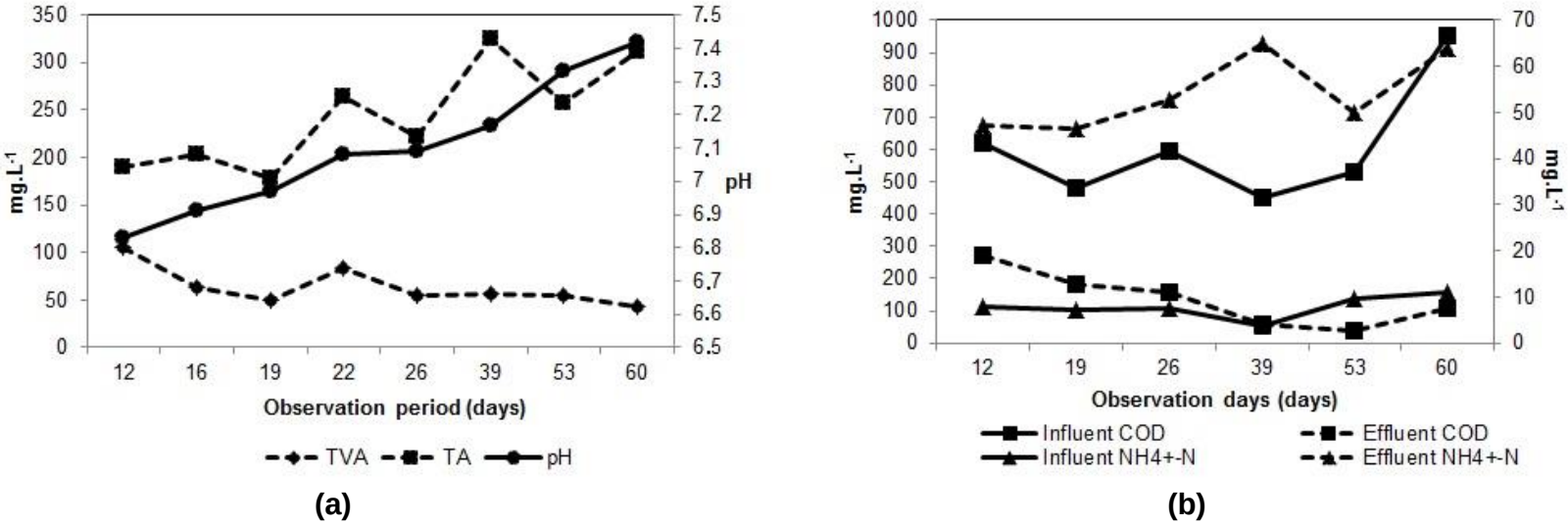

Figure 2. (a) Total Alkalinity (TA), Total Volatile Acids (TVA) and pH (secondary scale) profiles for the reactor effluent; (b) COD and NH_4^+-N (secondary scale) profiles for the reactor influents and effluents.

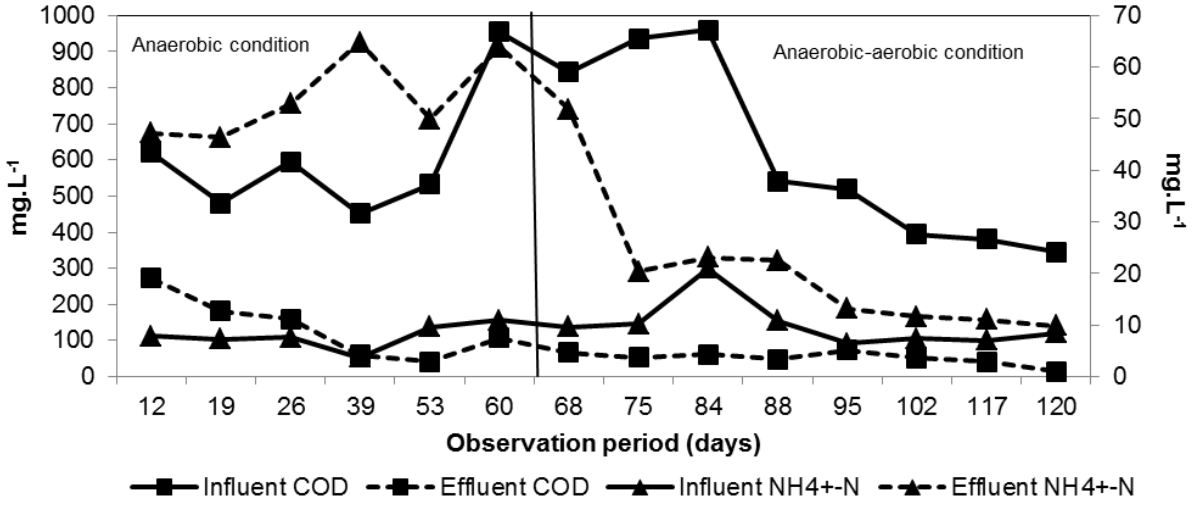

Figure 3. Organic matter concentration values expressed in terms of COD and ammonia nitrogen concentration (secondary scale) for the reactor influents and effluents for the biomass startup and adaptation periods.

Table 3. Results of the concentrations of the nitrogen compounds evaluated over time for the reactor influents and effluents during the system startup period.

Condition	Observation period (days)	Evaluated parameters				
		Influent TKN ($mg.L^{-1}$)	Effluent TKN ($mg.L^{-1}$)	Influent NH_4^--N ($mg.L^{-1}$)	Effluent NH_4^--N ($mg.L^{-1}$)	Effluent NO_3^--N ($mg.L^{-1}$)
Anaerobic	12	86.1	52.5	8.0	47.2	n.a.
	19	73.5	54.6	7.2	46.4	n.a.
	26	87.5	63.7	7.5	52.8	n.a.
	40	77.0	77.0	3.7	64.8	n.a.
	54	86.8	66.8	9.6	49.9	n.a.
	61	84.7	74.9	10.9	63.8	n.a.
Anaerobic-Aerobic	69	77.7	61.6	9.6	51.8	1.5
	76	95.2	28.0	10.2	20.3	13.3
	85	77.7	23.1	21.0	23.1	21.6
	89	81.2	32.9	10.8	22.9	21.6
	103	70.0	15.4	6.4	13.1	11.8
	110	52.5	14.7	7.3	11.6	12.7
	118	64.4	15.4	7.0	11.1	13.7
	120	73.5	23.8	8.4	13.6	23.4

n.a. – Not analyzed

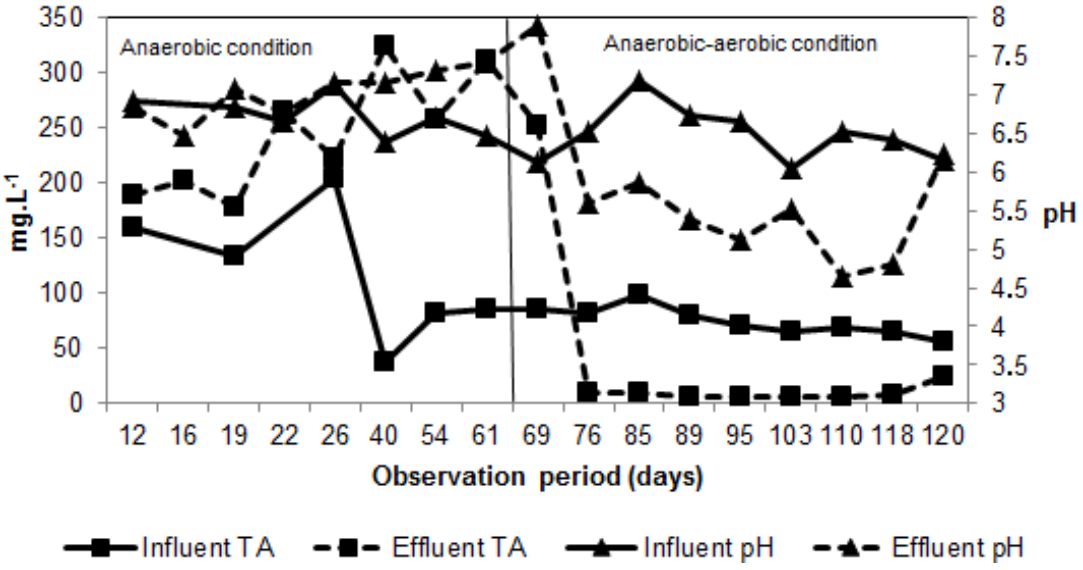

Figure 4. Input and output values of total alkalinity and pH (secondary scale) for the biomass startup and adaptation periods.

anaerobic-aerobic reactor operation promoted nitrification, conditions to promote denitrification were implemented in the next stage of the work. Thus, recirculation of the liquid phase from the aerobic compartment to anaerobic compartment II was contemplated to utilize the endogenous electron donors produced in the reactor during anaerobic digestion, such as nitrate reducers during the denitrification stage.

Nitrogen removal in the combined system with recirculation

After the adaptation period, the operation stages were initiated to maintain the HRT and recirculation conditions (Table 3) and were maintained for 73 days. The oxygen demand concentrations in the aerobic compartment for stages I, II and III, respectively, were 5.23±0.59, 4.96±0.66

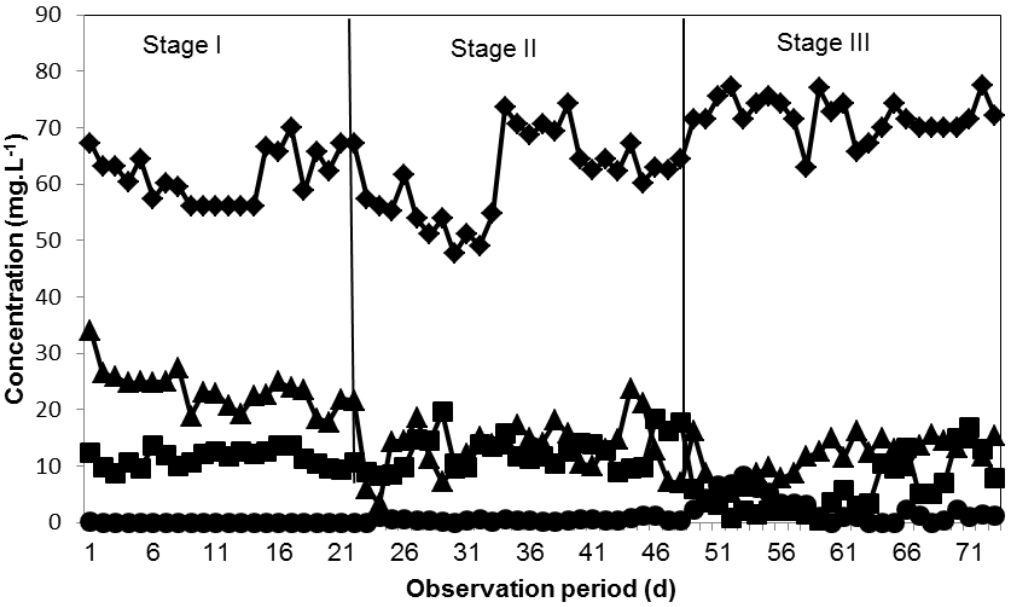

Figure 5. Temporal variations in the concentrations of influent TN (♦), effluent NH_4^+-N (■), effluent NO_3^--N (▲) and effluent NO_2^--N (●) during the three evaluated stages.

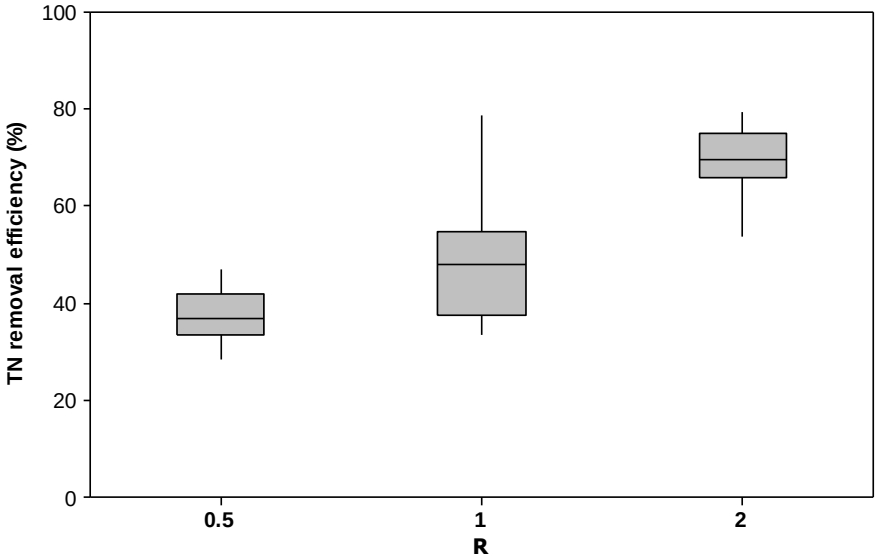

Figure 6. Box-plot of the distribution of TN removal efficiency at each stage, with maximum and minimum points, 1st and 3rd quartiles and medians.

and 4.69±0.21 mg L^{-1}. The oxygen needed for the oxidation of ammonia nitrogen is approximately 4.57 g O_2/g oxidized N-ammonia (Metcalf and Eddy, 2003). Oliveira Netto and Zaiat (2012) operated a combined anaerobic-aerobic fixed-bed reactor with liquid phase recirculation that was applied to the treatment of domestic sewage with an average concentration of dissolved oxygen of 5.7±0.7 mg L^{-1}. The temporal variations in the concentrations of TN, NH_4^+-N, NO_3^--N and NO_2^--N are illustrated in Figure 5. TN removal efficiencies of 37.6, 48.1 and 69.7% (Figure 6) were observed in stages I, II and III, respectively. The effective TN decrease was caused by the introduction of effluent recirculation, which aimed to direct the nitrate formed in the aerobic zone to anaerobic zone II of the reactor, allowing its denitrification by the use of electron donors

Table 4. Mean concentrations and standard deviations of total alkalinity (TA), bicarbonate alkalinity (BA) and pH evaluated in influents and effluents from stages I, II and III.

Parameter	Stage I		Stage II		Stage III	
	Influent ($mg.L^{-1}$)	Effluent ($mg.L^{-1}$)	Influent ($mg.L^{-1}$)	Effluent ($mg.L^{-1}$)	Influent ($mg.L^{-1}$)	Effluent ($mg.L^{-1}$)
TA	72	5	71	36	86	14
BA	32	0	28	24	32	6
pH	6.2	4.6	6.5	6.4	6.3	5.5

from the first compartment, such as organic matter, volatile organic acids and methane. The use of products from the anaerobic digestion as electron donors was considered satisfactory, and there was no external addition of carbon at any of the stages evaluated.

During stage III, where the highest TN removal efficiency occurred (69.7%), the effluent concentrations of NH_4^+-N, NO_3^--N and NO_2^--N were 5.6±4.7, 11±3 and 2.4±2.4 mg L^{-1}, respectively. In stages I and II, efficiency was decreased, mainly due to the reduction in the recirculation rate. Thus, it is noteworthy that the increase in internal recirculation of the nitrified effluent increased the amount of nitrate reduced to nitrogen gas and that there was a consequent decrease in the TN effluent concentration.

The increased recirculation rate promoted better mass transfer within the reactor. This improved transfer can be attributed to greater mixing due to the increase of the upflow velocity, which, according to Foladori et al. (2014), reduces channeling of preferential paths and improves the contact between the substrate and the bacterial biomass. In this experiment, the upward flow velocities were 0.113, 0.150 and 0.225 m h^{-1}, for stages I, II and III, respectively. Furthermore, an increased recirculation rate promotes liquid dilution within the reactor and may contribute to decrease the possible impacts caused by the variability of the input load (Jin et al., 2012).

Although stage III was operated with the highest input load (0.157±0.007 $kgNm^3$ d^{-1}) because of the variation in influent composition due to the fluctuation of the manufacturing process, it was at this stage that the system had the highest removal load: 0.139±0.01 kgm^3d^{-1}. This value is higher than that obtained by Oliveira Netto and Zaiat (2012), who operated a laboratory-scale anaerobic-aerobic fixed-bed reactor for the treatment of domestic sewage. When an HRT of 11.4 h and a recirculation rate of 1.5 were applied, the removed nitrogen load was 0.072 kgm^3 d^{-1}.

Araujo and Zaiat (2009) evaluated a combined anaerobic-aerobic fixed-bed reactor for the treatment of wastewater from lysine processing. They observed that under reactor operating conditions with a hydraulic retention time of 35 h (21 h in the anaerobic zone and 14 h in the aerobic zone) and recirculation ratios (r) of 0.5 and 1, the TN removal efficiencies were 42 and 54%, respectively. However, when these authors applied an r of 3.5 and an HRT of 35 h, the TN removal efficiency increased to 77%. Thus, they concluded that increasing the internal recirculation of treated effluent increases the amount of nitrate reduced to nitrogen gas through the denitrification in the anaerobic compartment of the reactor, with a subsequent decrease in the effluent TN concentration.

Although the TN removal was considered efficient in stage III, with the establishment of nitrification and denitrification, resupply of alkalinity to the medium by denitrification was not observed, as in this case, theoretically, for each 1 g of nitrate transformed to N_2, 3.57 g of alkalinity ($CaCO_3$) must be generated (Metcalf and Eddy, 2003). However, simultaneous nitrification and denitrification provided by the input of oxygen through recirculation prevented the quantification of alkalinity recovery in anaerobic compartment II. Thus, the effluent concentrations of total alkalinity, bicarbonate alkalinity and pH reached very low values (Table 4).

Alkalinity consumption resulted in decreased pH in the aerobic compartment in all evaluated stages. Despite the pH values being lower than 6.4, this parameter cannot be considered an inhibitor of nitrifying activity, but the absence of alkalinity was considered a limiting factor in the process. Throughout the reactor length, TA recovery in anaerobic compartment I was due to ammonification and conversion of volatile organic acids, which resulted in values of 247.3, 183.1 and 214.7 mg $CaCO_3$ L^{-1} for stages I, II and III, respectively. However, the TA recovery was not sufficient to meet the nitrification needs, and its deficit reached levels of 31, 34 and 35% for stages I, II and III, respectively. Furthermore, the industrial wastewater had low alkalinity concentrations of approximately 70 mg L^{-1}. For such cases, it is suggested that alkalinity be supplemented. However, it can be concluded that the higher recirculation rates provided the highest denitrification efficiencies (Table 5) and, theoretically, higher alkalinity recovery and TN removal.

Organic matter removal

The performance of the combined reactor relative to COD removal can be seen in Table 6. The most removal occurred in the first compartment due to anaerobic digestion; therefore, the COD removal was constant, and

Table 5. Effluent concentrations of nitrogen in the nitrite and nitrate forms (N_e), denitrification efficiencies (E_{DN}) and TN removal efficiencies (E_{TN}), in stages I, II and III.

Stage	Parameters		
	N_e (mg.L^{-1})	E_{DN} (%)	E_{TN} (%)
I	23.5	36.7	37.4
II	13.2	60.9	48.7
III	14.2	72.1	69.4

Table 6. Mean concentrations and standard deviations of COD evaluated in influents and effluents and the removal efficiencies of COD in stages I, II and III.

Stage	Influent COD (mg.L^{-1})	Effluent COD (mg.L^{-1})	COD Removal (%)
I	521±51	14±3	97±1
II	521±78	15±6	97±1
III	609±61	27±12	96±2

recirculation did not affect this parameter. Such values are relatively higher than those found by Kreutz et al. (2014) when evaluating a chambered anaerobic-aerobic fixed-bed reactor with a recirculation of the liquid phase of 0.5. The usable volume of that reactor was 4.75 L, and for an HRT of 11 h, the authors obtained a COD removal efficiency of 59%. However, the system was fed with cattle slaughterhouse wastewater and had an organic load of 2.288 kg.m^3.d^{-1}, higher than those applied in the present work.

Asadi et al. (2012) evaluated the simultaneous removal of carbon and nutrients from industrial wastewater in a single aerobic-anoxic upflow bioreactor with a sludge blanket. Optimized removals of 80 and 50% were obtained for COD and TKN, respectively, when the HRT was 12 h and the aeration time was 40 to 60 min/h. The low concentrations of total suspended solids in the effluent, approximately 26, 48 and 42 mg L^{-1}, with adequate efficiency in the removal of this parameter, indicated that the increased upflow velocity, due to recirculation, did not cause biomass detachment throughout the evaluation period.

Conclusions

The evaluated system was efficient in removing organic matter for all conditions applied and in removing TN, found with the application of an HRT of 11 h and a recirculation rate of 2. The effect of dilution and the favoring of the mass transfer caused by the increase in the recirculation rate contributed to better reactor performance. Therefore, the this system with recirculation of the liquid phase is advantageous for the treatment of effluent from poultry slaughterhouses, as it promotes the secondary treatment and removal of nutrients in a single reactor with no need for an external carbon source.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Comparison between Giemsa and Van Geison stains in demonstration of collagen fibers (Kosti-2016)

Ziyad Mudasir*, Salma Adil and Ahmed Ibn Edriss

Department of Histopathology and Cytology, Faculty of Medical laboratory Sciences, University of El-Imam El-Mahdi, Kosti, Sudan.

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Trichrome stain (such as Van Geison) is usually used in histopathology laboratory for demonstration of collagenic fibers. Lack of selectivity and tendency of stain to fade makes van Gieson not ideal for collagen demonstration. This study was aimed to compare between Giemsa's and van Gieson's stains in collagen fibers demonstration. Twenty biopsies were obtained from rabbit's skin after anesthesia by chloroform and immediately fixed by 10% neutral buffered formalin for 48 h. Then samples were processed using tissue processing machine and sectioned by rotary microtome. Two hundred (200) tissue sections of 5 micron thickness were prepared. A 100 tissue sections was stained by Van Geison and another 100 tissue sections stained by Giemsa. The stained section was compared with illustrated photomicrographs in order to assess staining quality. Best collagen staining quality was obtained by Van Geison's 60 (60%) and 40 (40%), mean 1.40, followed by Giemsa's stain excellent 55 (55%) and good 45 (45%), mean 1.45. Conclusively, Van Geison's is superior but Giemsa stain is rapid, sensitive without fading tendency, easy to perform and low cost and can be used as special stain under optimized conditions.

Key words: Collagen, Giemsa, Van Geison.

INTRODUCTION

One of the most important vital roles which collagen fibers play is maintaining structural integrity. Also collagen determines tissue function (Whittaker and Canham, 1991); so many pathological conditions are closely associated with collagen degradation and collagen deformity. Such pathological conditions are: Infarct expansion after myocardial infarction, decrease in renal function due to increased fibrosis after kidney transplantation which finally leads to eventual graft failure (Diaz Encarnacion et al., 2003; Grimm et al., 2003). Hence, increase in the need of quantification of fibrosis for prediction of graft survival makes accurate identification of collagen fibers of great importance.

Stains such as Van Gieson and the various forms of trichrome have been used traditionally to detect collagen fibers in corresponding tissue sections. The mechanism of these stains is not completely understood but they bind different tissue components differentially. Such

*Corresponding author. E-mail: ziyad.mu300@gmail.com.

differentiation depends on various differences in different factors such as size of the dye molecules, differences in the tissue physical structure (for example, tightly versus loosely packed), and the amino acid composition of the elements of the tissue (Kiernan, 2002). Regarding these factors, in addition to the lack of selectivity; makes van Gieson not ideal for collagen demonstration (Kiernan, 2002; Whittaker et al., 1994). This confounding reason (poor staining of collagen fibers by Van Gieson's stain) and the tendency of the stain to fade, prompted colleagues (Sweat et al., 1964) to seek a better method. Picrosirius red F3BA was found to consistently stain thin collagen fibers, did not fade, and was suitable with polarized light microscopy.

A neutral stain is made from the interaction of acidic and basic dyes. Both cation and anion contain chromophoric groups and there is colored dye in both parts of the dye molecule. Owing to the combination of already large molecules, solutions of neutral stains are often colloidal.

Neutral dyes are soluble in alcohol only, rarely in water, whilst basic and acidic dyes are usually soluble in both. The Romanowsky dyes are the best known of the neutral stains and are formed by the interaction of polychrome methylene blue and eosin. The original Romanowsky stain was prepared by chance with an oxidized methylene blue and it is the oxidation of methylene blue into methylene azure that gives the stain its special selectivity; this oxidation is analogous to the 'ripening' of other stains, such as hematoxylin.

Basic stains color acidic tissue components such as nuclei. Acidic stains will combine with basic structures such as cytoplasm. Neutral dyes have, as expected, an affinity for acidophilic and basophilic elements in the cell, and certain tissue components also react with the compound neutral stain, thus giving a triple staining effect (Drury and Weilngton, 1980).

Giemsa stain is one of the Romanowsky dyes which was introduced early by Gustav Giemsa as stain for malaria parasite. Also Giemsa stain has a wide application in neuropathology as a stain for detection of mast cells (Woronzoff-Dashkoff, 1993). Giemsa is used in hematological patterns in differentiating leukocytes (Wittekind, 1983). The stain, which is classified under the neutral dyes, is requiring neutral pH (6.8- 7.5), which is carried out by using buffer solution.

MATERIALS AND METHODS

Twenty skin biopsies were taken from a rabbit after anesthesia. All biopsies were 2× 2× 0.3 cm in dimension. After the collection of specimens, all of them were immediately fixed in a wide suitable container by 10% neutral buffered formalin ten times the size of specimen for 48 h.

After fixation of specimens, the cut- up was done, specimens were put in cassettes then bearded the unique cases number. The specimen then passed into a tissue processing machine (Leica, 2000) for further treatment in Table 1.

After tissue processing was completed the specimens placed in an embedding centre where they were removed from their cassettes and placed in wax-filled molds that best correspond to the size of the tissue. At this stage specimens were carefully orientated. The cassette in which the tissue has been processed was then placed on top of the mold and attached by adding further wax. The specimens "blocks" were allowed to solidify on a cold surface and when set the molds were removed. The cassette, already filled with wax and forming part of the block, provided a stable base for clamping in the microtome. The block containing the specimen was thereafter subjected to section cutting (Edriss, 2015). 20 blocks were prepared. The blocks were cooled to solidify to turn out their moulds and were then cut by rotary microtome (Diapath Galileo, fully automatic microtome Galileo, 2012). 10 sections of 5 µm thickness were sectioned from each block and kept in incubator with a temperature of 5 to 6ºC above the melting point of wax, that is, at 60ºC for 40 min.

Table 1. Tissue processing schedule.

10% buffered formalin	2
70 percent alcohol	3
90 percent alcohol	3
Absolute alcohol	1
Absolute alcohol	1
Absolute alcohol	2
Absolute alcohol	2
Xylene	2
Xylene	2
Wax bath	3
Wax bath	3

Staining

All sections were de-waxed by xylene for 10 min and rehydrated in descending alcohol concentrations of 100% through 90 and 70% to distilled water for 3 min in each stage. Each section was stained separately.

Verhöeff's Van Geison's method

1. Verhöeff's solution (freshly prepared) for 20 minutes.
2. Rinse in water.
3. Differentiate in 2% aqueous ferric chloride until elastic tissue fibers appear black on a gray background.
4. Rinse in water.
5. Rinse in 95% alcohol to remove any staining due to iodine alone.
6. Counter stain in van gieson for 3 to 5 min.
7. Blot to remove excess stain.
8. Dehydrate rapidly through ascending grades of alcohol.
9. Clear in xylene and mount in DPX (Verhöeff's 1908).

Giemsa staining method

1. Rinse in stock solution of acetic acid.
2. Stain in giemsa working solution in coplin jar for 10 minutes.
3. Wash with buffer.
4. Differentiate in 0.5% acetic acid three dips.
5. Rinse in 100% alcohol.
6. Clear in xylene and mount in permanent mounting medium.

Table 2. Microscopic evaluation of staining quality.

Stain	Excellent	Good	Bad	Total
Geimsa	55 (55%)	45 (45%)	0	100
Van Geison's	60 (60%)	40 (40%)	0	100

Table 3. Giemsa staining.

Entities	Color
Collagen	Pink
Nucleus	Blue

Table 4. Van Gieson staining.

Entities	Color
Collagen	Red
Nucleus	Black

Table 5. The Report of the Giemsa stain.

Std. Deviation	N	Mean	Giemsa
0.00000	55	1.0000	Excellent
0.31782	45	1.8889	Good
0.49237	100	1.4000	Total

Table 6. The correlations.

Correlations		Giemsa results	Van gieson results
Result Giemsa	Pearson Correlation	1	0.903**
	Sig. (2-tailed)		0.000
	N	100	100
Result Van gieson	Pearson Correlation	0.903**	1
	Sig. (2-tailed)	0.000	
	N	100	100

**, Correlation is significant at the 0.01 level (2-tailed).

RESULTS

All quality control measures were adopted throughout the study procedures. Sections were examined by light microscope (LABOMED, LaboAmerica, inc 2013) for the assessment of histomorphological appearance. The characteristics were compared with illustrated micro-photographs (Gartner and James, 2005).

Mean count for each procedure was calculated from 100 sections. Giemsa's stain collagen fibers exhibited excellent 55 (55%) and good 45 (45%) mean 1.5, while Van Geison's gave 60 (60%) and 40 (40%) excellent and good histomorphology respectively mean 1.6 (Table 2 to 6, Figure 1).

DISCUSSION

The routine stain in histopathology is hematoxylin and eosin stain. Any stain used to bring about histological

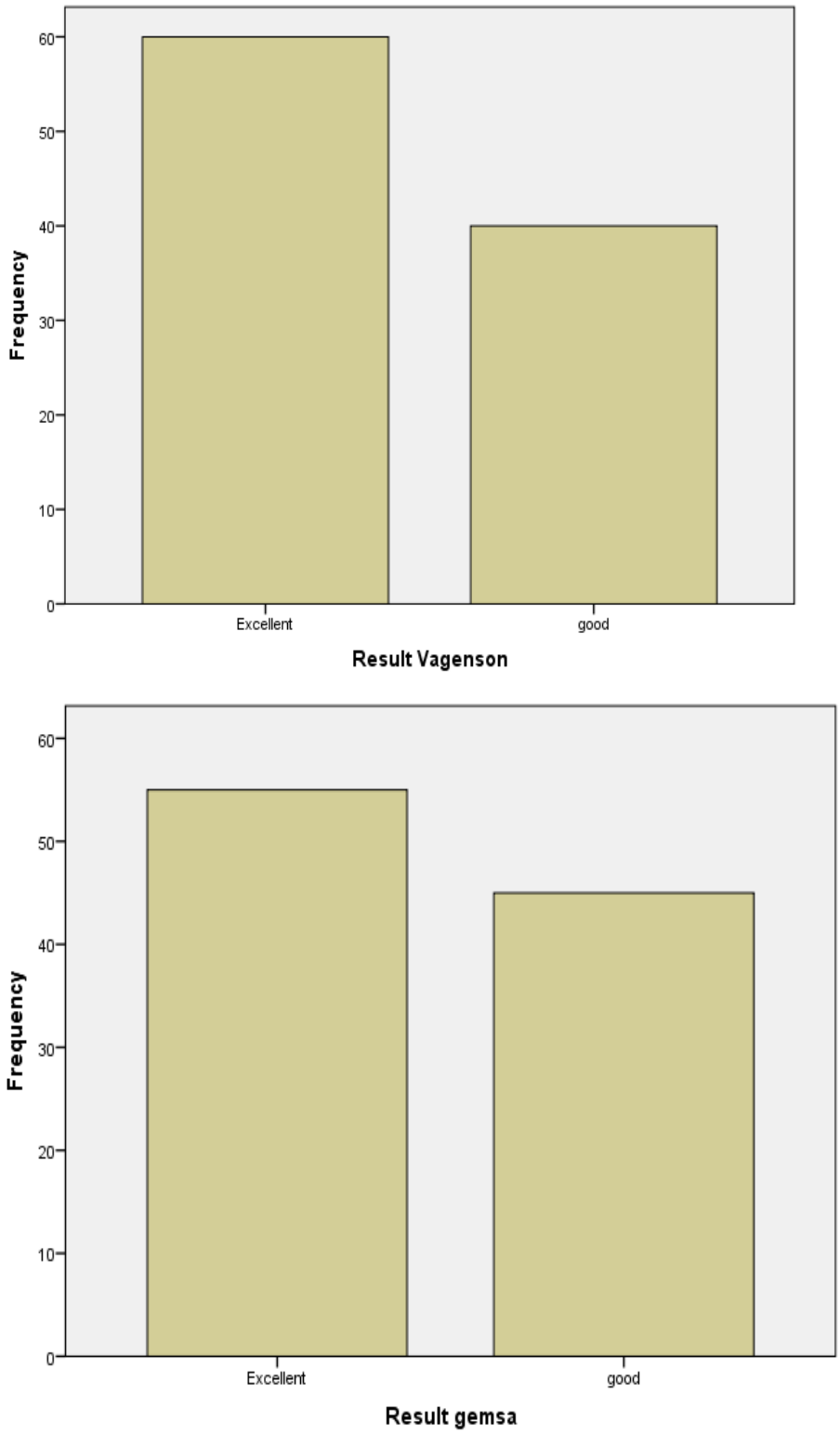

Figure 1. Van Gieson and Giemsa staining quality.

structure in tissue section rather than hematoxylin and eosin stain is termed "special stain". Immunohistochemical and *in situ* hybridization stains were included in this term. There are two broad areas of application: Research and

diagnosis utilize special stains. In research, special stains are used for identifying normal and abnormal cells in tissue section.

Although the Giemsa dye has been shown to work well with a wide variety of procedures, it does not gain wide acceptance (Iniguez et al., 1985). The colors are different from those seen in blood films fixed in alcohol. When Giemsa is used for staining bacteria in tissue section fixed by formaldehyde, the organism stains purple and pink cytoplasm will be seen (Kiernan, 2008).

Wittekind et al. (1991) found that Giemsa stain seems suitable to replace the Gomori-type trichrome stains under appropriate staining conditions. The staining result depends on many factors such as pH and differentiation and this is in line with current study (Wolf-Dieter, 2006).

In conclusion, though the results of van Geison's were superior, Giemsa stain has several properties, such as being rapid; sensitive without fading tendency; easy to perform and low cost, and when used for detection of collagen fibers, there is no need for counter stain (the nucleus takes up methylene blue thus stains blue); so the study recommends the use of Giemsa's as special stain under optimized conditions for skin biopsies in case of collagen demonstration and when infection is suspected.

Conflict of Interests

The authors have not declared any conflict of interests.

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