African Journal of Biotechnology

Volume 16 Number 49, 6 December, 2017

ISSN 1684-5315

Academic Journals

ABOUT AJB

The African Journal of Biotechnology (AJB) (ISSN 1684-5315) is published weekly (one volume per year) by Academic Journals.

African Journal of Biotechnology (AJB), a new broad-based journal, is an open access journal that was founded on two key tenets: To publish the most exciting research in all areas of applied biochemistry, industrial microbiology, molecular biology, genomics and proteomics, food and agricultural technologies, and metabolic engineering. Secondly, to provide the most rapid turn-around time possible for reviewing and publishing, and to disseminate the articles freely for teaching and reference purposes. All articles published in AJB are peer-reviewed.

Contact Us

Editorial Office: ajb@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJB

Submit manuscript online http://ms.academicjournals.me/

African Journal of Biotechnology

Table of Content: Volume 16 Number 49 6 December, 2017

ARTICLES

academicJournals

Vol. 16(49), pp. 2258-2266, 6 December, 2017
DOI: 10.5897/AJB2017.16146
Article Number: C9A8C7066961
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Anaerobic decomposition of submerged macrophytes in semiarid aquatic systems under different trophic states, Paraíba State, Brazil

Vanessa Virginia Barbosa[1], José Etham De Lucena Barbosa[2], Luiz Ubiratan Hepp[3], Marcela Bianchessi Cunha Santino[4] and Janiele França Nery[5*]

[1]Programa de Pós-graduação em Engenharia Ambiental, Universidade Estadual da Paraíba, Rua Juvêncio Arruda, s/n, 58109790, Campina Grande, PB, Brasil.
[2]Departamento de Biologia, Universidade Estadual da Paraíba, Rua Juvêncio Arruda, s/n, 58429600, Campina Grande, PB, Brasil.
[3]Universidade Regional Integrada do Alto Uruguai e das Missões, Avenida Sete de Setembro,1621, 99700000, Erechim, RS, Brasil.
[4]Centro de Ciências Biológicas, Universidade Federal de São Carlos, Via Washington Luiz, km 235,13565905, São Carlos, SP, Brasil.
[5]Instituto Federal de Ciência e Tecnologia da Paraíba, Acesso Rodovia PB 151, s/n, 58187000, Picuí, PB, Brasil.

Received 11 July, 2017; Accepted 18 September, 2017

The macrophytes play an important role in the regulation of biological and chemical processes in aquatic ecosystems, particularly in shallow lakes. They also play an important role in storage and nutrient cycling, serving as a source of organic matter to native environments. The aim of this study was to describe the kinetic aspects of the nutrients released during the anaerobic decomposition process of *Egeria densa* Planch and *Chara braunii* Gmnel macrophytes in waters with different trophic states. The study was conducted *in vitro* and under anaerobic conditions for determination of both particulate and dissolved fractions of nitrogenous, phosphorus and carbon, in predetermined days in oligotrophic and eutrophic water. Mathematical models were applied to describe the macrophytes decomposition process. Both species showed the same biphasic decay pattern of organic matter and carbon mineralization. The phosphorus, nitrogen and carbon released were high for both species, regardless of the trophic water state. The loss of mass was similar for both species and the nutrients concentration in the dam water did not represent a limiting factor for the mathematical model.

Key words: *Chara braunii*, *Egeria densa,* mathematical modeling, nitrogen, phosphorus.

INTRODUCTION

The submerged plants have a key role in biochemical processes regulation in aquatic ecosystems, especially in shallow lentic systems (Scheffer and Van Nes, 2007). They function as an autochthonous organic matter source and exert a relevant ecological role in nutrient and carbon storage as in their cycling (Palma-Silva et al., 2012). Submerged macrophytes species, *Egeria densa* Planch and *Chara braunii* Gmel are from northeastern Australia and the extreme south of South America (Sampaio and Oliveira, 2005). Once these plants present high ecological plasticity, they colonize aquatic systems in semi-arid regions of Brazil (Macêdo et al., 2012). *C.*

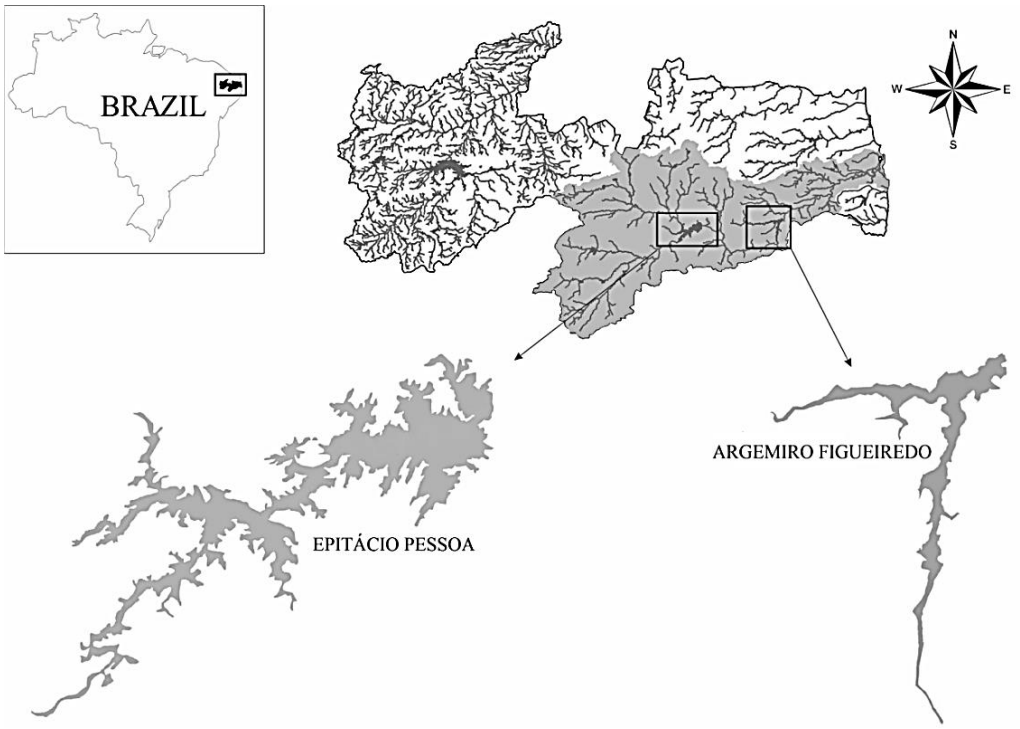

Figure 1. Localisation of sampling points, Epitácio Pessoa and Argemiro Figueiredo dams.

braunii belongs to the *Characeae* family and *Egeria densa* to the Hydrocharitaceae family, both rooted submerged macrophytes. *E. densa*, has a more fibrous plant structure (Rodrigues and Thomaz, 2007) as compared to *Chara braunii*, which like other macroalgae, has a protein-rich structure (Patarra et al., 2011).

With the senescence of macrophytes, the plant detritus enters through the carbon and nutrients cycle into the aquatic environment, which is incorporated in plant tissues during primary production (Kim and Rejmánková, 2004). However, depending on the colonization intensity, a relevant contribution of these organisms to eutrophication could occur, since the release of water-soluble compounds during senescence may act either as a nutrition or pollution to the water column (Anesio et al., 2003). During the biomass decomposition of submerged macrophytes, significant changes can occur in water, that is, increase of dissolved and particulate organic matter, aquatic system acidification, increased electrical conductivity (Bianchini and Cunha-Santino, 2010) and nutrient release (Wang and Fan, 2013). Such physical and chemical changes may be affected either by extrinsic factors like intrinsic ones, such as the species chemical composition (Bianchini and Cunha- Santino, 2008) and the nutrient concentration in the water which can accelerate the organic matter degradation or make it slower (Xie et al., 2004) due to the heterotrophic activity dynamics during the degradation process.

In situ and *in vitro* studies (Fonseca et al., 2014) on these kinetic aspects of macrophytes degradation, focus on the Brazilian Savanna (Cerrado) region and data on the Brazilian semiarid region decomposition kinetics of submerged macrophytes are still lacking. Therefore, the aim of this study was to describe the kinetic aspects of anaerobic decomposition of submerged macrophytes, *E. densa* and C. *braunii* in waters with different nutrients concentration. The hypotheses of this study is that the decomposition process occurs faster in eutrophic than in oligotrophic waters. Among the submerged species studied, *C. braunii* is expected to decompose rapidly, since it is an alga.

MATERIALS AND METHODS

The water samples used for the decomposition experiment were collected in two dams located in the Paraiba River basin in the Brazilian State of Paraiba, with varying trophic degrees. The “Epitácio Pessoa” dam (7° 30'41 "S 36° 11'52" W) is an oligotrophic dam (Vasconcelos et al., 2013). The “Argemiro Figueiredo” dam (07° 27'43 "S 35° 35'6" W), and its waters are characterized as eutrophic-hypertrophic status (Vasconcelos et al., 2013) (Figure 1).

*Corresponding author. E-mail: janiele.biologa@gmail.com.

Macrophytes decomposition and mass balance experiment

The macrophytes were collected in the Epitácio Pessoa dam using a dredge collector for particles excess removal and dried in low temperature (40°C) to achieve the constant mass and to avoid loss of volatile compounds. Anaerobic incubations were performed according to Bianchini et al. (2010). Macrophytes fragments (size average = 0.5mm; s = 0.14) from both species were incubated in glass bottles in a proportion of 10 g/L^{-1}, kept in the dark under anaerobic conditions (oxygen ≤ 1.00 mg/L) at 27°C (ranging from 26 to 27ºC), which is the average temperature of the studied dam. Different treatments (species × trophic level) were analyzed in triplicate using *E. densa* fragments placed in glass bottles with eutrophic (eutrophic environment) and oligotrophic waters (oligotrophic environment). The same procedure was performed with *C. braunii*. In the respective sampling days (1, 3, 5, 15, 30, 60 and 90 days), three incubations of each treatment had contents fractionated in particulate organic matter (POM) and dissolved organic matter (DOM) by pre-filtration fiber filter glass (Φ pore = 0.8 µm) to remove the coarser material and then by pore membrane filtration (0.45 µm) (Bianchini et al., 2010). The control treatment consisted of only incubations with water from each dam.

The particulate mass was determined by gravimetry (Wetzel, 2001) in an analytical scale and converted into carbon-based particulate organic carbon (POC). For the conversion of particulate organic matter into carbon, it was assumed that the macrophytes carbon content is 47% ash-free (Wetzel, 2001). The organic matter content of particulate detritus was obtained by muffle incineration of the samples at 550°C for two hours (Blindow et al., 2006).

In a filtered dissolved organic matter fraction, the dissolved organic and inorganic carbon concentrations were determined using a carbon analyzer (Shimadzu TOC-5000A). In the dissolved fraction of the incubations, the pH and the electrical conductivity were determined with a multiparameter probe (HORIBA U-50). The concentrations of total nitrogen and total phosphorus were determined spectrophotometrically according to APHA (1998).

Mathematical modeling

To describe the macrophytes decomposition kinetics, the double exponential model was adopted, which considers that the detritus is composed by two fractions as described in Equation 1 (Lousier and Parkinson, 1976).

$$COP = COPLS \times e^{(-kT*t)} + COPR \times e^{(-kR*t)} \quad (1)$$

Where: COP_{LS} = soluble labile carbon (%); COP_R = refractory organic carbon (%);$k_T = k_1 + k_2$; overall coefficient of mass loss (day^{-1}); k_1 = the coefficient of mineralization of the labile fraction (day^{-1}); k_2 = leaching coefficient (day^{-1}); k_R = mass loss coefficient of the refractory fraction (day^{-1}).

The formation and mineralization of DOC were fitted to the model described in Equation 2 (Cunha-Santino et al., 2010).

$$\frac{dC_{COD}}{dt} = k_T\left(\frac{k_2}{k_T} C_{COPLS}\right) - k_3 C_{COD} \quad (2)$$

Where, dC_{COD} = change per time unit in the DOC concentration; k_T = leaching rate(day^{-1}); k_3 = COD digestion coefficient (day^{-1}).

The half-life ($t_{1/2}$) of the decomposition process was calculated by Equation 3:

$$t^{1}/_{2} = \ln(0.5)/-k \quad (3)$$

Where: k = decay coefficient for each type of plant fraction (K_{LS} and k_R).

Data analysis

Statistical analyzes were conducted to check the differences in the loss of the mass between species and the relationship between macrophytes decomposition coefficients and the nutrients concentration in the water. ANOVA statistic test was used, two-way repeated and measured in the STATISTIC software 7. This test was also used to test the isolated effects and the macrophytes species treatments and the trophic level over the phosphorus concentrations. Analytical approach was used for the data, and the significance was assumed as different at p ≤0.05 level.

RESULTS

Decomposition kinetics and carbon balance

The decomposition kinetics for soluble-labile fraction of the *E. densa* detritus (k_{COPLS} = 0.58, $t_{1/2}$ = 1 day) as well as the refractory fraction (k_{COPR} = 0.009; $t_{1/2}$ = 77.0) were higher in oligotrophic water. The same occurred with the *C. braunii* with both decomposition kinetics (k_{COPLS} = 0.99 ($t_{1/2}$ = 0.77 days) and refractory detritus (k_{COPR} = 0.008 ($t_{1/2}$ = 87 days) being higher in oligotrophic environment. Thus, there was no influence of the decomposition trophic state kinetics on the studied macrophyte ($F_{2,1}$ = 0.003; p = 0.85), disproving the hypothesis.

As for the average levels of the remaining particulate carbon, *E. densa* lost 76.93 and 64.66% of its initial mass, in eutrophic and oligotrophic incubations, respectively, while the *C. braunii* detritus lost 64.58 and 66.47% in eutrophic and oligotrophic waters, respectively (Figure 2). Statistically, the decomposition between the two species was similar, not corroborating our hypothesis ($F_{2,1}$=0.00,p = 0.97).

Regarding the carbon balance, it was observed that decrease in particulate carbon occurred concomitantly with the leaching of dissolved organic carbon (DOC) (Figure 3). Regarding dissolved carbon formation, the content observed in the *E. densa* decomposition was 6.0% over the initial carbon content of 3.5% in the incubations with *C. braunii* detritus. The dissolved carbon values increased until the 30th day in incubations with *E. densa* and as a result, there was a decrease at the end of the experiment which is up to 2% lower than in eutrophic and 4% in oligotrophic waters. In the *C. braunii* incubations, the dissolved carbon increase occurred until the 15th day, then, decreased for concentrations less than 0.5% in both trophic states tested. The dissolved carbon content generated from the species decomposition were different ($F_{2.1}$ = 29.68; p≤0.05), the organic carbon dissolved formed in *E. densa* decay was higher than that from *C. braunii* from the beginning to the end of the experiment. For the treatments, however, there was no significant difference between the dissolved carbon content ($F_{2.1}$ = 0.38, p = 0.53).

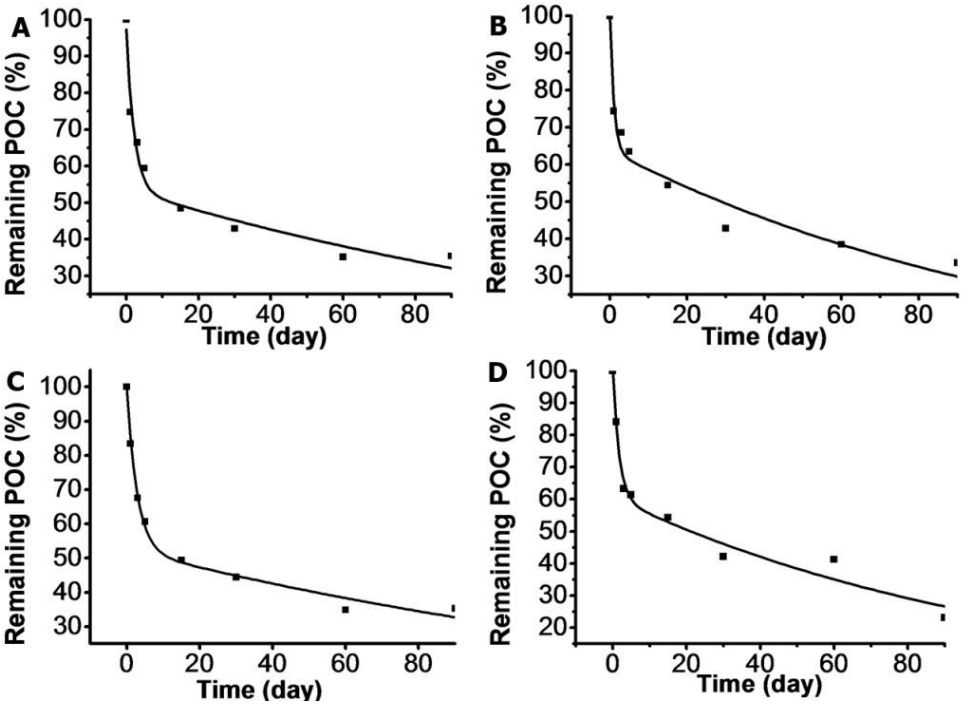

Figure 2. Temporal variation of particulate organic carbon (POC) during the anaerobic decomposition of *C. brauni* and *E. densa* in different states waters. A) Incubating in eutrophic water and *C. braunii;* B) Incubation in oligotrophic water and *C. braunii;* C) Incubation in eutrophic water and *E.densa;* D) incubation in oligotrophic water and *E. densa*.

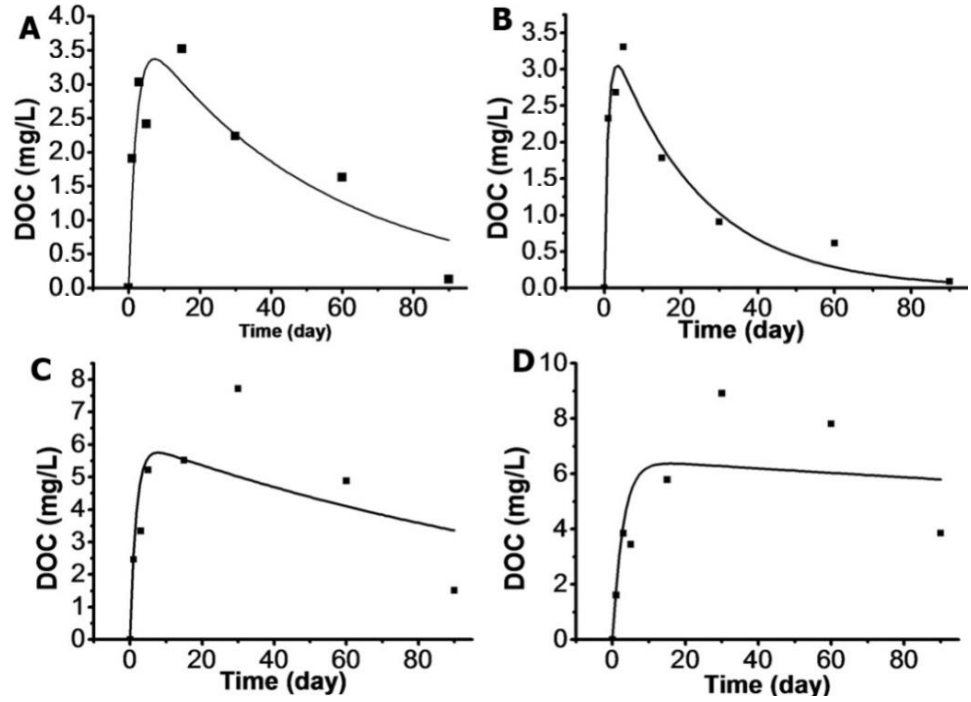

Figure 3. The DOC temporal change for *C. braunii* and *E. densa* decomposition process in oligotrophic and eutrophic media. A) Incubation with eutrophic water and the C. *braunii* B) Incubation with oligotrophic water and *C. braunii;* C) Incubation with eutrophic water and *E. densa* species D) Incubation with oligotrophic water and and *E. densa.*

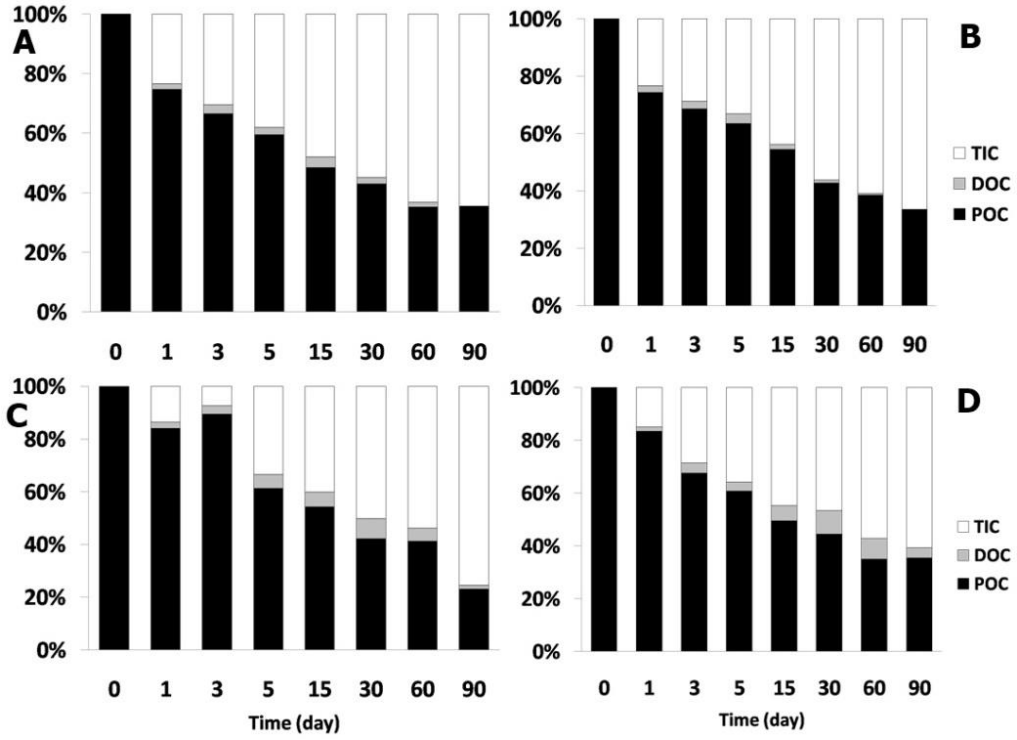

Figure 4. Carbon balance (total inorganic carbon (TIC), dissolved organic carbon (DOC) and particulate organic carbon (POC) (in %) in anaerobic incubations of *C. braunii* and *E. densa* decomposition). Incubation with eutrophic water and the C. *braunii*; B) Incubation with oligotrophic water and *C. braunii*; C) Incubation with eutrophic water and the *E. densa*; D) Incubation with oligotrophic water and *E. densa*.

The formation of dissolved carbon, mineralized carbon (TIC), and the decrease in the particulate carbon is shown in Figure 4. The dissolved carbon half-life was higher for decay of the *E. densa* in oligotrophic water ($t_{1/2}$ = 173.2 days). For both species, the dissolved carbon was mineralized with low coefficients (K_3): *E. densa*/ oligotrophic = 0.004 day^{-1}; *E. densa*/eutrophic = 0.020 day^{-1}; *C. braunii*/oligotrophic = 0.040 day^{-1} and *C. braunii*/eutrophic = 0.010 day^{-1}.

Dynamics of nutrients during decomposition

During the decomposition process, the phosphorus concentrations increased in the dissolved fractions of the decomposition incubations throughout the sampling period for both species (Figure 5). In the eutrophic incubations, the average phosphorus release values were higher than in the oligotrophic incubations, from 0.15 (initial concentration of phosphorus in water) to 7.5 ± 0.45 mg L^{-1} in the *C. braunii* decomposition incubations and 8.53 ± 0.07 mg l^{-1} in the *E. densa* incubations.

In oligotrophic treatments, the average P was 6.75 ± 0.35 mg l^{-1} in *C. braunii* decomposition and 7.85 ± 0.12 mg l^{-1} in *E. densa*. In both treatments, the *E. densa* detritus released higher phosphorus concentrations than *C. braunii*, but these differences were not significant ($F_{2,1}$ = 0.23; p = 0.62), the same occurred between treatments ($F_{2.1}$ = 0.00; p = 0.97), and with the combined effect of the species with treatment ($F_{2.1}$ = 0.13; p = 0.71).

Similarly, the N release (Figure 5) was not influenced by the species ($F_{2.1}$ = 0.03; p = 0.85). In the eutrophic environment, an increase in nitrogen concentration of 2.32 ± 0.58 mg l^{-1} was observed in the *E. densa* incubations and in the *C. braunii* incubations, the increase was 2.5 ± 0.23 mg l^{-1}. In the oligotrophic environment, the increase was 2.16 ± 0.03 mg l^{-1} (*E. densa*) and 1.4 ± 0.58 mg l^{-1} for *C. braunii*. There was no trophic state influence on the N release ($F_{2.1}$ = 0.02; p = 0.87).

With high nutrient concentrations, changes in the water physical parameters could be observed such as the electrical conductivity reaching higher values in eutrophic incubations, from 1.574 ± 5.18 to 6.346 ± 5.5 μScm^{-1} in *C. braunii* decomposition and 6.346 ± 5.5μScm^{-1} in *E. densa* decomposition. In the oligotrophic treatment, the increase was 1.135 ± 21.45 to 5.793 ± 5.7 μScm^{-1} (*C. braunii*) and 4.050 ± 26.43 μScm^{-1} (*E. densa*). There were significant differences in the electrical conductivity variation during decomposition considering the different

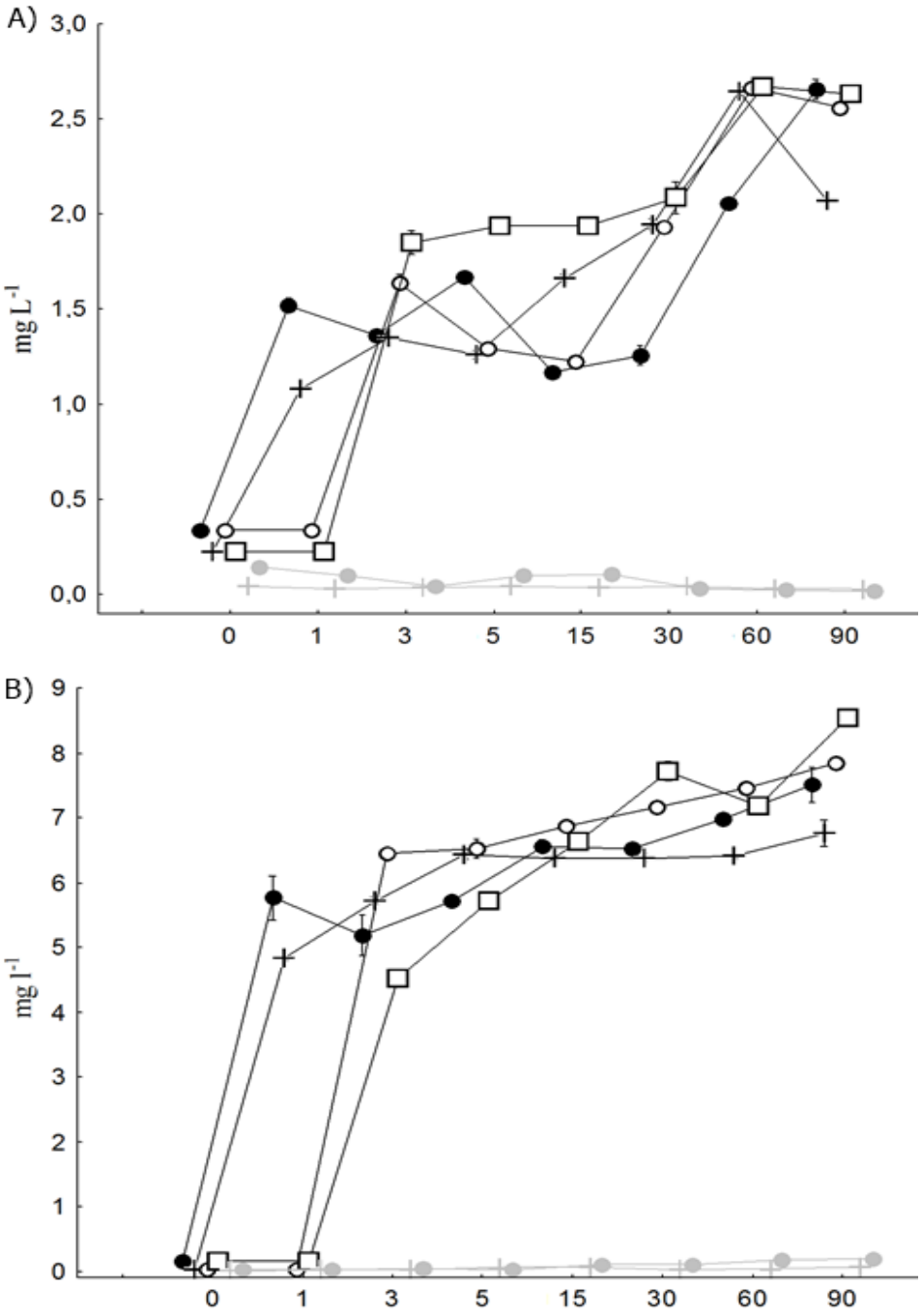

Figure 5. Temporal variation in nitrogen (A) and phosphorus (B) release of *E. densa* and *C. braunii* decomposition incubations. ● Eutrophic *C. braunii* incubation; ┼ Oligotrophic *C. braunii* incubation; ○ Eutrophic *E. densa* incubation; □ Oligotrophic *E. densa* incubation; ● No plant control group in eutrophic water; ┼ No plant control group in oligotrophic water.

species ($F_{2.1}$= 20.35; p ≤0.05) and also in the trophic degree ($F_{2.1}$ = 4.64, p = 0.03).

As in electrical conductivity, pH in the decomposition incubations also changed during the decomposition process, reaching slightly acidic values in the incubation in the first 24 h. In the *C. braunii* decomposition in oligotrophic treatment, the decomposition chamber pH decreased from 9.2 ± 0.40 to 5.7 ± 0.06 on the first day and in the end of the experiment, the pH was 6.3 ± 0 00. In the eutrophic environment, the pH decreased from 8.8 ± 0.48 to pH 6.7 ± 0.11 on day one, reaching the value of 6.4 ± 0.00 at the end of the experiment.

In the *E. densa* decomposition, a higher acidification was observed in the incubations than with *C. braunii* ($F_{2.1}$ = 6.18; p = 0.001). In the eutrophic treatment, the medium pH decreased from 8.8 ± 0.48 to pH 6.4 ± 0.03, with some values below 5.5 ± 0.59 between the 3rd and 15th days. In the oligotrophic incubations, the pH decreased from 9.2 ± 0.40 to pH 5.7 ± 0 on the first day, showing variation of 4.7 ± 0.17 to 5.5 ± 0.0. There were no significant differences between trophic state and pH change ($F_{2.1}$ = 1.66; p = 0.20).

DISCUSSION

Phosphorus and nitrogen-enriched water may accelerate the detritus decomposition (Rejmánkavá and Houdkavá, 2006); however, other studies suggest slower decay rates in eutrophic waters (Sarneel et al., 2010). In this study, the lack of relationship between the trophic state and the mass loss was due to the availability of water nutrients (nitrogen and phosphorus) and does not represent a limiting factor for the macrophytes decomposition and does not influence the chemical immobilization (Xie et al., 2004). The metabolic activities of microorganisms usually occur according to the quantity and especially the quality of the detritus (Cunha-Santino and Bianchini, 2009). In this case, eutrophic waters may have lower decomposition rates if the detritus quality (intrinsic factor) is a predominant constraint on the decomposition process as observed in *C. braunii*.

Overall, there were no differences between the remaining mass content of the two species, although belonging to different groups, both are submerged macrophytes, and have similar habits, with lower content of hard support tissues (Suzuki et al., 2013) than those found in emergent macrophyte. Thus, from the quantitative point of view, the decomposition of these species were similar and fast, since there is a proximity of mineralized carbon in both species (*E. densa* = 87.76% and *C. braunii* = 75.15%).

The k_r coefficients were low in the species, due to the slow degradation and the presence of fibers which can exert a barrier in the anaerobic degradation (Agoston-Szabó and Dinka, 2008). Cellulose fiber in *Chara* species biomass was determined at 9.67% (Muztar et al., 1978), whereas for *E. densa*, it was 29.2% (Little, 1979).

As they presented slower decomposition rates, the fibers are generally accumulated in limnic sediments, becoming possible precursors of humic compounds (Bianchini and Cunha-Santino, 2008), which allows us to affirm that the refractory fractions of *E. densa* and *C. braunii*, could act in the ecosystem metabolism as a possible precursor source in the humic substances genesis, due to the low coefficient of mineralization (k_R). The dissolved carbon presented a refractory potential during *E. densa* and *C. braunii* decomposition, with low mineralization coefficients (K_3). In aquatic environments, the dissolved carbon is mostly (up 60%) composed of humic substances (Bianchini et al., 2014). The refractability was primarily due to the type of synthesized by-product by decomposing microorganisms in the specific conditions adopted in this experiment, that is, anaerobic, temperature and substrate type (Cunha-Santino and Bianchini, 2009). In the decomposition process, transformations of plant tissues fractions, by leaching, in dissolved carbon, are very important because these compounds interfere with the organic carbon transfers to the water column organisms, as well as to that held on the particulate detritus (Sala and Gude, 1999).

In this study, P and N releases were observed throughout the *C. braunii* and *E. densa* decomposition process, indicating that these nutrients are part of these plants biomass, being raised in the aquatic environment from the growth phase to senescence. The amount of incorporated nutrients depends on the productivity rate and the particular species can interfere with decomposition, like *Chara* species, storing nutrient for long periods, especially during the low temperature periods, slowing the aging process (Kufel and Kufel, 2002).

The decay of detritus in *E. densa* and *C. braunii* provoked an intense release of P and N in the decomposition chamber and may have an impact during its death on the water column in the environment colonized by these species, especially in the first 15 days in which concentrations of these nutrients were higher. In the leaching phase, release of P was higher than N. In plant biomass decay processes, the fractions of P may be more soluble than the N fractions (Rejmánková and Houdkova, 2006). Throughout the experiment, P was accumulated in the water, and not consumed, unlike the study by Kroger et al. (2007) which reported a decrease of P concentration in water since they were added to the pellet (environmental route) by decay.

With regards to the released concentrations of N and P, the decomposition of these species is a potential source of nutrients, thereby contributing to the eutrophication process, since these nutrients act as one of the limiting factors causing the process (Mattar et al., 2009). As the submerged macrophyte have faster decay rates (Petersen and Cummin, 1974) than other macrophytes (emergent, foil floating and floating), nutrients stock in the biomass of these organisms is of short-term duration.

During decomposition, electrical conductivity increased due to the large accumulation and release of ions present in the leached materials (Mun, 2000). In this decomposition stage, an intense generation of inorganic carbonaceous compounds (e.g. CO_2) emissions from anaerobic mineralization also occurred. From the degradation phase of labile-soluble fraction, the electrical conductivity continued to increase without stabilization due to the ions released from the refractory fraction decomposition of detritus, mainly in *C. braunii* incubations.

Parallel to conductivity, pH decreased rapidly at the beginning of the experiment; this was due to the medium acidification, ammonium, bicarbonates and organic acids formation. Over time, the media pH increased due to the anaerobic ammonium oxidation reactions (Mulder et al., 1995). The frequency means of slightly acidic to softly (mean <4.7 and <6.9) were probably due to the balance between the buffering systems and constant input of intermediates during the process, which would tend to the medium acidification (Weimer and Zeikus, 1977).

The hypothesis of this study was that the decomposition process of the *E. densa* and *C.* braunii occurs faster in eutrophic than in oligotrophic waters, surprisingly, it was found in this study that there was no significant difference in mass loss of *E. densa* and *C. braunii* detritus in oligotrophic and eutrophic environment. Although, they are species with distinct chemical and structural evolution from a quantitative point of view of carbon, these species have similar ecosystem metabolic responses. The differences in the contents of labile and refractory-soluble compounds in the same species are due to the use of different proportions of the plant parts (stem and leaf) in the decomposition incubations.

Conclusion

The mass loss kinetics between *E. densa* and *C. braunii* was not significantly different in the present study, which rejects the hypothesis that these species represent distinct divisions (Chlorophyta x Spermatophyta) and present distinct kinetics of mass loss. The trophic state (eutrophic and oligotrophic media) of semiarid Paraiba did not represent a limiting or stimulatory factor for the decay of *E. densa* and *C. braunii* detritus. The decomposition of both species is a potential source of nutrients release that can cause eutrophication in water bodies where such weeds live. The accumulation of these refractory compounds (such as fibers) due to decomposition, occurred in long-term, which could generate accumulation of humic substances in the aquatic environment.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Agoston-Szabó E, Dinka M (2008). Decomposition of *Typha angustifolia* and *Phragmites australis* in the littoral zone of a shallow lake. Biologia 63(6):1104-1110.

Anesio AM, Abreu PC, Biddanda BA (2003). The role of free and attached microorganisms in the decomposition of estuarine macrophyte detritus. Estuar. Coast. Shelf Sci. 56(2):197-201.

APHA. Standard Methods for the Examination of Water and Wastewater (1998). American Public Health Association, American Water Works Association, Water Environmental Federation. no.20. Washington, D.C.

Bianchini JI, Cunha-Santino MB (2008). As rotas de liberação do carbono dos detritos de macrófitas aquáticas. Oecol. Bras.12:20-29.

Bianchini JI, Cunha-Santino MB, Ribeiro JU, Penteado DGJ (2014). Implication of anaerobic and aerobic decomposition of *Eichhornia azurea* (Sw.) Kunth. on the carbon cycling in a subtropical dam. Braz. J. Biol. 74(1):100-110.

Bianchini JI, Cunha-Santino MB, Romeiro F, Bitar AL (2010).Emissions of methane and carbon dioxide during anaerobic decomposition of aquatic macrophytes from a tropical lagoon (São Paulo, Brazil). Acta Limnol. Bras. 22(2):157-164.

Blindow I, Hargeby A, Meyercordt J, Schubert H (2006). Primary production in two shallow lakes with contrasting plant form dominance: a paradox of enrichment? Limn. Oceanog. J. 51(6):2711-2721.

Cunha-Santino MB, Bianchini J(2009). Humificação e mineralização de macrófitas aquáticas: uma revisão sobre esses processos. Oecol. Bras. 13(4):666-676.

Fonseca ALS, Bianchini JI, Pimenta CMM, Mangiavacchi N, Soares CBP (2014). Kinetics of aerobic decomposition in the leaching phase of allochthonous plant detritos. Acta Limnol. Bras 26(1):89-97.

Kim, JG, Rejmanková, E (2004). Decomposition of macrophytes and dynamics of enzyme actives in subalpine marshes in Lake Tahoe basin, U.S. A. Plant and soil, 266:303-313.

Kroger R, Holland MM, Moore MT, Cooper CM (2007). Plant senescence: a mechanism for nutrient release in temperate agricultural wetlands. Environ. Pollut. 146:114-119.

Kufel L, Kufel I (2002).Chara beds acting as nutrient sinks in shallow lakes – a review. Aquat. Bot. 72(3):249-260.

Little ECS (1979). Handbook of utilization of Aquatic Plants, FAO Fisheries Technical Paper 187:176.

Lousier JD, Parkinson D (1976). Litter decomposition in a cool temperate deciduous forest. Can. J. Bot. 54(5-6):419-436.

Mattar-Neto J, Krüger CM, Dziedzic M (2009). Análise de indicadores ambientais no reservatório do Passaúna. Eng. Sanit. Ambient 14(2):205-214.

Mulder A, Van De Graaf AA, Robertson LA, Kuenen JG (1995). Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiol. Ecol. 16(3):177-184.

Mun HT (2000). Mass loss and changes of mineral nutrients during decomposition of mushrooms, *Russulaalbo aerolata* and *Lactarius violascens*. Korean J. Biol. Sci. 4(1):51-55.

Muztar AJ, Slinger SJ, Burton JH (1978).Chemical composition of aquatic macrophytes: Investigation of organic constituents and nutritional potential. Can. J. Bot. 58(3):829-842.

Palma-Silva C, Albertoni EF, Trindade CRT, Furlanetto LM, Acosta MC (2012). Uso de *Eichhornia crassipes* (mart) Solms para fitorrremediação de ambientes eutrofizados subtropicais no sul do Brasil. Perpectivas 36(133):73-81

Patarra RFL, Paiva AI, Neto E, Lima J (2011). Nutricioonal value of selected macroalga. J. Appl. Physiol. 23(2):205-208.

Petersen RC, Cummins KW (1974). Leaf processing in a woodland stream. Freshw. Biol. 4(4):343-368.

Rejmánková E, Houdková K (2006). Wetland plant decomposition under different nutrient conditions: what is more important, litter quality or site quality. Biogeochemistry 80(3):245-262.

Rodrigues RB, Thomaz SM (2007). Photosynthetic and growth responses of *Egeria densa* to photosynthetic active radiation. Aquat. Bot. 92(4):281-284

Sala MM, Gude H (1999). Role of protozoans on the microbial ectoenzymatic activity during the degradation of macrophytes. Aquat. Microb. Ecol. 20:75-82.

Sampaio EVSB, Oliveira NMB (2005). Aproveitamento da macrófita aquática *Egeria densa* como adubo orgânico. Planta Daninha 23(2):169-174.

Sarneel JM, Geurts JJM, Beltman B, Lamers LPM, Nijzink MM, Soons MB, Verhoeven TA (2010). The effect of nutrient enrichment of either the banck or the surface water on shoreline vegetation and decomposition. Ecosystems 13(8):1275-1286.

Scheffer M, Van Nes EH (2007). Shallow lakes theory revisited: various alternative regimes driven by climate, nutrients, depth and lake size. Hydrobiologia 584:455-466.

Suzuki MS, Fonseca MN, Esteves BS, Chagas GG (2013).

Decomposition of *Egeria densa* Planchon (hydrocharitaceae) in a well oxygenated tropical aquatic ecosystem. J. Limnol. 74(2):278-285.

Vasconcelos JF, Barbosa JEL, Lira W, Azevedo SMFO (2013). Microcystin bioaccumulation can use potential mutagenic effects in farm fish. Egypt. J. Aquat. Res. 39(3):185-192.

Wang BLIF, Fan Z (2013). Nutrient release during the decomposition of submerged macrophyte (*Hydrilla verticillata*). J. Food Agric. Ecosyst. Environ. 1(3):30567-2572.

Weimer, PJ, Zeikus, JG (1977). Fermentation of cellulose and cellobiose by *Clostridium thermocellum* in the absence of *Methanobacterium thermoautotrophicum*. Appl. Environ Microbiol. 33(2):289-297.

Wetzel RG (2001). Limnology: Lake and river ecosystems. Academic Press, California, USA, (3):850.

Xie Y, Yu D, Ren B (2004). Effects of nitrogen and phosphorus availability on the decomposition of aquatic plants. Aquat. Bot. 80(1):29-37.

academicJournals

Vol. 16(49), pp. 2267-2277, 6 December, 2017
DOI: 10.5897/AJB2017.16202
Article Number: F22438366963
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

The impact of over-expression of NPK1 gene on growth and yield of sorghum under drought stress

Shireen K. Assem[1*], Mohamed M. Zamzam[2], Mohamed E. Saad[1,4], Basita A. Hussein[3] and Ebtissam H. A. Hussein[3]

[1]Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza, Egypt.
[2]Department of Agricultural Botany, Faculty of Agriculture, Al-Azhar University, Cairo, Egypt.
[3]Department of Genetics, Faculty of Agriculture, Cairo University, Giza, Egypt.
[4]Department of Biology, Faculty of Sciences, Taibah University, Almadinah Almonawarah, Kingdom of Saudi Arabia.

Received 12 August, 2017; Accepted 8 November, 2017

***Sorghum bicolor* L. is an important crop in many tropical regions, yet it has received little attention in applying modern biotechnologies for improvement due to transformation difficulties. Drought is among the most important factors limiting sorghum productivity. The *Nicotina* Protein Kinase 1 (*NPK1*) gene confers tolerance to adverse environmental conditions. The present work was conducted to determine the amenability of sorghum genotypes to *Agrobacterium*-mediated transformation with the *NPK1* gene and to characterize the role of that gene in *S. bicolor*. The *NPK1* gene along with the *bar* gene was successfully introduced into two sorghum genotypes: Dorado and SPGM94021. The transformation frequencies were 1.49 and 1.79% for Dorado and SPGM94021, respectively. Stable integration of the transgenes in T_0 and T_1 plants was confirmed through polymerase chain reaction (PCR) and Southern blotting. The *NPK1* gene expression was measured through real-time-PCR. T_1 plants tested *in vitro* for tolerance to mannitol osmotic stress maintaining a higher growth rate and showed increased tolerance to stress conditions compared to the non-transgenic plants. The transgenic sorghum had a significantly higher kernel weight under drought stress conditions than the control plants. Accordingly, the *NPK1* gene might induce a mechanism that protects sorghum plants against possible water-deficiency stress conditions.**

Key words: Abiotic stress related genes, regulatory genes, signal transduction, transgenic sorghum, plant transformation, quantitative real time-polymerase chain reaction (qRT-PCR), drought stress tolerance.

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench], a tropical plant of the family Poaceae, is the fifth most important cereal after wheat, rice, maize and barley and plays a unique role in food security and renewable energy (Belton and Taylor, 2004). The crop is well adapted to tropical and subtropical areas throughout the world. Beside its

*Corresponding author. E-mail: shireen_assem@yahoo.com or shireenassem@ageri.sci.eg.

principal uses as flour, in the preparation of porridge and unleavened bread, sorghum species are sources of fiber and fuel and are, also, used in the alcohol industry as they are rich in starch. Despite its importance, the yield and quality of sorghum are constrained by a range of biotic and abiotic factors. Nevertheless, abiotic stresses remain the greatest constraints of crop production with approximately 70% estimated yield reduction (Acquaah, 2007).

Drought is one of the priciest factors affecting a huge number of people every year (Wilhite, 2000). Drylands occupy about 41% of the global terrestrial surface and are home to more than a third of the world's population (Mortimore, 2009). Moreover, the increase of potential evapotranspiration and the changes of the precipitation patterns under a warming climate are leading to a growing global aridity and expansion of drylands (Milesi et al., 2010). Therefore, interest in crop tolerance to environmental stresses has been growing since the last few decades (Tuberosa and Salvi, 2006).

Sorghum is a drought tolerant crop and often grows where water stress conditions are expected. However, the yields, under dryland conditions, are severely affected and much less than that of irrigated sorghum (Assefa and Staggenborg, 2010). Several investigations have been conducted on grain sorghum under stress conditions. Seed mass, harvest index, and biomass were among the parameters affected, most severely, by moisture stress (Wenzel, 1999). Genetic engineering is a potential tool for crop improvement. Although grain sorghum is a very important cereal, particularly in the semi-arid tropic areas as a vital source of food for millions of people around the world, sorghum improvement through genetic engineering is progressing slowly. Like other cereal crops, the first fertile transgenic sorghum plants were obtained *via* particle bombardment (Casas et al., 1993). *Agrobacterium*-mediated transformation of sorghum was first reported seven years later (Zhao et al., 2000).

To improve sorghum abiotic stress tolerance, *HVA*1, thaumatin-like protein (TLP), *mtld*, *OsCDPK*-7 and *TPS*1 genes were successfully introduced into sorghum genome (Devi et al., 2004; Gao et al., 2005a; Maheswari et al., 2010; Mall et al., 2011; Yellisetty et al., 2015). In order to make plants more tolerant to stress and restore the cellular balance, transferring a single gene encoding a single specific stress protein, that is, "single-action" genes may not suffice the required tolerance levels. To overcome such constraint, plant transformation with regulatory genes has emerged (Bhatnagar-Mathur et al., 2008).

Nicotina Protein Kinase 1 (*NPK1*) is a regulatory single transduction gene located upstream of the oxidative pathway and can induce expression of HSPs and GSTs (Kovtun et al., 2000; Shou et al., 2004a). Under stress conditions, plants generate reactive oxygen species, including hydrogen peroxides, superoxide anion, and hydroxyl radical (Shou et al., 2004a). Hydrogen peroxide accumulation can initiate the expression of detoxification and stress protection genes, e.g. heat shock proteins (HSPs), glutathione-S-transferases (GSTs), peroxidases and pathogenesis-related proteins, thereby, guarding plants against stress damages (Kovtun et al., 2000). HSPs serve as molecular chaperones in the ATP-dependent protein assembly/disassembly and prevent protein denaturation during stress (Horwich, 2014). GSTs are enzymes that can detoxify endobiotic and xenobiotic compounds through covalent linkage of glutathione to hydrophobic substrates. Activation of these stress genes can reduce damage caused by chilling, heat, and drought; thus, protect plants from environmental stresses (Das and Roychoudhury, 2014; Li et al., 2003), thereby improving the yield potential of the major cereal crops.

The objectives of this study were to utilize *Agrobacterium*-mediated transformation method to develop water use efficient sorghum lines and to evaluate the regenerated transgenic lines at molecular level. An additional goal was to evaluate the first generation of the transgenic lines (T1) for survival to water deficiency *in vitro* and under greenhouse conditions.

MATERIALS AND METHODS

Plant materials, *Agrobacterium tumefaciens* strain and binary vector

Two *S. bicolor* genotypes, that is, Dorado and SPGM94021, obtained from the Department of Sorghum, Field Crops Research Institute, Agricultural Research Center, Egypt, were sown in the field during the sorghum season and plants have been self-pollinated. Panicles were harvested 10 to 12 days post pollination. *A. tumefaciens* strain LBA4404 containing the standard binary vector pSHX004 (Shou et al., 2004a) was used for sorghum transformation. The binary vector has been introduced into the *A. tumefaciens* strain LBA4404 by direct transformation of the competent cells with the pSHX004 DNA. The vector system, pSHX004 in LBA4404, was maintained on a yeast extract peptone (YEP) medium containing 50 mg/L spectinomycin and 25 mg/L rifampicin. Bacteria cultures, for weekly experiments, were initiated from stock plates stored for up to two weeks at 4°C.

Sorghum transformation

Sorghum panicles were surface sterilized as described in Assem et al. (2014). Immature embryos ranging in length from 1.0 to 1.5 mm were aseptically excised from kernels and prepared for transformation. The *A. tumefaciens* strain LBA4404 was used for the delivery of the binary vector pSHX004 into the dissected immature embryos.

For transformation, *A. tumefaciens* cultures were grown for 2 days at 28°C on YEP medium amended with 100 mg/L spectinomycin and 25 mg/L rifampicin. One loop of the culture was scrapped and suspended in 5 ml of liquid infection medium supplemented with 100 µM acetosyringone. Immature zygotic embryos were washed twice with bacteria-free infection medium, the final wash was discarded and 1 to 1.5 ml of *A. tumefaciens* suspension (OD_{550} = 0.4-0.5) was added to the embryos. The tubes were incubated, in dark, for 10 min at ambient temperature. After

Table 1. Composition of utilized media.

Media	Composition
Infection	4.3 g/L MS salts (Duchefa Biochemie, Prod. No M0221.0050), 0.1 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 10 mg/L thiamine HCl, 1.5 mg/L 2,4-D, 1 g/L vitamin assay casamino acid, 36 g/L glucose, 68.5 g/L sucrose, pH 5.2. Add 100 µM acetosyringone before using.
Co-cultivation	Infection media with reducing sucrose to 20 g/L and glucose to 10 g/L, increasing 2,4-D to 2 mg/L, adding 0.5 mg/L MES, 0.7 g/L L-proline, 10 mg/L ascorbic acid, pH 5.8, 3 g/L phytagel. Adding 100 µM acetosyringone after autoclaving.
Resting	Co-cultivation media with reducing 2,4-D to 1.5 mg/L. Adding 10 g/L PVP and 100 mg/L carbenicellin (After Autoclaving). Without glucose and acetosyringone.
Selection	Resting media with 1.5 mg/L bialaphose (After autoclaving).
R1 (shooting)	4.3 g/L MS salts (Duchefa Biochemie, Prod. No M0221.0050), 0.1 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 10 mg/L thiamine HCl, 0.7 g/L L-proline, 60 g/L sucrose, 10 g/L PVP, pH 5.8, 3 g/L phytagel. After autoclaving add 0.1 mg/L TDZ, 0.25 mg/L ABA, 0.5 mg/L Zeatin, 1 mg/L IAA, 100 mg/L carbenicellin.
R2 (rooting)	2.2 g/L MS including vitamins (Duchefa Biochemie, Prod. No M0222.0050), 20 g/L sucrose, 3 g/L phytagel, pH 5.8. After autoclaving, add 100 mg/L carbenicellin.

infection, embryos were transferred to co-cultivation medium with scutellum side up. Plates were incubated in the dark at 22°C for 4 days after which embryos were transferred to resting medium and incubated at 28°C for one week. The transformation process can be divided into 5 sequential steps: agro-infection, co-cultivation, resting, selection, and plant regeneration. The infection and the co-cultivation media were prepared as described by Zhao et al. (2000), without modification. The compositions of all media utilized in this study are shown in Table 1.

Selection and regeneration

For stable transformation, selection of transformed events was carried out using selection medium containing 1.5 mg/L bialaphos as a selective agent. After 8 weeks on selection medium, survived embryogenic calli were transferred to regeneration medium (R1) for shoot development. Calli-derived shoots were transferred to rooting medium (R2). Cultures of shooting and rooting stages were maintained at 28°C under 16 h photoperiod cool white fluorescent light (75 µmol/m^2/s). Regenerated plantlets have been transferred to soil for further development in the greenhouse. Putative transgenic and non-transgenic plants were subjected to leaf painting when 6 to 7 leaves had fully emerged from the whorl. Solution of the commercial Basta herbicide at concentration of 0.1% was applied to the upper and lower surfaces of the fifth fully emerged leaf.

Polymerase chain reaction (PCR)

PCR analysis was conducted to screen the putatively transgenic T_0 and T_1 plants. Genomic DNA was extracted from putatively transgenic sorghum plants and non-transformed plants, as control, using the cetyltrimethylammonium bromide (CTAB) protocol (Murray and Thompson, 1980). The forward and reverse primers, 5' TAACAAATGGATGCTGAAGC 3' and 5' CCATCCCAACATAGTGAGAT 3', were used to amplify a 605 bp fragment of DNA containing part of the *NPK1* transgene; whereas the forward and reverse primers, 5' TAC ATCGAGACAAGCACGGTCAACT 3' and 5' ACGTCATGCCAGT CCCGTG 3', were used to amplify a fragment of 484 bp for the *bar* gene. PCR reactions were carried out in a total volume of 25 µl containing 10 ng of genomic DNA, 1x PCR buffer, 3 mM $MgCl_2$, 0.2 µM of forward and reverse primers, 0.24 mM dNTP, and 1.5 U of Taq polymerase. Amplification was conducted in a T100 thermocycler (BIO-RAD, Singapore) using the following program for the *NPK1* fragment: An initial DNA denaturation for 5 min at 94°C followed by 35 amplification cycles (94°C, 1 min; 55°C, 1 min, 72°C, 1 min) and a final extension step at 72°C for 10 min. For the amplification of *bar* fragment, the annealing temperature was increased to 60°C for 1 min.

Southern blotting

Ten micrograms of genomic DNA from sorghum plants were used for Southern blot analysis. DNA from T_0 and T_1 *Agrobacterium*-derived events and control plants were digested with the restriction enzyme *Eco*RI, at 37°C overnight. Digested DNA was separated on a 0.8% (w:v) agarose gel and transferred by alkaline transfer method onto a positively charged nylon membrane (Boehringer, Mannheim, Germany) and then cross-linked to the membrane by UV irradiation. A fragment (605 bp) of the *NPK1* gene sequence, generated by PCR, was DIG-labeled and used as a probe. The membrane was hybridized overnight at 68°C with the labeled probe. The hybridization signals were detected by the colorimetric method. The labeling and signal detection were performed using the random priming DNA labeling and detection kit (Roche Cat. No.11093657910).

Quantitative Real-Time PCR

Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen,

Table 2. Summary of transformation experiments of two sorghum genotypes using pSHX004 harboring the *NPK1* gene.

Genotype	No. of embryos on Co-cultivation	No. of survived embryogenic calli after 8 weeks of selection	No. of regenerated plantlets	No. of plantlets (+PCR)	Transformation frequency (%)
Dorado	535	151[b]	14[a]	8[a]	1.49[a]
SPGM94021	334	125[a]	11[a]	6[a]	1.79[a]

Numbers with the same letters do not differ statistically between themselves at the level of 5% probability.

Hilden, Germany) following manufacturer's instructions. After removing DNA using TURBO DNAse following the manufacturer's instructions (Life Technologies), total RNA samples were quantified using the Qubit RNA assay (Life Technologies). One step-Taqman assay was used to detect the amount of *NPK1* mRNA in the samples. The 18S small-subunit ribosomal RNA gene was used as the endogenous control for RNA quantification. *NPK1* primers and probe were synthesized and labeled by Life Technologies (Carlsbad, CA). Real-time PCR *NPK1* primers and probe sequences were obtained from Shou et al. (2004b) and 18S rRNA TaqMan assay mix was obtained from Life Technologies. Real-time PCR was carried out using the 7500 Fast Real-Time PCR instrument (Life Technologies). Reactions were conducted in a total volume of 20 µl containing 100 ng of sorghum total RNA, 10 µl of 2X master mix from the TaqMan RNA-to-CT 1-step Kit (Life Technologies), 0.4 mM of each primer, 0.2 mM of probe, and 0.5 µl of reverse transcriptase from the same kit. PCR conditions were as follows: Reverse transcription on 48°C for 15 min, heat activation at 95°C for 10 min, followed by 40 amplification cycles (at 94°C for 30 s, annealing at 57°C for 1 min and extension at 72°C for 1 min). All samples were tested in triplicate. To eliminate the possibility of inherent variation in the amount of starting material between samples, the amounts of *NPK1* mRNA detected by real-time RT-PCR were normalized by dividing them with their corresponding amounts of 18S rRNA**.**

Preliminary evaluation of transgenic plants under water-deficit conditions

The performance of the transgenic plants expressing the *NPK1* gene was evaluated under mannitol stress conditions using seeds from the two transgenic lines Dorado and SPGM94021. Drought tolerance assay was applied as described in Kim et al. (2014) with modifications. Briefly, T_1 seeds from each transgenic line and non-transgenic lines were surface-sterilized and germinated for four days in the dark at 25°C on half-strength MS basal medium containing 1% sucrose with or without 5 mg/L bialaphos for transgenic and non-transgenic plants, respectively. Germinated seedlings were then transferred to a half-strength MS containing 400 mM mannitol medium and maintained at 28°C under 16 h photoperiod cool white fluorescent light (75 µmol/m^2/s). These experiments were replicated three times. The response of young seedlings to stress conditions was analyzed after 6 days. The parameters of shoot length, root length and fresh weight were scored for twenty randomly selected bialaphos resistant seedlings and plantlets were transferred to a soil mixture composed of peatmoss: soil: sand (1:1:1) in pots of 10 cm diameters. Plantlets were grown in greenhouse at 28°C and 16/8 h (light/darkness) photoperiod. Plantlets were grown under well-watered conditions of 100% field capacity for the first three weeks. Soil water content was maintained by weighing and adding water into pots, daily. After three weeks of well irrigation, water withholding was applied for 11 to 13 days (depending on the weather) until leaves wilted, at which point the plants were irrigated. Repeated drought cycles were imposed until plants reached anthesis. The yield potential was evaluated by counting the number of kernels per panicle and weighing 100 grains harvested from the transgenic and control plants grown under drought stress conditions in the green house.

Statistical analysis

Three replicates of each experiment were analyzed for significant difference between treatments. For transformation experiments, to eliminate the variation in the initial number of transformed embryos, the percentage values of each replicate were compared in both genotypes. For the preliminary evaluation of T1 plants, the mean values of each parameter from twenty randomly selected plants for each replicate were compared relatively to the other genotype and to the nontransgenic control. Significance was determined by the analysis of variance (ANOVA) employing ASSISTAT software version 7.7 using a complete randomized design with repetition and the difference between the means were compared using Tukey test at a level of 5% probability.

RESULTS

Sorghum transformation and regeneration

Agrobacterium-mediated transformation was utilized to develop transgenic sorghum plants with low transgene copy number. Two sorghum genotypes; Dorado and B-SPGM49021 were transformed with the standard binary vector pSHX004 in LBA4404. The transgene of interest was the kinase domain of the *NPK1* gene driven by a constitutive promoter (35S C4PPDK). The transformation data (Table 2) were obtained from three independent experiments for sorghum genotype Dorado and two independent experiments for genotype SPGM94021.

Most of the infected immature embryos showed normal growth on co-cultivation and resting media, although some embryos failed to grow after transformation. Selection stage was initiated by placing the embryo-derived calli on selection media (callus induction media containing 1.5 mg/l bialaphos). After two months of selection, survived embryogenic calli were used for shoot regeneration (Figure 1). Transgenic plants grew normally in the greenhouse like non-transgenic plants and showed normal fertility. The transformation frequencies based on PCR screening of

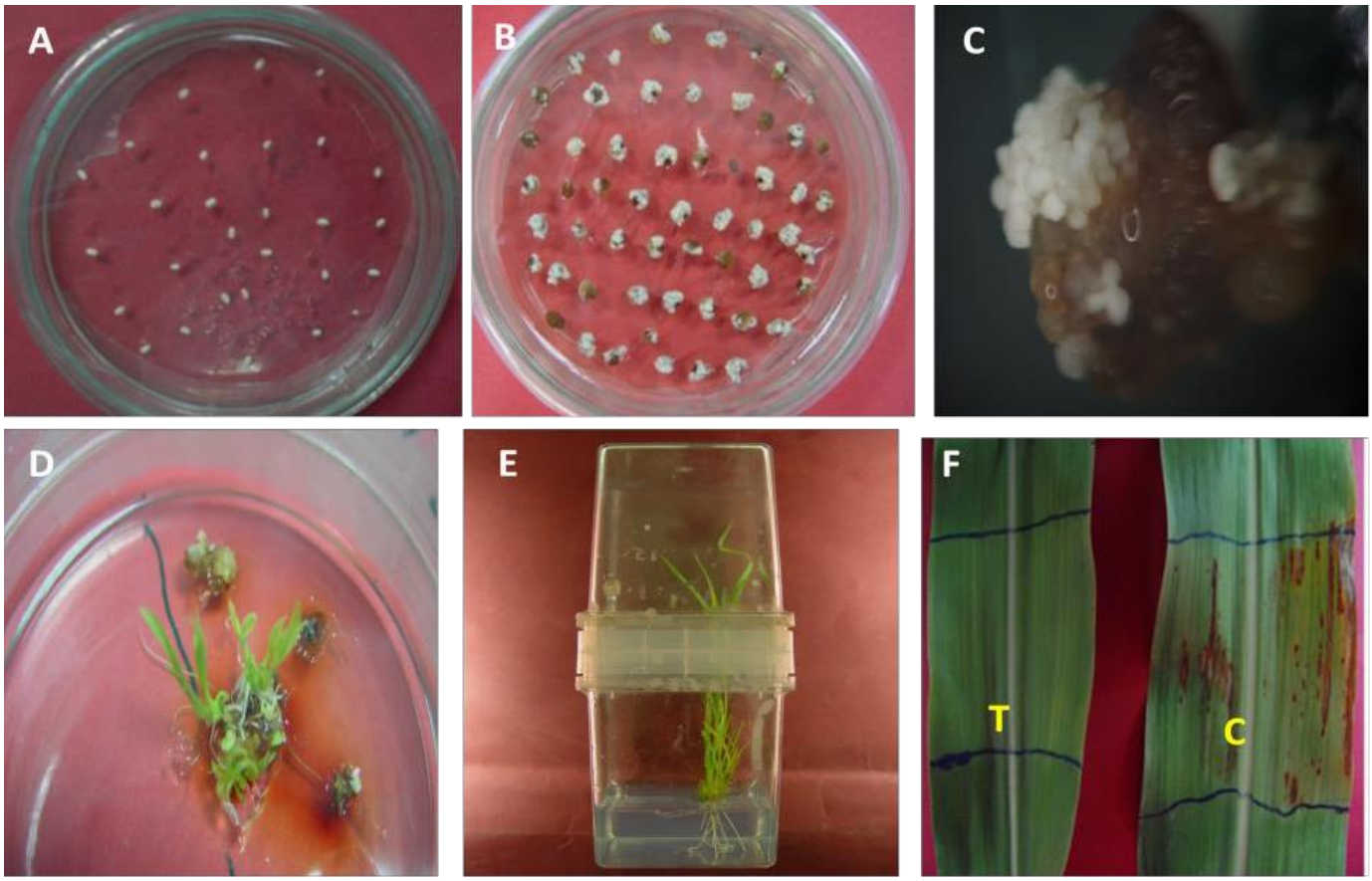

Figure 1. Different stages of transformation. (a) Immature embryos after *Agrobacterium* infection. (b&c) Embryogenic calli on selection medium. (d) Shoots formation on regeneration medium. (e) Putative transgenic sorghum plantlets on rooting medium. (f) Basta herbicide painting of transgenic "T" and non-transgenic control "C" leaves.

the putatively transgenic plantlets were 1.79 and 1.49% for B-SPGM94021 and Dorado, respectively. Transformation frequency was calculated as the number of regenerated PCR-positive plantlets/number of agro-infected embryos × 100. Leaf painting assay indicated improved tolerance of the transgenic plants to Basta herbicide as compared to the non-transgenic plants which showed wilting at the painted area (Figure 1f). Transgenic T_0 plants were harvested and T_1 seeds of both genotypes were grown in the bio-containment greenhouse to test the inheritance of the transgenes.

Analysis of the transgenic plants

PCR analysis (Figure 2) revealed the presence of the expected bands for *bar* and *NPK1* amplicons at 484 and 605 bp, respectively, in 14 out of a total of 25 individual events of the genotypes Dorado and SPGM94021 with no T-DNA truncation. Similarly, PCR analysis was conducted on the T_1 plants. As shown in Figure 2c, bands corresponding to the expected size of 605 bp for the *NPK1* gene were observed in some of the progeny, thus, confirming the inheritance of the transgene.

To confirm the integration of the transgene, Southern blot analysis of T_0 and T_1 transgenic plants for *NPK1* gene was conducted. The results confirmed the stable integration of the *NPK1* gene into the sorghum genome as well as its transmission to the T_1 plants (Figure 3). The estimation of transgene copy number revealed the presence of fewer than five copies of the *NPK1* transgene, e.g. two copies in sample No. 2, four copies in sample No. 8, and one copy in sample No. 9.

Further analysis of the putative transgenic plants using a quantitative real-time RT-PCR analysis indicated all the tested transgenic events expressed the *NPK1* gene, although they had different expression levels. Table 3 summarizes the results of the molecular evaluation of 6 independent transgenic events, 3 belonging to the Dorado genetic background while the other 3 were generated from transforming the SPMG94021 background. All 6 events were positive in PCR evaluation and in all, except for event 14 (not tested),

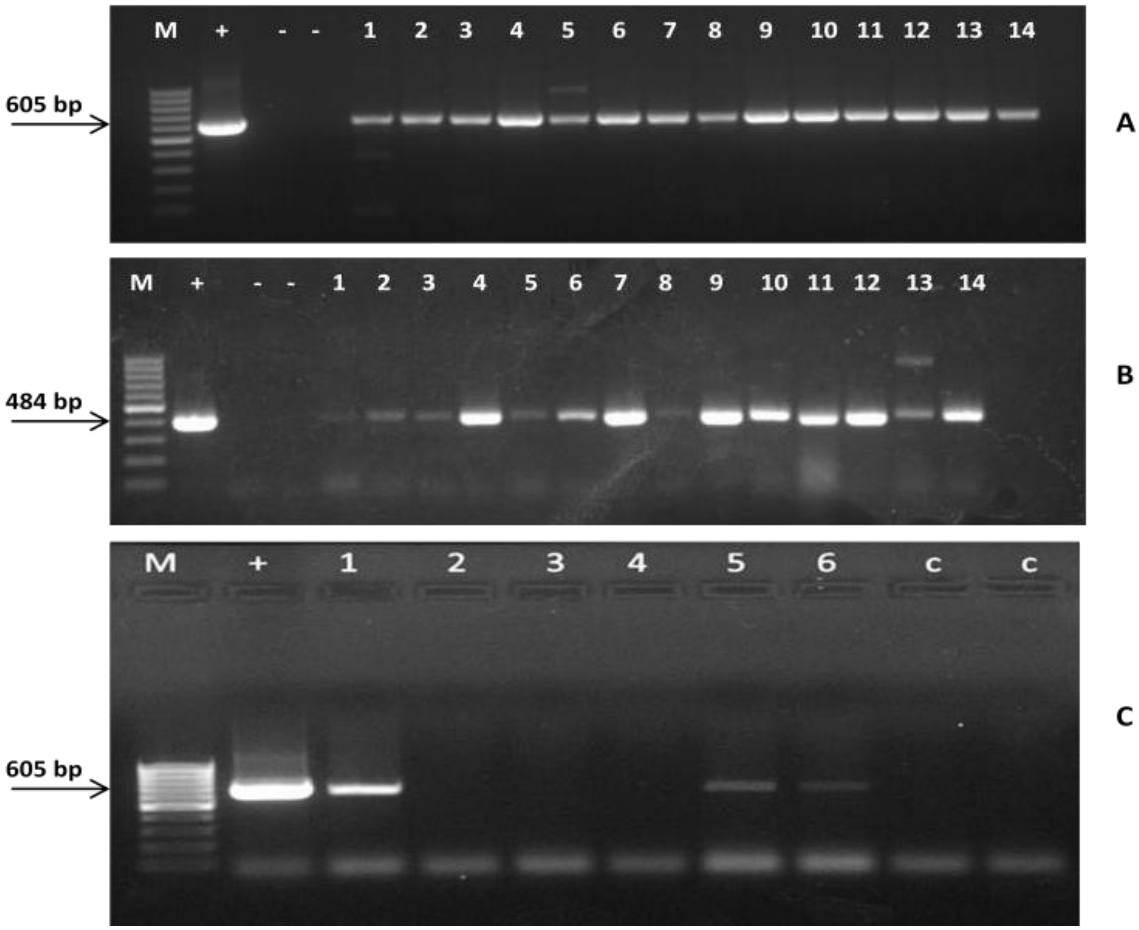

Figure 2. PCR verification of the transgenes in sorghum plants. Verification of *bar* gene (a) and *NPK1* gene (b) into putative transgenic T_0 plants. "M" Molecular weight size marker 100 pb; "-" negative control of un-transformed plant. "+" positive control of the plasmid pSHX004; Lanes 1-16 DNA samples from individual events of sorghum genotypes Dorado (1, 2, 3, 4, 6, 12, 13, 14, and 15) and SPGM94021 (5, 7, 8, 9, 10, 11, and 16). (c) Verification of the *NPK1* gene into transgenic T_1 plants. Lanes 1-6 DNA samples from Dorado (1-3) and SPGM94021 (4-6) T_1 plants.

the band specific for the *NPK1* transgene were detected in Southern blotting. Relative Quantification (RQ) represents the values of accumulated mRNA of the transgene compared to the endogenous gene of the calibrator sample (control non-transgenic) valued as 1.

Evaluation of T_1 plants under drought stress conditions

In the present investigation, transgenic seeds were recovered from both sorghum cultivars, Dorado and SPGM94021. A preliminary kill curve experiment has been carried out with control seedlings of both genotypes on 200, 400 and 600 mM mannitol. Results revealed that 400 mM concentration of mannitol was very critical for control seedlings (Zamzam, 2014). Thus, a culture medium containing 400 mM mannitol was employed to evaluate the performance of T_1 transgenic seedlings expressing *NPK1* gene.

As shown in Table 4 and Figure 4, transgenic plants maintained a relatively higher growth rate than that of the non-transgenic plants, under drought stress conditions. Significant differences in shoot length and fresh weight were found between non-transgenic and their counterparts' transgenic plants expressing the *NPK1* gene. However, the reduction in root lengths was not significant at 5% probability. In the greenhouse, three-week-old control and transgenic plants were subjected to water withholding. Normally, under our greenhouse conditions, the non-transgenic sorghum plants are usually irrigated every 6 to 7 days. In this experiment, watering was stopped for 11 to 13 days. The transgenic plants displayed a higher capacity of recovering after re-irrigation compared to the non-transgenic plants. Moreover, the weight of 100 grains

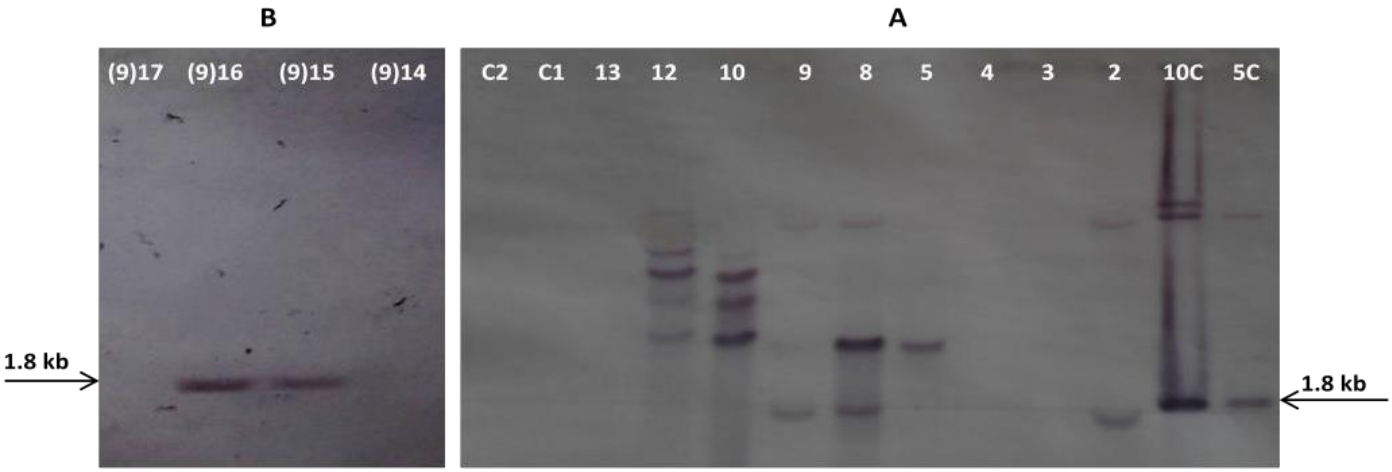

Figure 3. Southern blot analysis of transgenic sorghum plants. (a) T_0 plants probed with PCR product of the *NPK1* gene: Lanes 5C & 10C, five and ten copies of the plasmid pSHX004; lanes C1 & C2, negative control of untransformed Dorado and SPGM94021 plants, respectively; lanes 2, 3, 4, 12 and 13, samples of putative transgenic T_0 events of the genotype Dorado; lanes 5, 8, 9 and 10, samples of putative transgenic T_0 events of the genotype SPGM94021. (b) T_1 plants probed with PCR product of the *NPK1* gene: Lanes (9)14 - (9)17, four plants from the T_1 progeny of SPGM94021 transgenic event No. 9.

Table 3. Summary of the molecular evaluation of some *NPK1* transgenic events.

Genotype	Code	RQ	PCR	Southern blot
Dorado	2	0.022	+	+
Dorado	12	1.608	+	+
Dorado	14	2.019	+	Not tested
SPGM94021	9	2.324	+	+
SPGM94021	8	0.102	+	+
SPGM94021	5	0.523	+	+

Table 4. Effect of drought stress on the growth of seedlings and the yield potential of *NPK1* expressing transgenic sorghum.

Genotype	Fresh weight (g)	Shoot length (cm)	Root length (cm)	Weight of 100 grains (g)	No of grains per panicle
Dorado (NPK1)	0.18^{a}	9.40^{a}	10^{a}	2.21^{a}	1098.33^{a}
Dorado (control)	0.12^{b}	6.63^{ab}	8.76^{a}	1.62^{bc}	1076.66^{a}
SPGM94021 (NPK1)	0.14^{ab}	8.46^{ab}	8.83^{a}	1.97^{ab}	973.33^{a}
SPGM94021 (control)	0.10^{b}	5.81^{b}	7.1^{a}	1.22^{c}	970.00^{a}

Numbers with the same letters do not differ statistically between themselves at the level of 5% probability. Data of fresh weight, shoot length and root length were collected 10 days after seed germination (4 days on bialaphos and 6 days on mannitol stress).

and the number of grains per panicle were used as physiological parameters for yield potential under water-deficit conditions. Kernels were collected from both transgenic plants and few recovered control plants after repeated exposure to drought stress under greenhouse conditions. The transgenic sorghum plants had a significantly higher mean of grain weight, under drought stress conditions, than the control plants.

DISCUSSION

Regulatory genes are very important research interest. Transcription factors represent an important category of regulatory genes. Many genes involved in stress response can be simultaneously regulated by a single gene encoding stress inducible transcription factor (Kasuga et al., 1999). While the expression of *ZmDof1* transcription

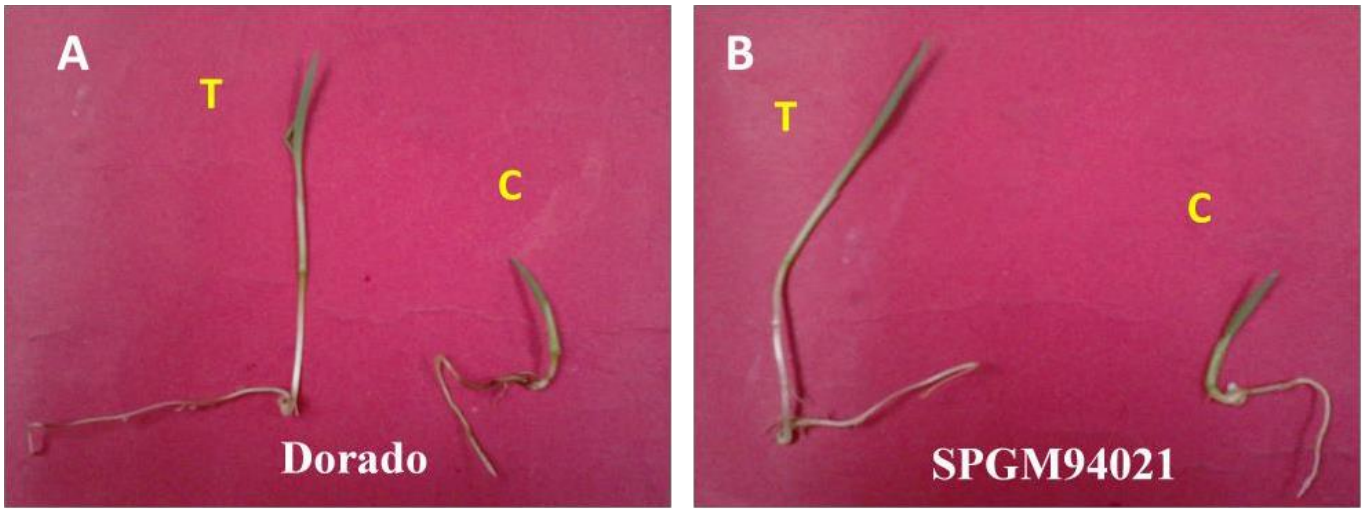

Figure 4. The effect of mannitol osmotic stress on the growth of sorghum seedling. (a) Dorado and (b) SPHM94021: transgenic "T" versus control "C".

factor in sorghum and wheat has activated the primary target, phospho*enol*pyruvate carboxylase (PEPC), which leads to the down-regulation of genes involved in photosynthesis and the functional apparatus of chloroplasts, and negatively impacted photosynthesis, and biomass (Peña et al., 2017). These results indicated that transcription factor strategies for crop improvement need to consider the downstream targets of the genetic elements to be introduced. Another category of regulatory genetic factors is stress signaling genes. Components of one signal transduction pathway may be shared by various stresses such as drought, salt and cold (Shinozaki and Yamaguchi-Shinozaki, 1999). Moreover, manipulation of signaling factors can control a broad range of downstream targets and result in superior tolerance to multiple stresses (Umezawa et al., 2006). Mitogen activated protein kinase (MAPK) cascade is an evolutionarily conserved signal transduction module involved in plant abiotic stress tolerance (Xiong and Zhu, 2001). The *NPK1* gene, a tobacco mitogen-activated protein kinase kinase kinase (MAPKKK), activated an oxidative signaling cascade and resulted in cold, heat, salinity and drought tolerant transgenic plants (Kovtun et al., 2000; Shou et al., 2004a).

Sorghum transformation and regeneration

To improve drought tolerance and validate the hypothesis that expression of *NPK1* gene in sorghum would improve drought tolerance in sorghum, two genotypes: Dorado and SPGM94021 were transformed with the pSHX004 construct. To the best of our knowledge, this is the first report of transforming sorghum plants with the *NPK1* gene. To select the transformed calli, the herbicide-resistant gene, *bar* (Thompson et al., 1987) was employed as a selectable marker. Selection of bialaphos-resistant callus is often conducted at 2 to 3 mg/L bialaphos (Casas et al., 1993; Frame et al., 2002; Grootboom et al., 2010). Bialaphos, as selection agent, has been found to hinder regeneration capacity of sorghum (Casas et al., 1993; Gao et al., 2005a; Lu et al., 2009; Grootboom et al., 2010). In the present investigation, bialaphos imposed a severe selection on sorghum callus. To sustain callus regenerability, bialaphos concentration was not increased beyond 1.5 mg/L. This is because non-transgenic plantlets may escape the *bar* selection system (Gao et al., 2005b).

Analysis of the transgenic plants

Further screening was conducted through PCR analysis, the transformation frequency was calculated and found to be within the cited range (Emani et al., 2002; Gao et al., 2005b; Zhao et al., 2000; Howe et al., 2006) from 0.2 to 4.5%, even though these reports were on highly regenerable and transformable genotypes, e.g. P898012 and TX430. The low transformation frequency may attribute to the detrimental effects of the *NPK1* gene. In this respect, Kovtun et al. (1998) reported that overexpression of *NPK1* gene causes detrimental effects on embryogenesis. Moreover, the constitutive expression of a calcium dependent protein kinases, *Os*CDPK-7, in sorghum induced apoptotic cell death in transgenic leaves (Mall et al., 2011). Further supporting this hypothesis is that the regeneration frequency for these genotypes is as high as 22 and 16% for Dorado and SPGM94021, respectively (Assem et al., 2014). Also, the *Agrobacterium*-mediated transformation

frequency of Dorado with similar construct, pTF102, was as high as 3.6% (Zamzam, 2014). In this respect, slight reduction in the transformation frequency with the pSHX004 construct compared to pTF102 was also reported in maize (Shou et al., 2004b). Taken together, the reduction in transformation frequency with pSHX004 construct in this study may be attributed to the death of the high *NPK1* expressers during the different transformation stages.

To further confirm the transgenic plants, Southern blotting was employed. Digesting the pSHX004 plasmid with the restriction enzyme *Eco*RI would liberate the *NPK1* gene from the T-DNA giving one band at the size of 1.8 Kb. Different Southern hybridization patterns seen with *Eco*RI-digested DNA implied that the transgenic lines resulted from independent transformation events. The number of hybridization bands ranged from one to four. The presence of more than one band and of bands larger than 1.8 Kb may be attributed to the occurrence of rearrangement or to duplication or amplification of transgenes. Duplication or amplification of the transgene was reported by Spencer et al. (1992) in maize and Cannell et al. (1999) in wheat. The transgene copy number in the recipient genome is an important determinant of the transgene expression and field performance of the transgenic plants. *Agrobacterium* is a potent transformation tool with the advantage of producing transgenic plants with low transgene copy number. In the present study, the low transgene copy number is consistent with other investigations using *Agrobacterium* on cereals (Hiei et al., 1994; Ishida et al., 1996; Cheng et al., 1997; Zhao et al., 2000; Gao et al., 2005a; Lu et al., 2009).

The expression of the *NPK1* transgene is the key of this investigation. Suppression and overexpression of *NPK1* in tobacco have resulted in some detrimental effects on cell division, embryogenesis, and seed development (Kovtun et al., 1998). In this study, although no abnormal transgenic plants were observed, the transformation efficiency was rather low. It is likely that only transgenic sorghum events with relatively low expression of the transgene were generated in this study. Although, quantitative real time PCR analysis revealed high expression of the *NPK1* transgene in some T_0 plants, that is, events No. 9, 12 and 14, low expression was found in other events (event No. 2 and 8). Transgenic event number 2 was almost silenced and it is likely that the T-DNA was inserted in a heterochromatin region of the chromosome and had low access to the RNA polymerase. In this respect, gene silencing has been previously reported in transgenic sorghum (Casas et al., 1993, 1997; Zhu et al., 1998; Able et al., 2001; Emani et al., 2002). Events 9 and 12 will be the main candidates for carrying out further investigation of the effects of overexpressing the *NPK1* gene on field performance. On the other hand, examining the field performance of event 2 may shed the light on the effects of attenuating the expression of this gene.

Evaluation of T_1 plants under drought stress conditions

The working hypothesis of this study was that expression of *NPK1* gene in sorghum would improve drought stress tolerance. To investigate this hypothesis, transgenic plants were evaluated for growth and yield potential under drought stress. Mannitol has been used to evaluate plant tolerance to drought stress. Mannitol affects the availability of water to plants by increasing the osmotic pressure outside the cells and causes plant symptoms like that of water-deficit (Rumpho et al., 1983). In this investigation, the relative reduction in the growth parameters: fresh weight and shoot length of the non-transgenic seedlings, under mannitol stress, suggests that the presence of the *NPK1* gene in transgenic sorghum plants aids in increasing the *in vitro* osmotic stress tolerance. The relative improvement in the growth of the transgenic plants, under mannitol stress, may be explained by the reduced sensitivity of transgenic cells to stress conditions. In this respect, Kovtun et al. (2000) reported that the ANP1, a class of MAPKKKs from *Arabidopsis* can be induced specifically by H_2O_2 and can activate a specific class of stress-induced MAPKs. The activated MAPK cascade activates stress-response genes that protect plants from diverse environmental stresses.

Moreover, Shou et al. (2004a) reported that the expression of *NPK1* in transgenic maize enhanced drought tolerance, suggesting that *NPK1* induced a mechanism that protected plants from dehydration damages. Similar results were, also, described, in Maize, by Muoma and Ombori (2014).

Over-expression of *NPK1* was found to impose detrimental effects on seed development in tobacco and the number of the defective seeds correlated with the level of the transgene expression, suggesting that seed phenotype was due to transgene expression and enhanced NPK1-dependent MAPK activity (Kovtun et al., 1998). In this study, we did not notice any reduction in seed germination or defect in seed development. Contrarily, under drought conditions, the transgenic plants had a higher means for grain weight despite the provision of equal amount of water. The relatively low expression of the NPK1 in this study may explain the normal development of seeds. The increased weight of transgenic kernels under drought condition in this study is consistent with the results of Shou et al. (2004a). Taken together, the results on the mannitol tolerance and the yield potential of transgenic sorghum plants expressing *NPK1*, demonstrate that *NPK1* gene might play a role in the protection of plants under water deficiency- stress conditions. Therefore, the *NPK1* gene can be successfully used for genetic improvement of

Egyptian sorghum inbred lines for osmotic tolerance.

CONFLICT OF INTERESTS

The authors have not declared any conflict interests.

ACKNOWLEDGEMENTS

This project was supported financially by Egypt's Science and Technology Development Fund (STDF), Egypt. The binary vector pSHX004 has been kindly provided by The Plant Transformation Facility, Iowa State University, USA. Sorghum lines have been provided by Shandaweel Research Station, Sorghum Department, Field Crops Research Institute, ARC, Egypt.

REFERENCES

Able JA, Rathus C, Godwin ID (2001). The investigation of optimal bombardment parameters for transient and stable transgene expression in sorghum. *In vitro* Cell. Dev. Biol. Plant 37:341-348.

Acquaah G (2007). Principles of Plant Genetics and Breeding. Wiley-Blackwell, Boston, Oxford, Chichester, Berlin, Singapore, Melbourne, Tokyo, Beijing.

Assefa Y, Staggenborg SA (2010). Grain sorghum yield with hybrid advancement and changes in agronomic practices from 1957 through 2008. Agron. J. 102:703-706.

Assem SK, Zamzam MM, Hussein BA, Hussein EHA (2014). Evaluation of somatic embryogenesis and plant regeneration in tissue culture of ten sorghum (*Sorghum bicolor* L.) genotypes. Afr. J. Biotechnol. 13:3672-3681.

Belton PS, Taylor JR (2004). Sorghum and millets: protein sources for Africa. Trends Food Sci. Technol. 15:94-98.

Bhatnagar-Mathur P, Vadez V, Sharma KK (2008). Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects. Plant Cell Rep. 27:411-424.

Cannell ME, Doherty A, Lazzeri PA, Barcelo P (1999). A population of wheat and tritordeum transformants showing a high degree of marker gene stability and heritability. Theor. Appl. Genet. 99:772-784.

Casas AM, Kononowicz AK, Haan TG, Zhang L, Tomes DT, Bressan RA, Hasegawa PM (1997). Transgenic sorghum plants obtained after microprojectile bombardment of immature inflorescences. In Vitro Cell. Dev. Biol. Plant 33:92-100.

Casas A.M., Kononowicz A.K., Zehr UB, Tomes DT, Axtell JD, Butler LG, Bressan RA, Hasegawa P (1993). Transgenic sorghum plants via microprojectile bombardment. Proc. Natl. Acad. Sci. USA. 90:11212-11216.

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, Wan Y (1997). Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiol. 115:971-980.

Das K. and Roychoudhury A. (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Frontiers Environ. Sci. 2(53):1-13.

Devi P, Zhong H, Sticklen M (2004). Production of transgenic sorghum plants with related *HVA1* gene. In: Seetharama N, Godwin ID. (eds), Sorghum Tissue Culture and Transformation. Oxford & IBH Publishing Co. Pvt. Ltd, New Delhi.

Emani C, Sunilkumar G, Rathore KS (2002). Transgene silencing and reactivation in sorghum. Plant Sci. 2:181-192.

Frame BR, Shou H, Chikwamba RK, Zhang Z, Xiang C, Fonger TM, Pegg SE, Li B, Nettleton DS, Pei D, Wang K (2002). *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system. Plant Physiol. 129:13-22.

Gao Z, Jayaraj J, Muthukrishnan S, Claflin L, Liang GH (2005a). Efficient genetic transformation of sorghum using a visual screening marker. Genome 48:321-333.

Gao Z, Xie X, Ling Y, Muthukrishnan S, Liang GH (2005b). *Agrobacterium tumefaciens*-mediated sorghum transformation using a mannose selection system. Plant Biotechnol. J. 3: 591-599.

Grootboom AW, Mkhonza NL, O`Kennedy MM, Chakauya E., Kunert K, Chikwamba R K (2010). Biolistic Mediated Sorghum (*Sorghum bicolor* L. Moench) Transformation via Mannose and Bialaphos Based Selection Systems. Int. J. Bot. 6: 89-94.

Hiei Y, Ohta S, Komari T, Kumashiro T (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6:271-282.

Horwich L. (2014). Molecular Chaperones in Cellular Protein Folding: The Birth of a Field. Cell 157:285-288.

Howe A, Sato S, Dweikat I, Fromm M, Clemente T (2006). Rapid and reproducible *Agrobacterium*-mediated transformation of sorghum. Plant Cell Rep. 25:784-791.

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996). High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nat. Biotechnol. 14:745-750.

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999). Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress inducible transcription factor. Nat. Biotechnol. 17:287-291.

Kim H, Lee K, Hwang H, Bhatnagar N, Kim DY, Yoon IS, Byun MO, Kim ST, Jung KH, Kim BG (2014). Overexpression of *PYL5* in rice enhances drought tolerance, inhibits growth, and modulates gene expression. J. Exp. Bot. 65:453-464.

Kovtun Y, Chiu WL, Tena G, Sheen J (2000). Functional analysis of oxidative stress-activated mitogen- activated protein kinase cascade in plants. Proc. Natl. Acad. Sci. USA 97:2940-2945.

Kovtun Y, Chiu WL, Zeng W, & Sheen J. (1998). Suppression of auxin signal transduction by a MAPK cascade in higher plants. Nature 395:716-720.

Li HS, Chang CS, Lu LS, Liu CA, Chan MT, Charng YY (2003). Over-expression of *Arabidopsis thaliana* heat shock factor gene (*AtHsfA1b*) enhances chilling tolerance in transgenic tomato. Bot. Bull. Acad. Sin. 44:129-140.

Lu L, Wu X, Yin X, Morrand J, Chen X, Folk WR, Zhang ZJ (2009). Development of marker-free transgenic sorghum [*Sorghum bicolor* (L.) Moench] using standard binary vectors with *bar* as a selectable marker. Plant Cell Tiss. Organ Cult. 99: 97-108.

Maheswari M, Varalaxmi Y, Vijayalakshmi A, Yadav SK, Sharmila P, Venkateswarlu B, Vanaja M, Saradhi PP (2010). Metabolic engineering using *mtlD* gene enhances tolerance to water deficit and salinity in sorghum. Biol. Plantarum 54: 647-652.

Mall TK, Dweikat I, Sato SJ, Neresian N, Xu K, Ge Z, Wang D, Elthon T, Clemente T (2011). Expression of the rice CDPK-7 in sorghum: molecular and phenotypic analyses. Plant Mol. Biol. 75:467-479.

Milesi C, Samanta A, Hashimoto H, Kumar KK, Ganguly S, Thenkabail P.S., Srivastava AN, Nemani RR, Myneni RB (2010). Decadal Variations in NDVI and Food Production in India. Remote Sens. 2:758-776.

Mortimore M (2009). Dryland Opportunities: A New Paradigm for People, Ecosystems and Development. IUCN, Gland, Switzerland, IIED, London, UK, UNDP, New York, USA.

Muoma JVO, Ombori O (2014). *Agrobacterium*-Mediated Transformation of Selected Kenyan Maize (*Zea mays* L.) Genotypes by Introgression of Nicotiana Protein Kinase (*npk1*) to Enhance Drought Tolerance. Am. J. Plant Sci. 5:863-883.

Murray MG, Thompson WF (1980). Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8:4321-4325.

Peña PA, Quach T, Sato S, Ge Z, Nersesian N, Changa T, Dweikat I, Soundararajan M, Clemente TE (2017). Expression of the Maize *Dof1* Transcription Factor in Wheat and Sorghum. Front. Plant Sci. 8:434-447.

Rumpho ME, Edwards GE, Loescher WH (1983). A pathway for photosynthetic carbon flow to mannitol in celery leaves: activity and localization of key enzymes. Plant Physiol. 73:869-873.

Shinozaki K, Yamaguchi-Shinozaki K (1999). Molecular responses to drought stress. In: Shinozaki K. & Yamaguchi-Shinozaki K. (eds), Molecular Responses to Cold, Drought, Heat and Salt Stress in

Higher Plants. RG Landes Co, Austin.
Shou H, Bordallo P, Wang K (2004a). Expression of the *Nicotiana* protein kinase (NPK1) enhanced drought tolerance in transgenic maize. J. Exp. Bot. 55:1013-1019.
Shou H, Bordallo P, Fan JB, Yeakley JM, Bibikova M, Sheen J, Wang K (2004b). Expression of an active tobacco mitogen-activated protein kinase kinase kinase enhances freezing tolerance in transgenic maize. Proc. Natl. Acad. Sci. USA 101:3298-3303.
Spencer TM, O'Brien JV, Start WG, Adams TR, Gordon-Kamm WJ & Lemaux, PG (1992). Segregation of transgenes in maize. Plant Mol. Biol. 18:201-210.
Thompson CJ, Movva NR, Tizard R, Crameri R, Davies JE, Lauwereys M, Botterman J (1987). Characterization of the herbicide-resistance gene *bar* from Streptomyces hygroscopicus. EMBO J. 6:2519-2523.
Tuberosa R, Salvi S (2006). Genomics-based approaches to improve drought tolerance of crops. Trends Plant Sci. 11:405-412.
Umezawa T, Fujita M, Fujita Y, Yamaguchi-Shinozaki K, Shinozaki K. (2006). Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. Curr. Opin. Biotechnol. 17:113-122.
Wenzel WG (1999). Effect of moisture stress on sorghum yield and its components. South Afr. J. Plant Soil 16:153-157.
Wilhite DA (2000). Drought as a natural hazard in concepts and definitions. In: Wilhite D.A. (ed), Drought: A Global Assessment. Routledge, Oxfordshire, New York City, Melbourne, New Delhi, Singapore, Beijing.
Xiong L, Zhu JK (2001). Abiotic stress signal transduction: molecular and genetic perspectives. Physiol. Plantarum 112:152-166.
Yellisetty V, Reddy LA, Mandapaka M (2015). *In planta* transformation of sorghum (*Sorghum bicolor* (L.) Moench) using *TPS1* gene for enhancing tolerance to abiotic stresses. J. Genet. 94:425-434.
Zamzam MM (2014). Genetic Improvement of Some Sorghum Lines via Genetic Engineering Approaches. M.Sc. Thesis, Faculty of Agriculture, Cairo University, Egypt. 170p.
Zhao Z, Cai T, Tagliani L, Miller M, Wang N, Pang H, Rudert M, Schroeder S, Hondred D, Seltzer J, Pierce D (2000). *Agrobacterium*-mediated sorghum transformation. Plant Mol. Biol. 44:789-798.
Zhu H, Muthukrishnan S, Krishnaveni S., Jeoung JM, Liang, GH (1998). Biolistic transformation of sorghum using a rice chitinase gene. J. Genet. Breed. 52:243-252.

academicJournals

Vol. 16(49), pp. 2278-2288, 6 December, 2017
DOI: 10.5897/AJB2017.16157
Article Number: 6DFC43666965
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Peroxidase and polyphenol oxidase activities associated to somatic embryogenesis potential in an elite hybrid genotype of *Theobroma cacao* L.

Minyaka Emile[1,2*], Niemenak Nicolas[3], Ngangue Latta Tanguy Armand[1], Madina Banen Colette Vanessa[1], Bahoya Joseph Aime[1] and Omokolo Ndoumou Denis[3]

[1]Biochemistry Laboratory, Faculty of Science, University of Douala, P. O. Box 24157 Douala, Cameroon.
[2]Laboratory of Plant Physiology and Biochemistry, Department of Biological Science, Higher Teachers Training, College of Yaoundé, P.O. Box 47 Yaoundé, Cameroon.

Received 18 July, 2017; Accepted 16 November, 2017

Cross-fertilization and somatic embryogenesis stand as potential useful tools for genetic improvement and vulgarization of agronomical interesting cocoa planting material for sustainable cocoa culture. Manual cross-fertilization (♀SNK13×♂UPA143) was conducted. ♀SNK13×♂UPA143 derived hybrid genotypes were tested for their susceptibility to black pods disease (BPD). The most tolerant hybrid genotype (KHACa12) was monitored for its precocity, yielding and somatic embryo potential. Peroxidase and polyphenol oxidase activities were evaluated in association to embryogenesis in KHACa12. The cross-fertilization ♀SNK13×♂UPA143 generated around 60% of offprint's tolerant or less susceptible to BPD. When transferred in experimental farm, KHACa12 produced flowers 18 months after planting. Mature pods from KHACa12 bearded 55±5 seeds/pod. Seventy-five pods were harvested from KHACa12 during the first season. These pods generated 6 kg of fermented-dried cocoa seeds. Somatic embryo responsive revealed that 71.041 ± 7.91% and 50.64 ± 9.48% staminodes and petals derived explants, respectively produced direct and indirect somatic embryos. Morphologically, the somatic embryos obtained were similar to zygotic embryos from the same hybrid genotype (KHACa12). Peroxidase and polyphenol oxidase activities profiles in zygotic and somatic embryos increase during the first stages of embryogenesis, then decrease when cotyledons developed. These results might indicate that, ♀SNK13×♂UPA143 could be used to develop cocoa (*Theobroma cacao* L.) hybrid genotypes tolerant or less susceptible to BPD. KHACa12 is an agronomic interesting hybrid genotype that could be produced in large scale and vulgarized using somatic embryogenesis process. The initiation and development of somatic embryo in KHACa12 can be monitored and modulated biochemically through peroxidase and polyphenol oxidase activities profiles.

Key words: Breeding, tissue culture, somatic, explants, hybrid.

INTRODUCTION

The chocolate and cocoa butter tree (*Theobroma cacao* L.) is a perennial tropical crop widely cultivated in Africa, Asia and America. Consumption of cocoa increases by 7% each year (ICCO, 2016). However, cocoa production is challenged with reliable planting material crisis. Mostly used planting material are low yielding and highly sensitive to disease (*Phytophthora megakary* specially). Improved planting material is not available or accessible to farmers. In many African cocoa producing countries, 25% of cocoa farms are made of improved planting

material. Additionally, most cocoa farms are old and need to be renewed with elite cocoa genotypes. Moreover, the ongoing cocoa culture practices that use chemical fertilizers and pesticides are costly and environmentally unfriendly. Therefore, developed and vulgarized elite cocoa genotypes are the ways out for sustainable cocoa production and profitability. Tremendous efforts have been put on cross-fertilization of targets *T*. *cacao* clones in order to provide farmers with elite cocoa genotypes. Breeding for resistance to black pods disease, precocity and yield are some of the characters expected in offprint (Efombagn et al., 2007). Since the parental clones are always heterozygote for these characters, the offprints from cross-fertilization of targets *T*. *cacao* clones are highly heterozygote. Additionally, the proportion of hybrid genotypes for the character such as tolerance *P. megakarya* did not exceed 1/3 (Tahi et al., 2000; Nyasse et al., 2003).

There is still much cross-fertilization to be tested. ♀SNK13×♂UPA143 has not been tested. Usually, pods or plantlets from interesting crossing are distributed to farmers. However, the allogamous character of *T*. *cacao* limits the vulgarization of elite hybrids genotypes. These explain why in cocoa producing country such as Cameroon, in spite of the distribution of these cultivars, cocoa plantations continue to be low yielding, with an average of 200 to 600 kg of dry cocoa per ha (Efombagn et al., 2007). An efficient and reliable vegetative multiplication of elite genotype was needed. Classic vegetative multiplication methods (grafting and the rooted cuttings) were studied. However, these vegetative propagation methods of cacao lead to plants highly sensitive to winds and dryness in African countries (Tahi et al., 2006). Somatic embryogenesis has been studied by many research teams (Omokolo et al., 1997; Li et al., 1998; Maximova et al., 2002; Traoré et al., 2003; Minyaka, et al., 2008, 2010; Minyaka, 2009). Cacao somatic embryogenesis allows rapid vegetative and large-scale multiplication of elite genotypes, genetic improvement, collection and conservation of cacao germoplasm, efficient diffusion of plant material as plantlets to farmers, etc. However, the limiting factor in using somatic embryogenesis to vulgarize an elite cocoa genotype is its somatic embryogenic responsive/ recalcitrance.

Peroxidase and polyphenol oxidase enzymes participate in the metabolism of auxins, coumaric acid and other phenolic compounds which are important in plant embryogenesis (Duchovskis et al., 2009). Changes in peroxidase and polyphenol oxidase acitivities in *T*. *cacao* embryogenesis (zygotic and somatic embryogenesis) have never been investigated. Such investigation enhances our understanding of the developmental processes underlying the formation of somatic and zygotic embryos in *T*. *cacao*. It could also aid in the development of stage-specific biochemical markers that might be used to optimize somatic embryogenesis protocols (Kormuťák et al., 2003).

In the present investigation, an agronomical interesting hybrid genotype from ♀SNK13×♂UPA143 was developed. Somatic embryogenesis potential, peroxidase and polyphenol oxidase activities during embryogenesis of this hybrid genotype (♀SNK13×♂UPA143) were monitored.

MATERIALS AND METHODS

The present study was conducted from September 2012 to October 2016.

Screening for susceptibility to *P. megakarya* of the progeny ♀SNK13×♂UPA143

Plant

T. cacao seeds from two pods of ♀SNK13×♂UPA143 (KHA) obtained by manual pollination, which were used to establish a nursery. Three months old plant leaves were used as plant biological material in leaf disc test (Nyasse et al., 1995) to evaluate the susceptibility of *T*. *cacao* plants to black pod disease.

Pathogen

The pathogen material used in this investigation was *P. megakarya* strain ELEG-8 (characterized by RADP at CIRAD, Montpellier-France). This strain was graciously offered by the laboratory of plant pathology of Institut de Recherche Agronomique pour le Developpement (IRAD) at Nkolbisson (Yaoundé, Cameroon). In the laboratory (Biochemistry Laboratory, Faculty of Sciences, University of Douala, Cameroon), the strain of *P. megakarya* was preserved by frequent subcultures on 1.5% (w/v) pea-based agar medium. To maintain its virulence, the strain was periodically inoculated onto cocoa pods.

Zoospore production

Zoospores (or inoculums) were obtained according to Nyasse et al. (1995) adapted method. Zoospores were obtained from l0-day-old cultures. Cultures with sporangia were induced to liberate zoospores by adding sterile distilled water at 4°C. After 1 h at room temperature, the zoospore concentration was adjusted to 3×10^5 zoospores/ml with a MALASSEZ hemati-meter.

Screening for susceptibility to P. megakarya of ♀SNK13×♂UPA143 (KHA) progeny

Leaf discs test was used for screening for susceptibility of hybrid genotypes from ♀SNK13×♂UPA143 progeny according to Nyasse

*Corresponding author. E-mail: minyakae@yahoo.fr.

et al. (1995) adapted method. The experimental design was made of three replicates and completely randomized 7 blocks of leaf discs (Ø = 1.5 cm) per hybrid. Hence, a total of 27 discs were used per hybrid. For each hybrid of the progeny, leaf discs were obtained from the slightly lignified young leaves (3 months old). Leaf discs were placed in trays and incubated for 24 h (at 25±1°C) in darkness prior to inoculation. After the 24 h, leaf discs were inoculated by depositing 10 µL (3×10^5 zoospores/mL) of zoospores suspension on either side in the middle of each leaf disc and incubated in darkness (at 25±1°C). The necrosis rate (from 0: "tolerant" to 5: "highly sensitive") of susceptibility (through the necrosis size) of each leaf discs (for each hybrid) was registered on 4, 5, 6, 7 and 8th day after inoculation.

Field transfer of seeds-derived plantlets

All seeds-derived plantlets with disease score ranging from 0 to 0.2 were planted in an experimental plot (Faculty of Science, University of Douala, Cameroon). The transfer was made when plantlets were six months old. Growing parameters were recorded every three months. The date of first flowers was registered. For the hybrid genotype with the best precocity, the number of seeds per mature pods and weight of dried seed were registered. The hybrid with the best agronomic characters was submitted to embryogenesis investigation.

Somatic embryogenesis

Plant and explants

Plant material used in this investigation was made of immature flower buds of an elite hybrid genotype ♀SNK13×♂UPA143 from an experimental plot (Faculty of Science, University of Douala, Cameroon). Staminodes and petals isolated from immature flower buds were as explants.

Explants culture

This experiment used the protocol developed by Minyaka et al. (2008). Explants were cultured in induction medium and incubated for 14 days in darkness at 25±1°C. The induction medium was made of DKW basal salts as described by Driver and Kuniyuki (1984) and supplemented with 250 $mg.L^{-1}$ glutamine, 100 $mg.L^{-1}$ myoinositol, 1 $ml.L^{-1}$ DKW vitamin stock (100 $mg.ml^{-1}$ myo-inositol, 2 $mg.ml^{-1}$ thiamine-HCl, 1 $mg.ml^{-1}$ nicotinic acid, and 2 $mg.ml^{-1}$ glycine), 20 $g.l^{-1}$ glucose, 18 µM 2,4-dichlorophenoxyacetic acid (2,4-D), and 45.4 nM TDZ.

After 14 days in induction medium, explants were transferred in maintenance medium and incubated in the same condition as previously. The maintenance medium was similar to induction medium except that, glutamine and myoinositol were absent; 45.4 nM TDZ were replaced by 250 $\mu g.L^{-1}$ kinetin and the concentration of 2,4-D was 9 µM. The pH of induction and maintenance media was at 5.8 prior to addition of 0.2% (w/v) phytagel.

In vitro morphogenetic structures from maintenance medium were transferred into embryo development medium and incubated for 21 days (darkness, 25±1°C). After the 21 days, two additional subcultures were done in the same medium at 21 days' intervals and incubated in darkness (25±1°C). The embryo development medium was made of DKW basal salts, 0.7% (w/v) $MgSO_4$, 3% (w/v) sucrose and 1 $ml.L^{-1}$ DKW vitamin stock. The pH of embryo development medium was at 5.7 prior to addition of 0.2% (w/v) phytagel.

Collection of data

The aspect of explants was recorded in induction, maintenance and embryo development media. At the end of the culture cycle (day 91), percentages of staminodes and petal-derived structures bearing embryo were estimated. Additionally, the date of observation of first embryo was written down.

Zygotic embryogenesis

Pods at different maturation stages were harvested from an elite hybrid genotype ♀SNK13×♂UPA143 at an experimental plot (Faculty of Science, University of Douala, Cameroon). For each pod, three portions were considered viz., apical, median and distal. After fixations on gallows, pods were sectioned (2 mm teak) transversally from distal to apical end using a blade of lancet. At each transversal of a pod, morphological characteristic of a giving ovum (axial diameter), embryo (developmental stage) and endosperm (consistence of endosperm) were noted down.

The developmental stage of embryo was appreciated with arbitrary symbols, from E.0 to E.14: E.0 (no visible embryo), E.1 (globular stage), E.2 (torpedo stage), E.3 (hearth shape stage) and E.4 to E.14 (different cotyledonary stages). Endosperm and embryos at different maturation stage were collected separately and kept at -20°C and used for peroxidase and polyphenol oxidase extraction and analysis.

Peroxidase and polyphenol oxidase in endosperm, zygotic embryo and *in vitro* morphogenetic structures

Peroxidase and polyphenol oxidase extraction

Peroxidase and polyphenol oxidase extracts were obtained from different *in vitro* morphogenetic structures, endosperm and zygotic embryos (at different developmental stages). Peroxidase and polyphenol oxidase extracts preparation were obtained from the aforementioned biological material. Fresh biological material tissues (1 g) were homogenized 1 g in mortar with 3 ml of potassium phosphate buffer (50 mM, pH 6.0). The homogenate was then centrifuged (4°C, 30 min, 6000 g) and the supernatant (soluble peroxidase and polyphenol oxidase extract) was collected. The pellet was re-suspended in potassium-phosphate buffer and re-centrifuged under the same conditions, and the new supernatant was added to the first.

Protein quantification

Protein contents in soluble peroxidase and polyphenol oxidase extracts were determined according to the method of Bradford (1976) and bovine serum albumin was used as standard.

Polyphenol oxidase assay

Polyphenol oxidase (PPO) activity was determined by measuring the increase in absorbance at 330 nm according to the method of Van Kammen and Broumer (1964). The reaction mixture incubated at 25°C contains: 2.7 ml of 1/15 M phosphate buffer pH 6.1 and 0.3 ml of 10 mM catechol. The reaction was initiated by adding 40 µl of enzymatic extract. The enzyme activity was determined according to the change in optical density at 330 nm after 30 s. This activity was expressed on a fresh weight basis, compared to the protein content.

Table 1. Average disease score of the progeny ♀SNK13×♂UPA143.

Progeny	♀SNK13×♂UPA143
Average disease score	3.68±1.47

Peroxidase assay

Peroxidase activity was determined according to Thorpe and Gaspar (1978) method by monitoring the formation of guaïacol at 420 nm. Five milliliters of reaction mixture (1V of 0.2% H_2O_2; 2V of 1% guaiacol; 5V of 1/15 M phosphate buffer, pH 6) was added to 10 µl of the extract. One unit of enzyme activity corresponded to 0.1 is degraded per min, at 420 nm. Peroxidase activity was expressed on a fresh weight basis (unit g^{-1} FW).

Data analysis

Collected data were submitted to descriptive statistics which generated means and standard deviation. Means were compared using the Student Newman and Keuls test. Correlations between variables were evaluated with Pearson correlation test. All statistical analysis was done using SPSS 17.0 software.

RESULTS

Screening for susceptibility to *P. megakarya* of the progeny ♀SNK13×♂UPA143

The screening for susceptibility to *P. megakarya* of the progeny ♀SNK13×♂UPA143 revealed a mean disease score of 3.68±1.47 (Table 1). The classification of hierarchical clusters of disease scores of the progeny displayed three mains groups of hybrid genotypes susceptibility: (1) A group of tolerant or less susceptible hybrid genotypes with disease scores [0; 2]. This group is subdivided into two disease scores subgroups ([0.12; 0.62] and [1.12; 2]); (2) A group of middle susceptible hybrid genotypes exhibiting disease scores [2.0; 3.6]. In this group, two subgroups were observed ([2.25; 2.62] and [3; 3.57]); (3) A group of highly susceptible hybrid genotypes displaying disease scores [3.75; 5].

In this progeny, the most tolerant hybrid genotypes were: KHACa12 (0.12), KHACb8 (0.37), KHACb26 (0.62), KHACb3 (1.12), KHACb9 (1.25), KHACb24 (1.25), KHACb22 (1.62), KHACb1 (1.71) and KHACa35 (1.71) (Figure 1 and Table 2). The hybrid genotype KHACa12 (0.12) appeared therefore to be the most hybrid genotype of the progeny ♀SNK13×♂UPA143.

Field transfer of seed-derived plantlet from ♀SNK13×♂UPA143

The seeds-derived plantlets from ♀SNK13×♂UPA143 were transferred in an experimental plot when plantlets were 4 months old. The monitoring of the plantlets on the field revealed that, the hybrid genotype (KHACa12 with disease score 0.12) submitted in the present investigation produced first flowers 18 months after planting. Mature pods from this hybrid were entirely harvested. A total of 75 pods were harvest during the first cocoa season. The number of cocoa seeds per pod was 55±5. After fermentation, the cumulate weight of dried cocoa seeds from the 75 pods was 6.0 kg. During this cocoa production season, none of the pods were attacked by black pod disease due to *P. megakarya* (Table 3).

Somatic embryogenesis potential

Morphogenesis and somatic embryo responsive of floral explants from hybrid genotype KHACa12

Staminodes and petals explants when cultured in induction medium, developed calli between 10th and 14th day of culture incubation. Immature explants appeared to be the most callogenic. The development of calli was amplified in maintenance medium. The differentiation of somatic embryo was observed when explants from maintenance medium were transferred in embryo development medium. Two types of somatic embryogenesis were observed: direct somatic embryogenesis and indirect somatic embryogenesis. Both types emerged from browning explants. Somatic cotyledonary embryos were morphological similar to zygotic cotyledonary embryos (Figure 2).

The staminods-derived explants appeared to be more embryogenic (71.041±7.91%) than petals-derived explants (50.64±9.48). The number of embryo per explants was higher in staminods-derived explants (4 to 10) compared to petals-derived explants (Table 4).

Zygotic embryogenesis

The developmental stage of embryo was appreciated with arbitrary symbols (from E.0 to E.14). The consistence of endosperm and sizes of ovum were also inspected. The sizes of the embryos ranged between 0.2 (for globular stage) to 1.6 cm in cotyledonary stages. The ovum sizes were embryo development stage-independent. Endosperm consistence was liquid in globular stage of embryo and viscous, colloidal or cellularized as embryo develops cotyledons (Figure 3).

Peroxidase and polyphenol oxidase activities in endosperm, zygotic embryo and in vitro morphogenetic structures

Peroxidase activities were monitored in endosperm and zygotic embryos at different developmental stages. In the endosperm, peroxidase activities remained low and quasi constant during zygotic embryo development. Reversely,

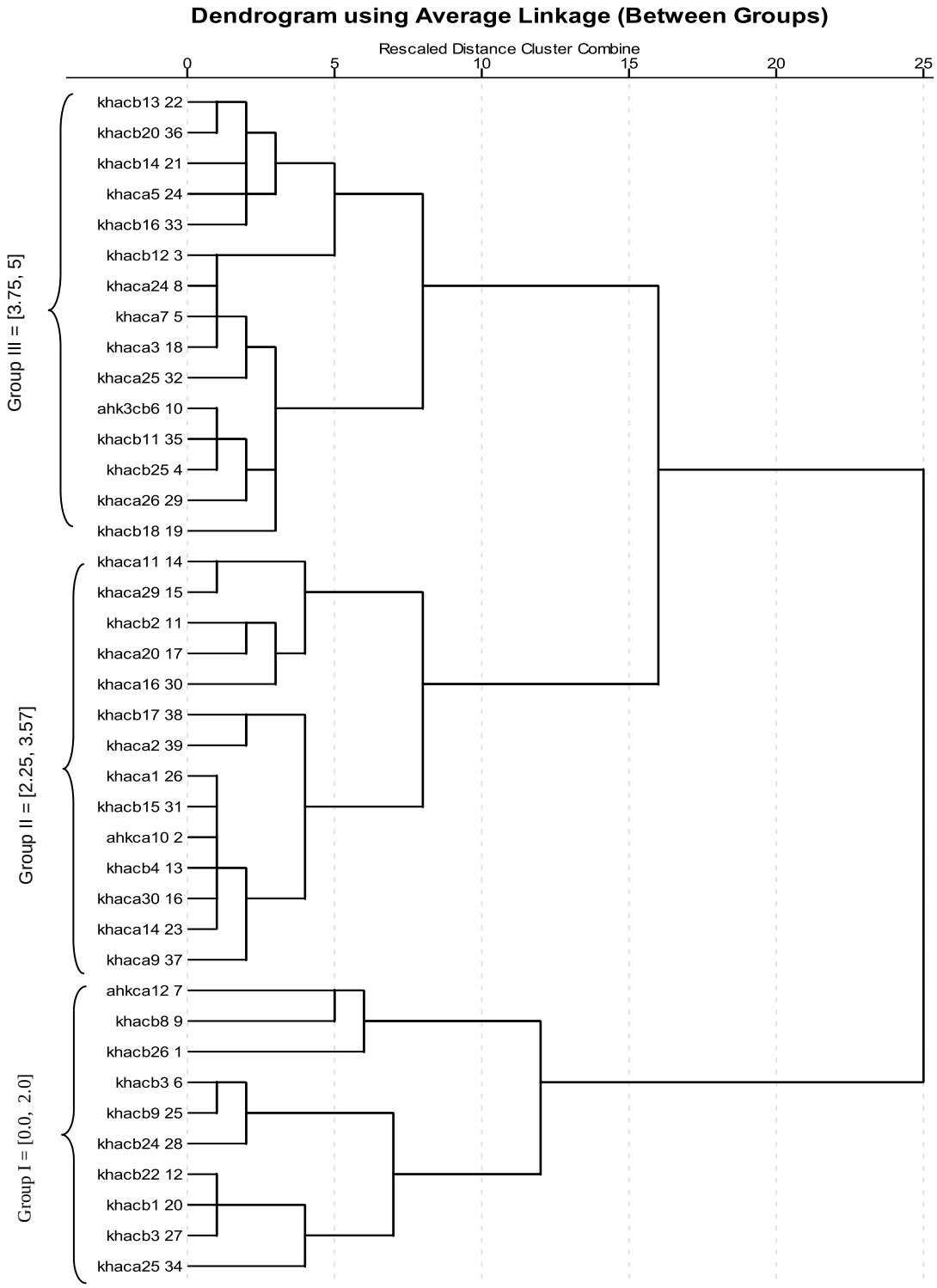

Figure 1. Dendrogram of disease score of hybrid family KHA. $khaca_i$=family KHA, pod a, seed i; $khacb_j$=family KHA, pod b, seed j.

in zygotic embryo, peroxidase activities increased from stage E0 to stage E2. There was no significant difference in the peroxidase activities between stages E5 and E14 (Figure 4).

Polyphenol oxidase (PPO) in endosperm exhibited a profile not quite different from peroxidase activities. PPO appeared to be low almost low in endosperm (compared zygotic embryo) during zygotic embryo maturation. In

Table 2. Disease scores and percentages of hybrid genotypes per group of susceptibility of progeny ♀SNK13×♂UPA143.

Group	Hybrid genotypes susceptibility categories	Disease scores interval	Percentages
I	Less susceptible	[0.0, 2.0]	25.64
II	Middle susceptible	[2.0, 3.6]	35.90
III	Highly susceptible	[3.75, 5]	38.46

Table 3. Agronomic characteristics of the selected hybrid genotype.

Age of hybrid genotype at first flowers buds	Number of seeds/mature pod	Weight of dried seeds from 75 pods (kg)	Number of pods during first cocoa season	Number of pods attacked by BPD
18 months	55±5	6.0	75	0

zygotic embryos, PPO activities increase from 0+ to E2, then decrease from E2 to E4. No significant fluctuation in PPO activities was observed between stages E4 and E14 (Figure 5).

At day 28 of cultures incubation, peroxidase and polyphenol oxidase activities appeared to be higher in staminods than petals. At day 56 of cultures incubation, embryogenic calli exhibited higher peroxidase and polyphenol oxidase activities than none embryogenic calli.

During somatic embryo maturation, it appeared that, peroxidase activities were significantly higher in globular stage of somatic embryo than cotyledonary stage. The same profile was observed with polyphenol oxidase. The activities of this enzyme were significantly higher in globular stage than cotyledonary stage of somatic embryo development (Figures 6 and 7).

DISCUSSION

Disease score evaluation of the offprint from ♀SNK13×♂UPA143 was conducted for the selection of hybrid genotypes tolerant to *P. megakarya*. Variability in disease scores between hybrid genotypes was observed. The heterogeneity in disease scores in the offprint might result from the heterozygote statute of the parents (♀SNK13 and ♂UPA143) for "resistance to black pod disease" character which has been reported to be additive and probably polygenic (Nyasse et al., 2007).

The proportion of less and middle susceptible hybrid genotypes was above 60% of the offprint. Usually, the proportion of cocoa tolerant offprint did not exceed 1/3 of the progeny (Thai et al., 2000). The high proportion of tolerant hybrid genotypes from ♀SNK13×♂UPA143 might be due to SNK13 which was reported among tolerant cocoa clones (Nyasse et al., 1995). This set of results might indicate that the present tested cross-fertilization (♀SNK13×♂UPA143) could be used to develop less or middle susceptible (to black pod disease) hybrid genotypes. Hybrid genotypes from cross-fertilization ♀SNK13×♂UPA143 could be used by farmers in other to reduce the use of pesticides and preserve the environment.

The less susceptible hybrid genotype, KHACa12, was monitored in the field. This hybrid genotype was able to produce flowers at the age of 18 months. This finding seems to be atypical. Most *T. cacao* genotypes produce first flowers between 28 and 36 months (Ndoumbe-Nkeng et al., 2001). KHACa12 hybrid genotype seems to be an outstanding hybrid genotype for its precocity. The number of seeds per pod ranged between 50 and 60. Additionally, 75 pods were harvested from KHACa12; the first year generated 6.0 kg of fermented dried 75 pods.

Somatic embryogenesis is reported as a reliable vegetative multiplication process for the vulgarization of allogamous plant such as *T. cacao* (Minyaka et al., 2008, 2010). However, the limiting factor in the use of this process in *T. cacao* is the recalcitrance and low responsive of many elites *T. cacao* genotypes to somatic embryogenesis (Tan et al., 2003). Somatic embryogenesis potential of KHACa12 hybrid genotype investigation was conducted in this study. KHACa12 hybrid genotype differentiated somatic embryos. Direct and indirect somatic embryos were observed from staminodes and petals-derived explants. Direct somatic embryogenesis is uncommon in *T. cacao* genotypes. The occurrence of direct somatic embryo in KHACa12 hybrid genotype implies the inhibition of callogenesis and ability of explants-cells to be directly competent and determined to change their differentiation pathways in other to become embryogenic (Minyaka, 2009). This might be due to stress-related compounds which are required for somatic cells to become embryogenic cells (Verdeil et al., 2007; Minyaka et al., 2008). Staminodes and petals explants were able to differentiate somatic embryos at different rates. Staminodes were more embryogenic than petals. This observation underlines the explants-type-dependence of somatic embryogenesis in *T. cacao* (Li et al., 1998; Minyaka, 2009). Somatic embryo responsive of both staminodes and petals explants was significantly

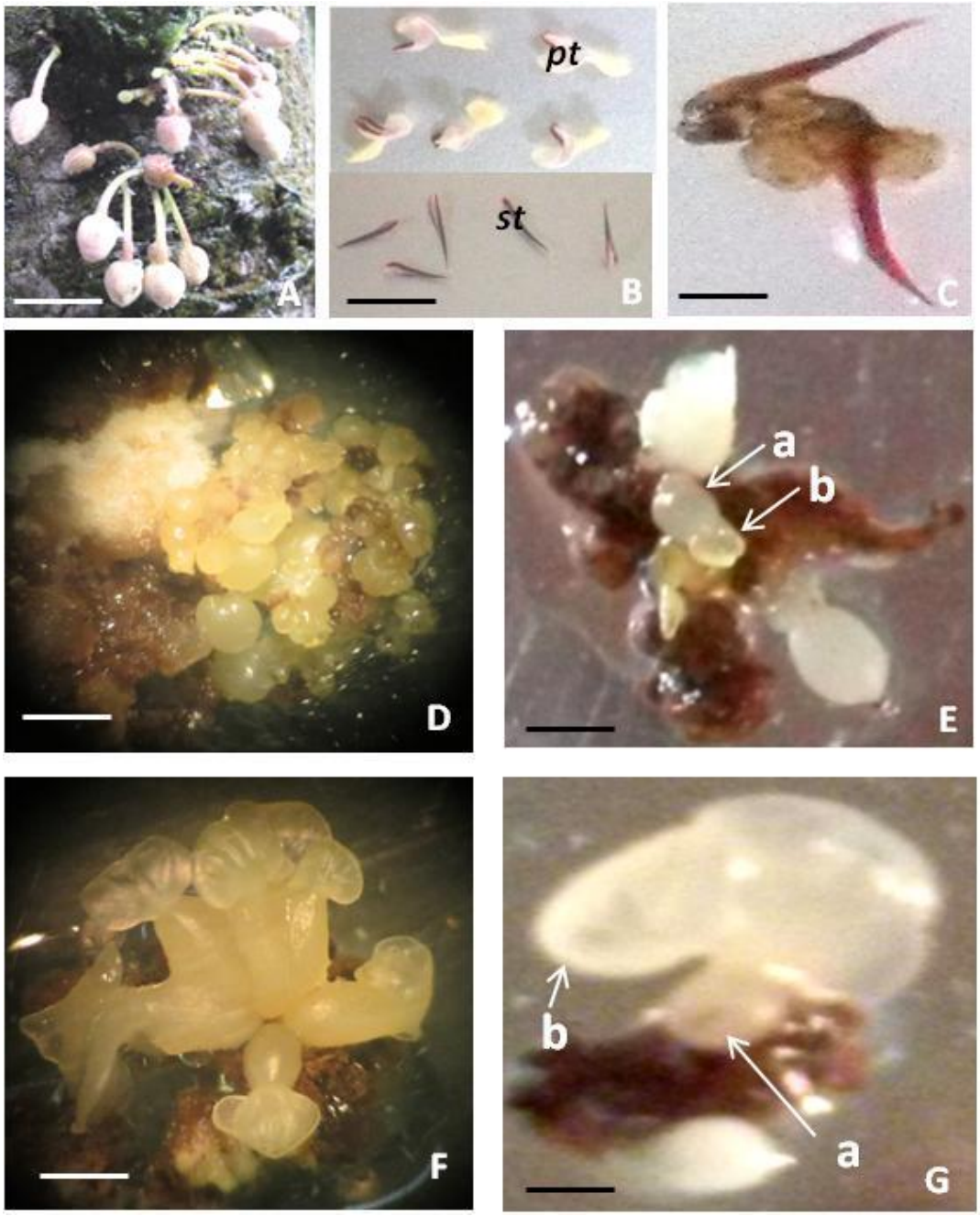

Figure 2. Different morphogenetic structures obtained from staminods and petals explants from hybrid genotype KHACa12. A. Flowers buds; B. staminods (*st*) and petals (*pt*) explants; C. staminods explants developing calli at day 10 of culture; D. callus bearing globular somatic embryo; E. callus bearing indirect somatic embryos at different maturation stages (a = radicul, b = cotyledons); F. callus bearing cotyledonary somatic embryo; G. petal-derived explant with direct cotyledonary somatic embryo (a: radicule, b: cotyledons). Bar = 0.25 cm.

Table 4. Embryo responsive of staminods and petals-derived explants.

Explant types	Number of embryos/explant	Embryogenic explants (%)
Staminods	4 - 10	71.041 ± 7.91
Petals	2 - 7	50.64 ± 9.48

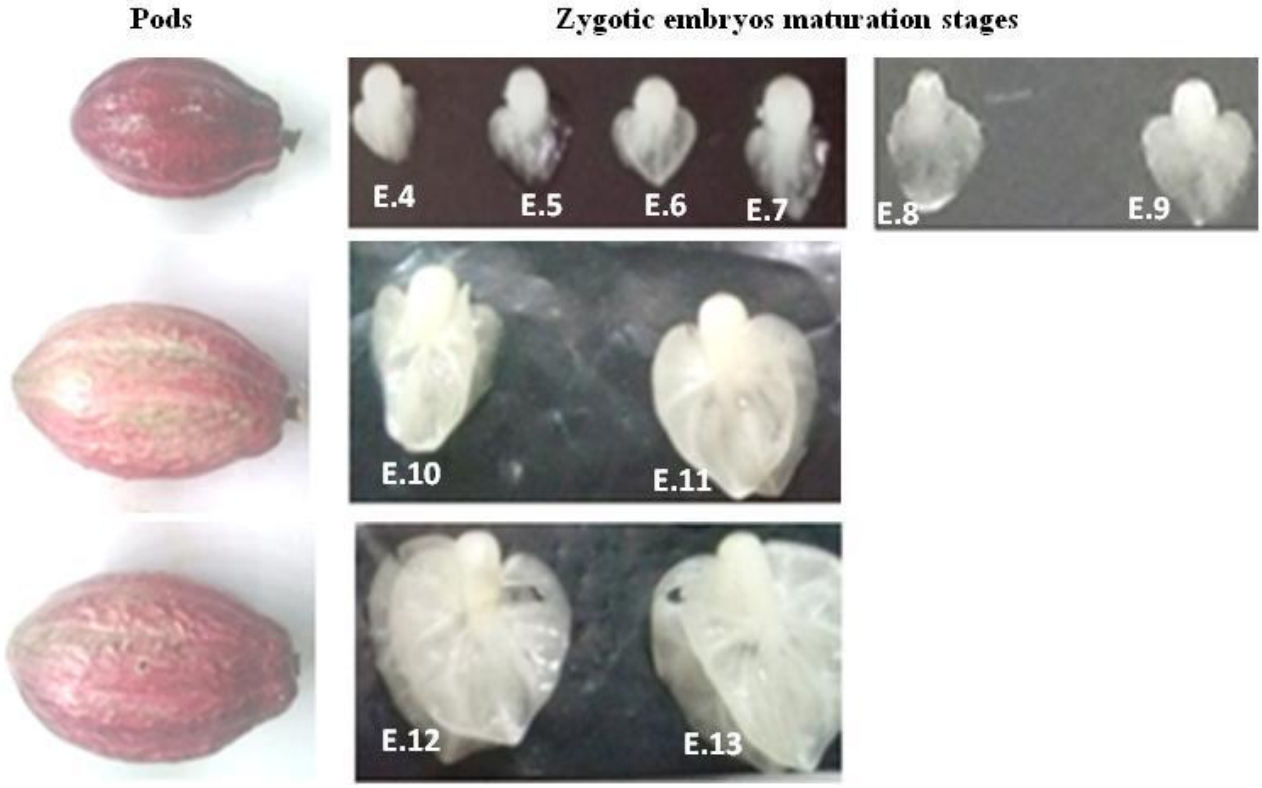

Figure 3. Different cotyledonary stages of zygotic embryo from hybrid genotype KHACa12 of *T. cacao*.

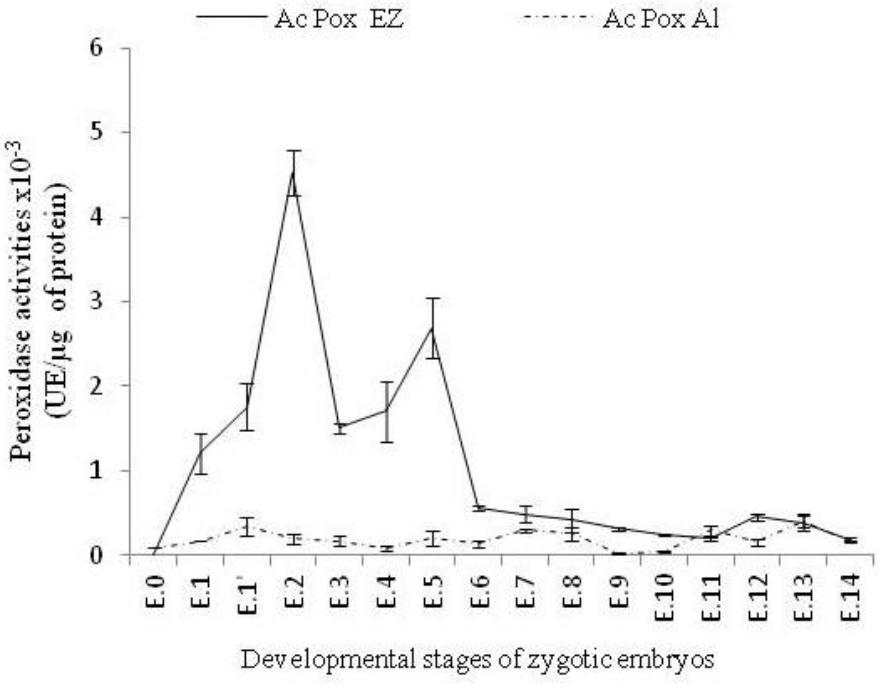

Figure 4. Peroxidase activities in zygotic embryos at different developmental stages and associated endosperm (albumen). Values are given in terms of mean ± SD (n = 9). Ac.Pox EZ, Peroxidase activities in zygotic embryo; Ac.Pox Al, peroxidase activities in endosperm.

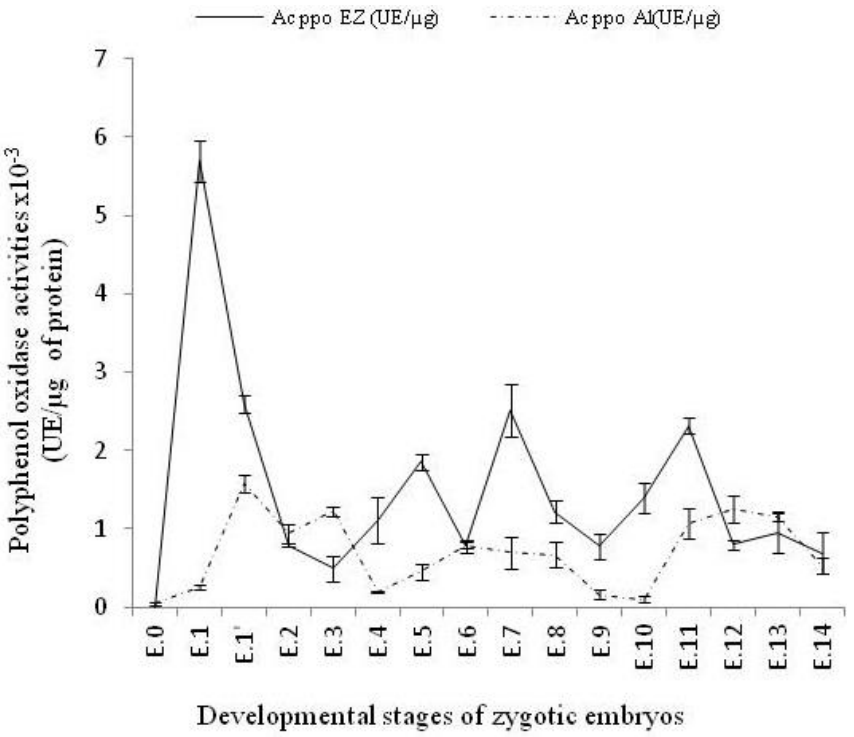

Figure 5. Polyphenol oxidase activities in zygotic embryos at different developmental stages and associated endosperm (albumen). Values are given in term of Mean ± SD (n = 9). Ac.ppo EZ (UE/µg), polyphenol oxidases activities in zygotic embryo; Ac.ppo Al (UE/µg), polyphenol oxidases activities in endosperm.

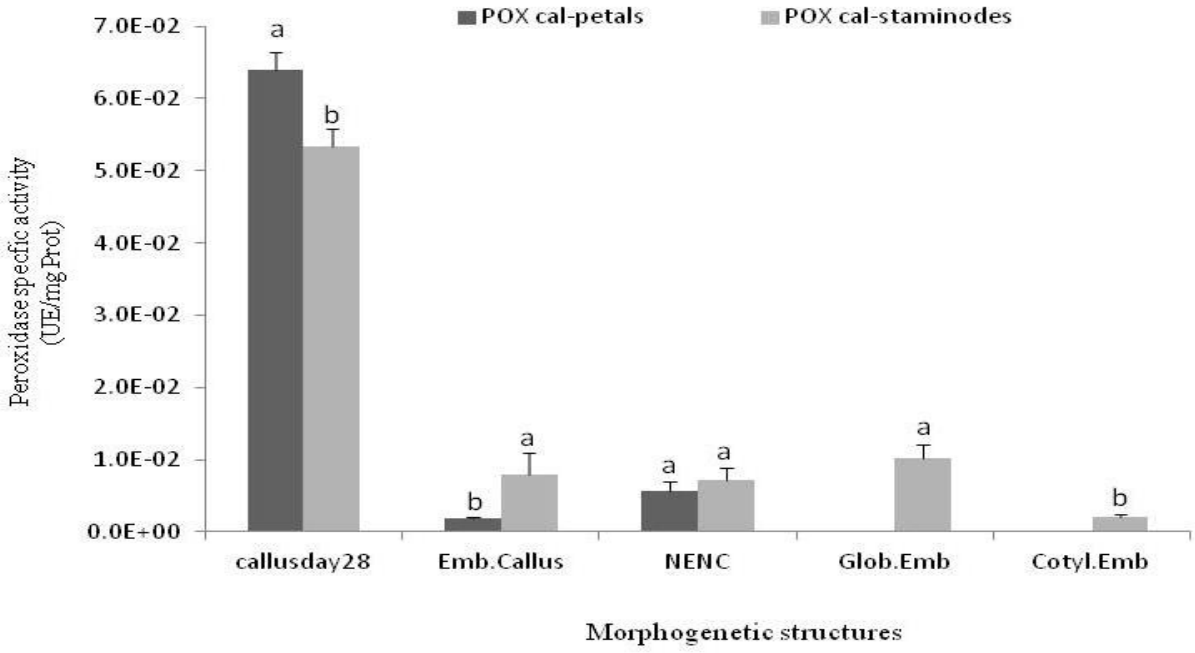

Figure 6. Peroxidase specific activities in different morphogenetic structures. Values are given in term of Mean ± SD (n = 9). Means were compared with Student Newman and Keuls test (5%). Callus day28, Callus at day 28 of cultures incubation; Emb.callus, embryogenic callus; NENC, non embryogenic necrotic callus; Glob.Emb, globular embryo; Cotyl.Emb, embryo with cotyledon. In callus day 28, Emb.callus and NENC significant difference between staminodes and petals are indicated in different letters. Significant difference between Glob.Emb and Cotyl.Emb are indicated by different letters.

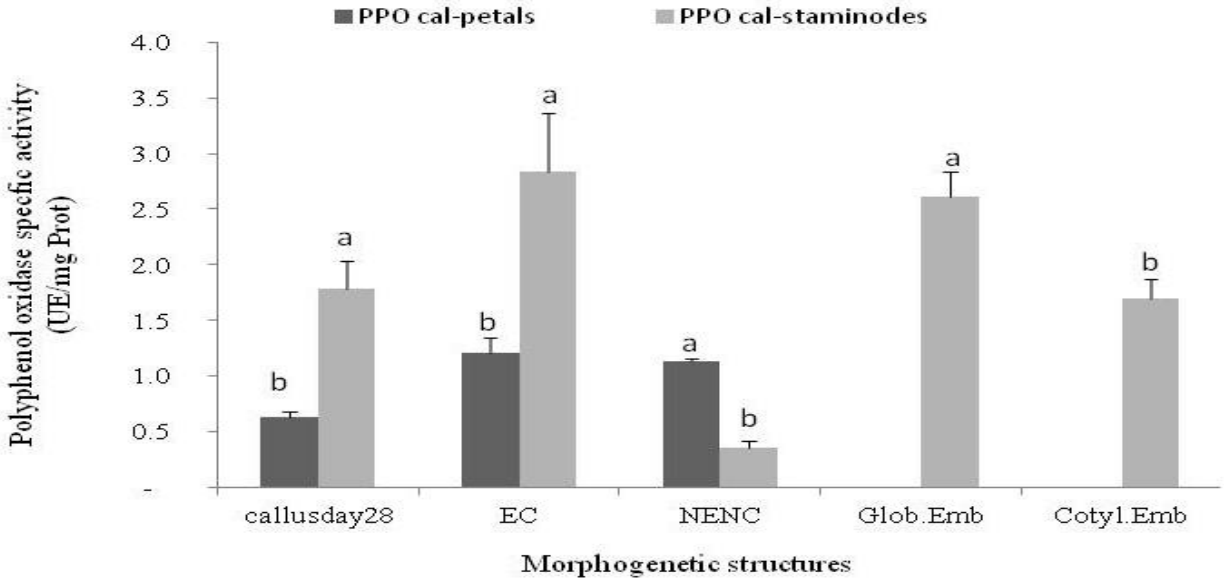

Figure 7. Polyphenol oxidase specific activities in different morphogenetic structures. Values are given in term of Mean ± SD (n = 9). Means were compared with Student Newman and Keuls test (5 %). Callusday28, Callus at day 28 of cultures incubation; Emb.callus, embryogenic callus; NENC, non embryogenic necrotic callus; Glob.Emb, globular embryo; Cotyl.Emb, embryo with cotyledon. In callus day 28, Emb.callus and NENC significant difference between staminodes and petals indicated different letters. Significant difference between Glob.Emb and Cotyl.Emb are indicated by different letters.

high in KHACa12 hybrid genotype compared to results reported by Tan and Furtek (2003) and Minyaka et al. (2008). In fact, these authors reported somatic embryogenesis responsive hanging between 10 and 40%. Hence, KHACa12 hybrid genotype appeared to be vulgarized through somatic embryogenesis.

Biochemical characterization of some woody plants zygotic and somatic embryogenesis is useful in the development of stage-specific biochemical markers that might be used to optimize somatic embryogenesis protocols (Kormuťák et al., 2003). Peroxidases and polyphenol oxidase activities were monitored during somatic and zygotitic embryos development in KHACa12 hybrid genotype. During zygotic embryogenesis in *T. cacao*, peroxidases and polyphenol oxidase activities increased during the earlier stages of zygotic embryogenesis, then decrease while cotyledons expanded. In somatic embryos, a decrease pattern of peroxidases and polyphenol oxidase activities was also observed from globular to cotyledonary embryos. Generally, somatic embryos (direct and indirect) emerged from brown staminodes and petals-derived explants. In *Fraxinus mandshurica*, Chun-Ping et al. (2015) reported the implication of peroxidases during somatic embryos differentiation in browning explants. The profile of peroxidase and polyphenol oxidase reported here in *T. cacao* for the first time matches with the observation reported by Amal and Hemmat (2015). These authors indicated that, in date palm, peroxidase activity was the highest level at embryogenic callus and then decreased gradually during the subsequent developmental stages. This set of results might reveal the participation of peroxidases and polyphenol oxidase in the initiation of zygotic and somatic embryogenesis in *T. cacao*. Hence, the stimulation of both enzymes in explants during *T. cacao* tissue culture could improve the somatic embryos responsive of cocoa genotypes.

Conclusion

The set of results presented in this investigation, might indicate that, ♀SNK13×♂UPA143 could be used to develop *T. cacao* hybrid genotypes tolerant or less susceptible to BPD. KHACa12 is an agronomic interesting hybrid genotype that could be produced in large scale and vulgarized by using somatic embryogenesis process. The initiation and development of somatic embryos from KHACa12 can be monitored and modulated biochemically through peroxidase and polyphenol oxidase activities profiles.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Amal FMZE, Hemmat AI (2015). Some biochemical changes and

activities of antioxidant enzymes in developing date palm somatic and zygotic embryos *in vitro*. Ann Agric. Sci. 60 (1):11-130.
Bradford MM (1976). A rapid and sensitive method for the quantitative of microgram quantities of protein utilizing the principle of protein-Dye binding. Ann. Biochem. 2:248-254.
Chun-Ping L, Ling Y, Hai-Long S (2015). Proteomic Analysis of Immature Fraxinus mandshurica Cotyledon Tissues during Somatic Embryogenesis: Effects of Explant Browning on Somatic Embryogenesis. Int. J. Mol. Sci. 16:13692-13713.
Driver, J. A. and A. H. Kuniyuki. 1984. In vitro propagation of Paradox walnut root stock. Hortic. Sci. 19:507-509.
Duchovskis P, Stanys V, Šikšnianienė BJ, Stanienė G, Šikšnianas T, Česlovas B (2009). Peroxidase and polyphenoloxidase polymorphism during embryogenesis in *Eucoreosma* section currants. Žemės Ūkio Mokslai, T.16. Nr. 1-2. pp. 47-52.
Efombagn, MIB, Nyasse S, Sounigo O, Kolesnikova-Allen M, Eskes AB (2007). Participatory cocoa (Theobroma cacao) selection in Cameroon: Phytophthora pod rot resistant accessions identified in farmers' fields. Crop Prot. 26:1467-1473.
ICCO (2016). ICCO Quarterly Bulletin of Cocoa Statistics, Vol. XLII, No. 3, Cocoa year 2015/2016.
Kormuťák A, Salaj T, Matúšová R, Vooková B (2003). Biochemistry of zygotic and somatic embryogenesis in silver fir (*Abies Alba* Mill.). Acta Biol. Cra. Series Bot. 45(1):59-62.
Li Z, Traore A, Maximova S, Guiltinan M (1998). Somatic embryogenesis and plant regeneration from floral explant of cocao (*Theobroma cacao* L.) using thidiazuron. *In vitro* Cell Dev. Biol. Plant. 34:293-299.
Maximova SN, Alemanno L, Young A, Ferriere N, Traore A, Guiltinan MJ (2002). Efficiency, genotypic variability, and cellular origin of primary and secondary somatic embryogenesis ofTheobroma cacaoL. In Vitro Cell Dev. Biol-Plant 38:252-259.
Minyaka E (2009). Métabolisme du soufre et embryogenèse somatique chez *Theobroma cacao* L. (Malvaceae). Thèse de Doctorat/Ph.D, Université de Yaoundé I, Université de Cocody/Abidjan. 132p.
Minyaka E, Niemenak N, Fotso A, Sangaré A, Omokolo ND (2008). Effect of $MgSO_4$ and K_2SO_4 on somatic embryo differentiation in *Theobroma cacao* L. Plant Cell Tis. Organ Cult. 94(2):149-160.
Minyaka E, Niemenak N, Issali EA, Sangare A, Omokolo ND (2010). Sulphur depletion altered somatic embryogenesis in *Theobroma cacao* L. Biochemical difference related to sulphur metabolism between embryogenic and non embryogenic calli. Afr. J. Biotechnol. 9(35):5665-5675.
Ndoumbe`-Nkeng M, Bieysse D, Cilas C (2001). Multi trait selection in a diallel crossing scheme of cocoa (*Theobroma cacao* L.). Plant Breed. 120:365-367.
Nyasse S, Efombagn MIB, Kebe BI, Tahi M, Despreaux D, Cilas C (2007). Integrated management of *Phytophthora* diseases on cocoa (Theobroma cacaoL): Impact of plant breeding on pod rot incidence. Crop Prot. 26:40-45.
Nyasse S, Efombagn MIB, Bouambi E, Ndoumbe-Nkeng M, Eskes AB (2003). Early selection for resistance to *Phytophthora megakarya* in local and introduced cocoa varieties in Cameroon. Trop. Sci. 43:96-102.
Nyasse S, Cilas C, Herail C, Blaha G (1995). Leaf inoculation as early screening test for cocoa (*Theobroma cacao* L.) resistance to Phytophthorablack pod disease. Crop Protect. 14:657-663.
Omokolo ND, Tsala NG, Niemenak N (1997). Phenol content, acidic peroxides and IAA-Oxides during somatic embryogenesis in *Theobroma cacao* L. Biol. Plant 39:337-347.
Tahi M, Kébé I, Eskes AB, Ouattara S, Sangare A, Mondeil F (2000). Rapid screening of cocoa genotypes for field resistance to *Phytophthora palmivora* using leaves, twigs and roots. Eur. J. Plant Pathol. 106:87-94.
Tahi GM, Kébé BI, N'Goran JAK, Sangare A, Mondeil F, Cilas C, Eskes AB (2006). Expected selection efficiency for resistance to cacao pod rot (*Phytophthora palmivora*) comparing leaf disc inoculation with field observation. Euphytica 149:35-44.
Tan CL, Furtek DB (2003). Development of an *in vitro* regeneration system for *Theobroma cacao* L. from mature tissues. Plant Sci. 164:407-412.
Thorpe TA, Gaspar T (1978). Changes in isoperoxydase during shoot formation in tobacco callus. *In vitro* 14:552-539.
Traore A, Maximova SN, Guiltinan MJ (2003). Micropropagation of *Theobroma cacao* using somatic embryo-derived plants: *In Vitro* Cell. Dev. Biol. Plant. 39:332-337.
Van Kammen A, Broumer D (1964). Increase of polyphenol oxidase activity by the local virus infection in uninoculated parts of leaves. Virology 22:9-14.
Verdeil J-L, Alemanno L, Niemenak N, Tranbager TJ (2007). Pluripotent versus totipotent plant cells: dependence versus autonomy. Trends Plant Sci. 12(6):245-252.

academicJournals

Vol. 16(49), pp. 2289-2298, 6 December, 2017
DOI: 10.5897/AJB2017.16083
Article Number: 79087C066966
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Evaluation of productivity of sweet potato genotypes for first and second generation bioethanol production

Daniel José Silva Viana[1], Silvânia Saldanha da Silva Pinto[2], Karen Paola Fróes Dias[3], David Lee Nelson[3], Lílian de Araújo Pantoja[3], Nísia Andrade Villela Dessimoni Pinto[4], Valter Carvalho de Andrade Junior[5] and Alexandre Soares dos Santos[2*]

[1]Department of Biology, Federal University of the Jequitinhonha and Mucuri Valleys, Diamantina, MG, Brazil.
[2] Department of Basic Sciences, Federal University of the Jequitinhonha and Mucuri Valleys, Diamantina, MG, Brazil.
[3]Institute of Science and Technology, Federal University of the Jequitinhonha and Mucuri Valleys, Diamantina, MG, Brazil.
[4]Department of Nutrition, Federal University of the Jequitinhonha and Mucuri Valleys, Diamantina, MG, Brazil.
[5]Department of Agronomy, Federal University of the Jequitinhonha and Mucuri Valleys, Diamantina, MG, Brazil.

Received 24 May, 2017; Accepted 17 August, 2017

The increased supply of ethanol fuel and its spread as an alternative to fossil fuels depend on the exploration of biomasses that can be regarded as alternatives to sugarcane and maize. In this study, the agricultural productivity and the potential for production of ethanol from roots and the aerial portion of twenty sweet potato genotypes were evaluated. The roots and branches were harvested 180 days after planting. Starch, hemicellulose, cellulose, lignin and soluble sugars from sweet potato roots or branches were determined and expressed as percentage of dry matter produced per hectare. The genotype UFVJM 28 stood out for the production of root and branches (fresh material), yielding 43.5 and 31.7 t ha^{-1}, respectively. The roots of the Palmas, Batata Mandioca, Cariru Vermelha and UFVJM 45 genotypes had the highest total soluble sugar concentrations, between 4.1 and 5.4%. The enzymatic digestibility for starch contained in the roots ranged from 58.2 to 91.2%, and when related to the cellulose contained in the branches, it ranged from 14.2 to 42.4%. The estimates for the production of ethanol from the roots ranged between 1120 and 4940 L ha^{-1}. The estimated production of bioethanol from sweet potato branches of the genotypes varied from 240 to 995 L ha^{-1}. Considering the combined use of roots and branches, at least four sweet potatoes genotypes presented a potential for ethanol production greater than 4000 L ha^{-1} in a 180-day cycle.

Key words: *Ipomoea batatas*, ethanol, biofuels, second generation ethanol, starch.

INTRODUCTION

The sweet potato (*Ipomoea batatas* (L.) Lam) is one of the most widely cultivated plant crops in the world. The plant root is the main product; it is used both for human consumption and animal feed. The sweet potato stands out for its ease of cultivation, hardiness, adaptability to different types of soil and climate, high drought tolerance, short production cycle and low production cost (Andrade Júnior et al., 2012). In 2014, 104.4 million tons of sweet

potato roots were produced worldwide, and China accounted for 68% of the world production (FAO, 2016). According to FAO (2016), the average worldwide productivity of sweet potatoes was only 13 tons per hectare in 2014, reaching 45.5 tons per hectare in Ethiopia. In general, the low agricultural productivity of this crop is associated with a low technification index and the lack of adoption of appropriate cultivation practices (Widodo et al., 2015). The mean starch content of fresh sweet potato roots is 20%, and it may range between 9.8 and 27.5% or between 40.0 and 83.1% on a dry weight basis (Dangler et al., 1984; Suarez et al., 2016). Because of its significant starch content, the sweet potato is also being evaluated as an alternative to sugarcane and maize for the production of ethanol (Ziska et al., 2009; Masiero et al., 2014). The literature has presented projections and case studies that report a productivity between 1250 and 8839 L of ethanol per hectare per year (Ziska et al., 2009). The great challenge for the incorporation of this starchy biomass for ethanol production is the selection of super-productive varieties with high starch contents. Two crops per year are likely, and saccharification of the starch should be accomplished by a small investment and positive energy balance. In addition, genotypes that are adapted to climatic and soil diversity in different regions of the globe must be selected. The use of the aerial portion of the sweet potato plant for the production of ethanol is also possible through use of the technology employed for second-generation ethanol production (Aditiya et al., 2016). However, few studies exist that address this perspective.

There are many varieties of sweet potato, with different sizes, shapes, textures, colors and compositions. Although extremely productive, some are not suitable for marketing. Even among the varieties already selected to satisfy the food market, there are considerable losses caused by rot, mechanical injury, sprouting, formation defect or pest damage and they are responsible for the lack of compliance with commercial specifications (Ray and Tomlins, 2010; Parmar et a., 2017). Because of these characteristics, one can envisage the simultaneous allocation of sweet potatoes to the food market and to biofuel industries, with economic advantages for the producer.

To furnish information that could facilitate the use of sweet potatoes as an alternative biomass for the ethanol fuel production chain, this study evaluated the agricultural productivity of twenty genotypes of *Ipomoea batatas* under the soil and climatic conditions of the Alto Vale do Jequitinhonha, central Minas Gerais State, Brazil, and estimated the productivity of ethanol, considering the carbohydrate contents of the roots and branches and the use of the whole plant.

MATERIALS AND METHODS

Cultivation of sweet potatoes

The sweet potato genotypes studied were Brazlândia Branca, Brazlândia Rosada, Batata Mandioca, Marmel, Cariru Vermelha, Palmas, Cambraia, Princesa, Tomba Carro I, UFVJM 45, UFVJM 20, UFVJM 46, UFVJM 23, UFVJM 48, UFVJM 01, UFVJM 04, UFVJM 06, UFVJM 14, UFVJM 21, and UFVJM 28. The planting of sweet potato genotypes was conducted in the city of Diamantina, MG, Brazil, a town with an average altitude of 1219 m, at 18°31'31"S and 43°51'19"W, and a Cwb climate according to the Köppen classification, characterized as humid temperate with a dry winter and rainy summer. The experiment was conducted in a randomized block design with 20 treatments and 4 repetitions, totaling 80 plots of 3.9 m^3 each. The spacing used was 1.0 m between rows (ridges) and 0.30 m between plants. Each plot contained 13 plants. The seedlings from clones maintained in the germplasm bank of the Federal University of the Vales do Jequitinhonha and Mucuri (UFVJM) were produced from branches with eight nodes, collected and planted for rooting in a greenhouse for 30 days in a commercial substrate (Bioplant - Bioplant Agrícola Ltda). The seedlings were distributed in trays containing 72 cells. Planting was accomplished in March 2014, and the harvest occurred in September of the same year after 180 days of cultivation. Fertilization, covering and other cultural practices employed were those recommended by Filgueira (2008). The area for the planting was plowed and fenced, after which the soil was formed into rows. The area was irrigated with a sprinkler every day in the morning during the first three months and then three times a week.

The production parameters

The productivity of fresh roots and branches per plot was expressed in tons per hectare. The percentage dry matter in the roots and branches of the genotypes was obtained after crushing the biomass in a Croton knife mill (TE - 625 – Tecnal - Brazil) with a No. 10 mesh screen and drying in a forced-air oven at 65°C (TE - 394/3 – Tecnal - Brazil) to constant weight. The productivity of the dry matter from the roots and branches was also expressed in tons per hectare. All the data were submitted for analysis of variance and the Scott-Knott test was applied to the means at $p < 0.05$.

Determination of carbohydrates present in the roots and branches of the sweet potato

About 400 g per plot of roots and branches of each genotype was analyzed. Fresh, sliced roots and fresh branches were dried in a forced-air oven (TE - 394/3 – Tecnal - Brazil) at 60°C for 96 h and ground in a Croton knife mill (TE - 625 – Tecnal - Brazil) with a No. 10 mesh screen. The samples were then labeled and placed in

*Corresponding author. E-mail: alexandre.soares@ufvjm.edu.br. Tel: +55 38 35321200.

plastic pots for further analysis. Total soluble sugar content of the roots was analyzed by the anthrone-sulfuric acid method (McCready et al., 1950); glucose concentration was determined by the enzymatic-colorimetric GOD-POD method (Trinder, 1969); reducing sugars were analyzed by the method proposed by Miller (1959); and the amount of starch in the roots was determined by the colorimetric enzymatic method described as follows. Dried and crushed root samples (2.0 g) were weighed in 2.0-mL polypropylene microtubes to which were added 500 µl of distilled water and 10 µl of alpha-amylase (Liquozyme® Novozymes). The tubes were incubated in a water bath at 90°C for a period of 30 min. The bath temperature was lowered to 60°C, 10 µl of amyloglucosidase (Spirizyme® Novozymes) was added, and the mixture was incubated for 1 h. The enzyme hydrolyzate was completed to 100 ml with distilled water, and the glucose released was determined as described earlier. The starch content was calculated on the basis of the conversion of the glucose to its equivalent in starch, multiplying the first value by 0.9, and then expressed as percentage of starch on a dry matter basis. Standard corn starch (Megazyme) was used as a reference to validate the method. The hemicellulose, cellulose and lignin contents of the aerial portions of the plants were quantified according to the gravimetric method proposed by Van Soest (1967). The concentration of total soluble sugars was determined by the anthrone-sulfuric acid method (McCready et al., 1950). All analytical determinations were performed in triplicate.

Calculation of the projected yields of alcohol from each genotype

On the basis of the carbohydrate (starch, soluble sugars, hemicellulose and cellulose) contents of the roots and branches of the sweet potato genotypes and the dry biomass yield per hectare, the theoretical production of ethanol per hectare was estimated. A 90% yield for the conversion of carbohydrates into alcohol was considered for the purpose of estimation. Equation 1 was used for the projection of ethanol production from sweet potato roots (EP_{roots}). For the projection of ethanol production from the branches ($EP_{branches}$), Equation 2 was used. The total estimated ethanol production per hectare of planted area, considering the use of both the roots and the branches, was calculated by summing the values obtained from the two previous equations.

$$EP_{roots} = \frac{(S \times 1.1 \times 0.511) + (T_R \times 0.511)}{0.789} \times 0.9 \quad (1)$$

$$EP_{branches} = \frac{(C_H \times 1.1 \times 0.511) + (T_B \times 0.511)}{0.789} \times 0.9 \quad (2)$$

where EP is the estimated ethanol production in liters per hectare; S is the kilograms of starch per hectare; T_R is the kilograms of total soluble sugars from roots per hectare; T_B is the kilograms of total soluble sugars from aerial portion per hectare; C_H is the kilograms of cellulose and hemicellulose per hectare; (1.1) is the factor for the conversion of starch, cellulose or hemicellulose into monosaccharides; (0.511) is the theoretical yield for the fermentative conversion of monosaccharides into ethanol; (0.789) is the density of ethanol; and (0.9) is the yield adopted for the conversion of total carbohydrates into ethanol.

Enzymatic digestibility of sweet potato starch

The enzymatic digestibility assay of starch present in the roots of sweet potato genotypes was evaluated using commercial enzymes and conducted according to the following method: 0.5 g of dried and crushed sweet potatoes, 3.5 ml of 100 mM acetate buffer, pH 4.0, 10 µl of alpha-amylase (AGXXL® - Novozymes) and 10 µl of amyloglucosidase (Spirizyme® - Novozymes) were transferred to glass tubes with screw caps and incubated in a water bath at 60°C for 15 min. The tubes were transferred to a water bath at 100°C for 5 min for denaturation of the enzymes; the contents of each tube was completed to 100 ml with distilled water and glucose concentrations were determined by the GOD-POD method, as described earlier. The enzymatic digestibility (ED_{starch}) was calculated according to Equation 3.

$$ED_{starch} = \frac{G \times 0.9}{S} \times 100 \quad (3)$$

where G is the amount of glucose released after enzymatic hydrolysis of dried roots, in grams; S is the amount of starch contained in dried root sample, in grams; and 0.9 is the factor for conversion of glucose to starch.

Enzymatic digestibility of cellulose in sweet potato branches

Enzymatic digestibility of cellulose in the aerial portions of sweet potato plants was conducted in two stages. The first step involved the pre-treatment of dried and ground samples, which were immersed in 4% sulfuric acid (w/v) with a solid:liquid ratio of 1:4 and held at 121°C in an autoclave for 30 min. After the pretreatment, the insoluble fraction was washed with water to remove sulfuric acid and recovered by vacuum filtration through Whatman No. 1 filter paper. The residue was dried to constant weight at 65°C in an oven with forced air circulation and reserved for the second stage of the assay. In the second step, 0.5 g of the pretreated and dried material was placed in glass tubes with screw caps to which were added 4.4 ml of 50 mM bicarbonate buffer, pH 5.0, and 100 µl of cellulolytic preparation (Celluclast® - Novozymes). The reaction medium was incubated at 50°C for 24 h. After digestion, the tube contents were completed to 100 ml with distilled water, and the glucose concentration was determined according to the methods already described. The enzymatic digestibility of cellulose ($ED_{cellulose}$) was calculated according to Equation 4.

$$ED_{cellulose} = \frac{G \times 0.9}{C} \times 100 \quad (4)$$

where G is the amount of glucose release after enzymatic hydrolysis of dried branches, in grams; C is the amount of cellulose contained in dried branch sample, in grams; and 0.9 is the factor for conversion of glucose to cellulose.

RESULTS AND DISCUSSION

The best results for agricultural productivity in this study, expressed in terms of total material (Table 1), were obtained with the Cariru Vermelha, UFVJM 21, Tomba Carro I, and UFVJM 28 genotypes. These genotypes were not statistically different from one another. The mean productivity was 36.6 t ha^{-1}. Azevedo et al. (2015) obtained mean productivities of 13.8 and 7.3 t ha^{-1} for the

Table 1. Agricultural productivity in total material (PTM), dry matter content (DM) and agricultural productivity in dry matter (PDM) of the roots from the different sweet potato genotypes studied.

Genotypes	PTM (t ha^{-1})	DM (%)	PDM (t ha^{-1})
Batata Mandioca	25.92 ± 5.30^{b}	29.98 ± 2.04^{a}	7.83 ± 1.91^{a}
Brazlândia Branca	9.88 ± 5.81^{b}	28.39 ± 4.56^{a}	2.62 ± 1.22^{b}
Brazlândia Rosada	24.71 ± 12.37^{b}	24.23 ± 2.93^{a}	6.22 ± 3.38^{b}
Cambraia	14.99 ± 3.05^{b}	26.31 ± 1.90^{a}	3.93 ± 0.77^{b}
Cariru Vermelha	32.36 ± 11.14^{a}	28.21 ± 3.28^{a}	8.89 ± 2.33^{a}
Marmel	22.30 ± 4.19^{b}	27.87 ± 4.08^{a}	6.32 ± 2.14^{b}
Palmas	18.35 ± 9.26^{b}	27.56 ± 2.08^{a}	5.04 ± 2.46^{b}
Princesa	19.82 ± 1.81^{b}	27.49 ± 2.80^{a}	5.48 ± 1.01^{b}
Tomba Carro I	35.78 ± 11.06^{a}	27.56 ± 4.34^{a}	9.61 ± 2.15^{a}
UFVJM 01	20.66 ± 10.36^{b}	26.55 ± 1.52^{a}	5.59 ± 3.13^{b}
UFVJM 04	20.72 ± 12.74^{b}	27.09 ± 1.82^{a}	5.72 ± 3.79^{b}
UFVJM 06	21.43 ± 9.34^{b}	27.14 ± 0.16^{a}	5.82 ± 2.55^{b}
UFVJM 14	21.89 ± 17.19^{b}	27.79 ± 4.37^{a}	5.72 ± 3.74^{b}
UFVJM 20	14.42 ± 3.34^{b}	28.49 ± 2.33^{a}	4.15 ± 1.19^{b}
UFVJM 21	34.67 ± 14.11^{a}	27.27 ± 3.15^{a}	9.31 ± 3.25^{a}
UFVJM 23	18.53 ± 3.62^{b}	25.48 ± 4.34^{a}	4.76 ± 1.38^{b}
UFVJM 28	43.48 ± 12.68^{a}	25.91 ± 3.03^{a}	11.07 ± 2.42^{a}
UFVJM 45	12.30 ± 4.51^{b}	29.55 ± 2.03^{a}	3.59 ± 1.17^{b}
UFVJM 46	15.94 ± 3.54^{b}	29.48 ± 2.92^{a}	4.75 ± 1.49^{b}
UFVJM 48	19.23 ± 7.57^{b}	27.37 ± 1.57^{a}	5.22 ± 1.87^{b}
CV (%)	38.61	9.11	37.90
Mean	22.37	27.48	6.08

*Means followed by the same lower case letter in the columns do not differ by the Scott-Knott test at $p < 0.05$.

Tomba Carro I and UFVJM 28 genotypes, respectively, cultivated in the municipality of Diamantina, MG, Brazil (18°12'01"S, 43°34'20"W and altitude of 1387 m) and harvested five months after planting in December 2007. The main differences between the cultivation conditions used by Azevedo et al. (2015) and those used in the present study were the planting season, the age of the plant at harvest and the use of irrigation. The use of irrigation and the later harvesting period probably favored the increase of productivity in the present study.

As for the dry matter content of the roots (Table 1), a range from 24.2 to 29.9% and an average of 27.5% was observed for the genotypes, and they did not differ significantly from one another. Shumbusha et al. (2014) evaluated the dry matter content of sweet potato clones in Uganda and encountered values between 23.5 and 35.2%. Regarding the productivity of the roots expressed as dry matter (Table 1), Batata Mandioca, Cariru Vermelha, UFVJM 21, Tomba Carro I, and UFVJM 28 contained the highest statistically equivalent levels, with an average of 9.3 t ha^{-1}.

As for the agricultural productivity of the branches from the sweet potato genotypes (Table 2), there were significant differences for all the variables. Agricultural productivities in terms of total plant material from the more productive genotypes were 31.7 (UFVJM 28) and 26.7 t ha^{-1} (UFVJM 46) (Table 2). The average dry matter content of all the genotypes was 14.8%, with emphasis on the UFVJM 48, Cariru Vermelha, UFVJM 28, UFVJM 14, UFVJM 06, UFVJM 45, and UFVJM 46 genotypes (Table 2). The branches of the UFVJM 28 genotype furnished the highest yield of dry matter (5.7 t ha^{-1}). Andrade Júnior et al. (2012), evaluating the production of branches of sweet potatoes for use as animal feed, reported the production of 1.4 to 3.5 tons of dry mass per hectare for seven genotypes originating in the region of the Vale do Jequitinhonha (Minas Gerais, Brazil).

There were significant differences between genotypes with regard to the concentrations of starch and soluble sugars (Table 3). The mean starch content of the genotypes was 54.2%. There were no significant differences between the starch yields for Cariru Vermelha, UFVJM 21, Tomba Carro I, and UFVJM 28 genotypes, and the highest productivities were obtained for these genotypes, between 4.9 and 6.5 tons of starch per hectare. Waluyo et al. (2015) reported yields between 0.9 and 7.4 tons of starch per hectare of sweet potato clones classified as promising for bioethanol production in

Table 2. Agricultural productivity of total plant material (PTM), dry matter content (DM) and agricultural productivity in dry matter (PDM) of branches from the different sweet potato genotypes studied.

Genotype	PTM (t ha^{-1})	DM (%)	PDM (t ha^{-1})
Batata Mandioca	19.34 ± 8.63^{b}	12.82 ± 1.12^{b}	2.51 ± 1.28^{c}
Brazlândia Branca	16.76 ±10.05^{c}	13.50 ± 0.83^{b}	2.21 ± 1.26^{c}
Brazlândia Rosada	21.30 ± 2.44^{b}	13.76 ± 2.78^{b}	2.92 ± 0.62^{b}
Cambraia	15.50 ± 5.40^{c}	14.15 ± 1.18^{b}	2.16 ± 0.71^{c}
Cariru Vermelha	16.50 ± 4.28^{c}	15.66 ± 1.14^{a}	2.61 ± 0.78^{c}
Marmel	13.17 ± 1.97^{c}	13.56 ± 1.30^{b}	1.79 ± 0.40^{c}
Palmas	13.37 ± 5.10^{c}	14.52 ± 1.55^{b}	1.92 ± 0.73^{c}
Princesa	15.33 ± 3.87^{c}	14.93 ± 1.60^{b}	2.24 ± 0.42^{b}
Tomba Carro I	22.74 ± 5.02^{b}	15.06 ± 1.26^{b}	3.39 ± 0.65^{b}
UFVJM 01	22. 89 ± 6.34^{b}	13.78 ± 1.09^{b}	3.10 ± 0.71^{b}
UFVJM 04	15.18 ± 6.45^{c}	14.30 ± 1.28^{b}	2.11 ± 0.77^{c}
UFVJM 06	19.76 ± 6.90^{b}	16.57 ± 2.17^{a}	3.17 ± 0.75^{b}
UFVJM 14	21.04 ±11.55^{b}	15.63 ± 1.43^{a}	3.29 ± 1.86^{b}
UFVJM 20	15.73 ± 3.93^{c}	13.92 ± 2.50^{b}	2.25 ± 0.92^{c}
UFVJM 21	20.91 ± 3.73^{b}	14.03 ± 0.75^{b}	2.94 ± 0.60^{b}
UFVJM 23	11.86 ± 1.11^{c}	14.09 ± 0.86^{b}	1.66 ± 0.08^{c}
UFVJM 28	31.68 ± 8.94^{a}	17.84 ± 5.22^{a}	5.66 ± 2.20^{a}
UFVJM 45	21.42 ± 6.84^{b}	17.51 ± 2.95^{a}	3.64 ± 0.91^{b}
UFVJM 46	26.70 ± 3.36^{a}	15.6 2± 1.30^{a}	4.18 ± 0.60^{b}
UFVJM 48	10.27 ± 2.44^{c}	15.37 ± 1.45^{a}	1.56 ± 0.30^{c}
Mean	18.57	14.83	2.76
C.V %	28.07	12.76	33.41

Means followed by the same lower case letter in the columns do not differ by the Scott-Knott test at $p < 0.05$.

Indonesia. Oliveira et al. (2017) related starch yields from 4.0 to 6.6 t ha^{-1} in a study with six sweet potato genotypes targeted as raw material for bioethanol.

The highest total soluble sugar concentrations were obtained with the Palmas, Batata Mandioca, Cariru Vermelha, and UFVJM 45 genotypes. There were no significant differences among them, and the concentrations ranged from 4.1 to 5.4%. Adu-Kwarteng et al. (2014) found total soluble sugar concentrations in sweet potato genotypes higher than those found in this work, ranging from 7.4% for the Sauti genotype to 10.3% for the Otoo genotype five months after planting. These sugars, consisting mainly of sucrose, can easily be converted to ethanol during fermentation.

There were no significant differences between the genotypes with respect to the different carbohydrate fractions present in the branches of the sweet potato genotypes evaluated (Table 4). However, the average hemicellulose content found in the twenty genotypes (13.7%) was greater than the average value recorded for the cellulose content (8.9%). The concentration of soluble sugars found in Brazlândia Branca and Tomba Carro I genotypes was at least 25% higher than the values recorded for the other genotypes (Table 4). The lignin contents of the sweet potato branches ranged from 1.5 to 4.0%, with no significant differences (Table 4). However, the average value of the lignin found in this study (2.6%) was lower than the values recorded for some lingo-cellulosic biomasses that exhibit a potential for the production of second generation ethanol, such as wheat straw, with 13 to 15% lignin, and bagasse from sugarcane, containing 23 to 32% lignin (Saini et al., 2015). Lignin is a key limiting factor in the saccharification of lignocellulosic feedstocks (Van der Weijde et al., 2016).

Although there were no significant differences in the quantities of cellulose and hemicellulose found in the branches of the sweet potato genotypes studied, there were significant differences in the concentrations of these carbohydrate fractions multiplied by the agronomic yield (dry matter per hectare). The highest hemicellulose productivities in the aerial portion by planted area, between 0.45 and 0.74 t ha^{-1}, were observed for the UFVJM 14, UFVJM 28, UFVJM 45 and UFVJM 46 genotypes (Table 5). The highest cellulose productivities in branches per planted area, 0.62 and 0.52 t ha^{-1}, were obtained for the UFVJM 28 and UFVJM 46 genotypes, respectively. The highest yields of soluble sugars from the branches by planted area, 0.067 and 0.073 t ha^{-1},

Table 3. Levels of starch and total soluble sugars (TSS) in the dry matter of sweet potato roots from the different genotypes studied and their yields per hectare.

Genotype	Starch (%)	TSS (%)	Starch (t ha^{-1})	TSS (t ha^{-1})
Batata Mandioca	49.37 ± 1.69^{b}	4.25 ± 2.03^{a}	3.87 ± 0.97^{b}	0.34 ± 0.20^{a}
Brazlândia Branca	54.75 ± 7.76^{a}	2.34 ± 1.72^{b}	1.50 ± 0.90^{b}	0.05 ± 0.02^{b}
Brazlândia Rosada	57.05 ± 1.48^{a}	3.25 ± 1.37^{b}	3.52 ± 1.90^{b}	0.22 ± 0.18^{b}
Cambraia	53.66 ± 0.48^{a}	2.18 ± 0.82^{b}	2.11 ± 0.43^{b}	0.08 ± 0.03^{b}
Cariru Vermelha	55.71 ± 2.65^{a}	4.08 ± 0.90^{a}	4.94 ± 1.29^{a}	0.37 ± 0.12^{a}
Marmel	44.39 ± 12.15^{c}	2.78 ± 0.23^{b}	2.92 ± 1.60^{b}	0.17 ± 0.04^{b}
Palmas	52.72 ± 0.88^{b}	5.45 ± 1.57^{a}	2.65 ± 1.30^{b}	0.25 ± 0.10^{a}
Princesa	53.04 ± 0.74^{b}	3.31 ± 0.96^{b}	2.91 ± 0.57^{b}	0.18 ± 0.05^{b}
Tomba Carro I	56.60 ± 0.31^{a}	3.23 ± 0.80^{b}	5.45 ± 1.24^{a}	0.30 ± 0.05^{a}
UFVJM 01	51.56 ± 1.88^{b}	2.66 ± 0.52^{b}	2.92 ± 1.70^{b}	0.16 ± 0.11^{b}
UFVJM 04	51.02 ± 0.94^{b}	3.86 ± 1.98^{a}	2.93 ± 1.95^{b}	0.21 ± 0.14^{b}
UFVJM 06	50.25 ± 1.05^{b}	3.75 ± 0.74^{a}	2.94 ± 1.33^{b}	0.23 ± 0.13^{b}
UFVJM 14	56.33 ± 1.34^{a}	3.94 ± 0.72^{a}	3.26 ± 2.22^{b}	0.21 ± 0.13^{b}
UFVJM 20	53.70 ± 0.53^{a}	2.54 ± 0.57^{b}	2.23 ± 0.65^{b}	0.11 ± 0.05^{b}
UFVJM 21	56.82 ± 1.13^{a}	2.15 ± 0.11^{b}	5.27 ± 1.79^{a}	0.20 ± 0.07^{b}
UFVJM 23	54.03 ± 0.73^{a}	1.61 ± 0.69^{b}	2.57 ± 0.73^{b}	0.07 ± 0.02^{b}
UFVJM 28	59.05 ± 1.53^{a}	2.79 ± 0.51^{b}	6.56 ± 1.56^{a}	0.32 ± 0.13^{a}
UFVJM 45	56.47 ± 1.27^{a}	4.38 ± 0.18^{a}	2.02 ± 0.65^{b}	0.16 ± 0.05^{b}
UFVJM 46	61.21 ± 0.87^{a}	3.80 ± 0.66^{a}	2.90 ± 0.89^{b}	0.18 ± 0.04^{b}
UFVJM 48	56.18 ± 2.50^{a}	2.72 ± 0.37^{b}	2.94 ± 1.08^{b}	0.15 ± 0.06^{b}
Mean	54.20	3.25	3.32	0.20
CV (%)	6.23	32.39	39.33	49.37

Means followed by the same lower case letter in the columns do not differ by the Scott-Knott test at $p < 0.05$.

Table 4. Hemicellulose (HC), cellulose (CL), lignin (LG) and total soluble sugars (TSS) in the dry matter of branches from the different sweet potato genotypes.

Genotype	HC (%)	CL (%)	LG (%)	TSS (%)
Batata Mandioca	12.38 ± 2.36^{a}	8.21 ± 3.72^{a}	1.77 ± 0.49^{a}	0.43 ± 0.01d
Brazlândia branca	11.71 ± 1.48^{a}	9.33 ± 1.22^{a}	2.26 ± 0.28^{a}	1.60 ± 0.05^{a}
Brazlandia rosada	11.92 ± 2.25^{a}	9.01 ± 2.08^{a}	3.11 ± 2.01^{a}	0.97 ± 0.03^{c}
Cambraia	14.32 ± 4.37^{a}	8.01 ± 1.85^{a}	2.46 ± 0.79^{a}	1.33 ± 0.03^{c}
Cariru Vermelha	13.79 ±3.60^{a}	7.24 ± 4.01^{a}	2.04 ± 0.29^{a}	1.12 ± 0.04^{c}
Marmel	14.96 ± 3.02^{a}	10.19 ± 3.71^{a}	3.05 ± 2.14^{a}	0.80 ± 0.03^{c}
Palmas	14.06 ± 2.29^{a}	7.96 ± 5.02^{a}	3.29 ± 0.66^{a}	0.79 ± 0.06^{c}
Princesa	13.83 ± 3.90^{a}	7.98 ± 2.81^{a}	4.04 ± 3.29^{a}	1.05 ± 0.08^{c}
Tomba Carro I	14.60 ± 3.47^{a}	6.43 ± 3.01^{a}	3.54 ± 2.70^{a}	1.99 ± 0.14^{a}
UFVJM 01	11.30 ± 2.36^{a}	6.08 ± 2.00^{a}	2.44 ± 0.37^{a}	1.31 ± 0.04^{c}
UFVJM 04	14.09 ± 3.53^{a}	8.49 ± 2.00^{a}	2.30 ± 0.66^{a}	0.64 ± 0.02^{c}
UFVJM 06	16.14 ± 5.25^{a}	10.11 ± 2.84^{a}	2.85 ± 0.96^{a}	0.61 ± 0.03^{c}
UFVJM 14	13.53 ± 2.23^{a}	11.18 ± 6.17^{a}	2.16 ± 1.05^{a}	0.77 ± 0.04^{c}
UFVJM 20	14.91 ± 1.47^{a}	9.73 ± 4.43^{a}	2.51 ± 0.94^{a}	0.73 ± 0.08^{c}
UFVJM 21	12.94 ± 2.47^{a}	8.61 ± 1.97^{a}	2.63 ± 0.68^{a}	0.72 ± 0.06^{c}
UFVJM 23	8.91 ± 2.65^{a}	9.86 ± 3.66^{a}	2.41 ± 0.37^{a}	0.85 ± 0.04^{c}
UFVJM 28	13.22 ± 3.32^{a}	11.19± 2.46^{a}	2.30 ± 0.53^{a}	1.29 ± 0.08^{c}
UFVJM 45	17.47 ± 1.72^{a}	9.12 ± 2.00^{a}	2.99 ± 0.85^{a}	1.29 ± 0.28^{b}
UFVJM 46	15.81 ± 2.97^{a}	12.46 ± 4.25^{a}	2.29 ± 0.93^{a}	1.31 ± 0.05^{c}

Table 4. Contd.

UFVJM 48	14.64 ± 0.85[a]	7.08 ± 3.10[a]	1.50 ± 0.80[a]	1.06 ± 0.02[c]
Mean (%)	13.73	8.9	2.61	1.06
CV (%)	22.16	32.54	48.23	10.52

Means followed by the same lower case letter in the columns do not differ by the Scott-Knott test at $p < 0.05$.

Table 5. Productivity of hemicellulose (HC), cellulose (CL) and total soluble sugars (TSS) in the dry matter of branches from different sweet potato genotypes.

Genotypes	HC (t ha^{-1})	CL (t ha^{-1})	TSS (t ha^{-1})
Batata Mandioca	0.32 ± 0.18[c]	0.17 ± 0.05[b]	0.011 ± 0.005[d]
Brazlândia branca	0.26 ± 0.14[c]	0.21 ± 0.12[b]	0.035 ± 0.007[c]
Brazlândia rosada	0.35 ± 0.05[c]	0.27 ± 0.10[b]	0.028 ± 0.006[c]
Cambraia	0.31 ± 0.12[c]	0.17 ± 0.07[b]	0.029 ± 0.003[c]
Cariru Vermelha	0.35 ± 0.08[c]	0.20 ± 0.16[b]	0.029 ± 0.011[c]
Marmel	0.27 ± 0.06[c]	0.19 ± 0.10[b]	0.014 ± 0.005[d]
Palmas	0.26 ± 0.08[c]	0.13 ± 0.07[b]	0.015 ± 0.005[d]
Princesa	0.32 ± 0.09[c]	0.17 ± 0.05[b]	0.024 ± 0.009[c]
Tomba Carro I	0.50 ± 0.12[b]	0.21 ± 0.11[b]	0.067 ± 0.021[a]
UFVJM 01	0.35 ± 0.07[c]	0.19 ± 0.07[b]	0.041 ± 0.008[b]
UFVJM 04	0.30 ± 0.13[c]	0.18 ± 0.09[b]	0.014 ± 0.004[d]
UFVJM 06	0.51 ± 0.13[b]	0.31 ± 0.04[b]	0.019 ± 0.002[d]
UFVJM 14	0.45 ± 0.25[a]	0.37 ± 0.25[b]	0.025 ± 0.002[c]
UFVJM 20	0.33 ± 0.14[c]	0.19 ± 0.06[b]	0.016 ± 0.002[d]
UFVJM 21	0.38 ± 0.09[c]	0.25 ± 0.08[b]	0.021 ± 0.001[c]
UFVJM 23	0.26 ± 0.02[c]	0.16 ± 0.06[b]	0.014 ± 0.001[d]
UFVJM 28	0.74 ± 0.25[a]	0.62 ± 0.26[a]	0.073 ± 0.005[a]
UFVJM 45	0.64 ± 0.16[a]	0.33 ± 0.08[b]	0.047 ± 0.005[b]
UFVJM 46	0.65 ± 0.06[a]	0.52 ± 0.21[a]	0.055 ± 0.009[b]
UFVJM 48	0.23 ± 0.05[c]	0.11 ± 0.06[b]	0.017 ± 0.006[d]
Mean (%)	0.38	0.25	0.03
CV (%)	30.83	41.37	26.99

Means followed by the same lower case letter in the columns do not differ by the Scott-Knott test at $p < 0.05$.

were obtained for the Tomba Carro I and UFVJM 28 genotypes, respectively. The highest productivity of total carbohydrates (cellulose, hemicellulose and total soluble sugars) in the aerial portion by planted area, 1.4 t ha^{-1}, was observed for UFVJM 28.

The estimated ethanol yields per hectare obtainable from the Cariru Vermelha, UFVJM 21, Tomba Carro I and UFVJM 28 genotypes varied from 3810 to 4940 L ha^{-1}. These values are higher than those achieved for the production of ethanol from maize, the main starchy raw material used by the biofuel alcohol industry, with a productivity between 2800 and 3800 L ha^{-1} (Duvernay et al., 2013; Ziska et al., 2009).

The productivity of sweet potatoes can vary greatly with the chosen genotypes, cultivation and soil and climatic conditions. Santana et al. (2013) identified sweet potato genotypes that were grown in Tocantins, a semi-humid tropical region of Brazil, yielded up to 181.6 L of ethanol per ton of roots and furnished a productivity of 3122 to 10007 L ha^{-1}. Waluyo et al. (2015) worked with sweet potato clones in Indonesia and obtained estimated ethanol yields ranging from 3320.1 to 5364.5 L ha^{-1} from the roots. Oliveira et al. (2017) estimated ethanol productivities from 2667 to 4379 L ha^{-1} for six sweet potato genotypes cultivated in Sergipe state, a tropical semiarid region of Brazil.

In addition to the production of ethanol estimated from the use of roots, the best projection for alcohol yield obtained from the branches, 995 L ha^{-1}, was calculated for the UFVJM 28 genotype. Considering the projections

Table 6. Theoretical projection of ethanol yield from the roots and branches of different sweet potato genotypes.

Genotype	Ethanol from roots (L ha^{-1})	Ethanol from branches (L ha^{-1})	Total Ethanol (L ha^{-1})
Batata Mandioca	3030 ± 810^{b}	370 ± 50^{j}	3400 ± 800^{c}
Brazlândia Branca	1120 ± 660^{b}	330 ± 80^{l}	1450 ± 650^{c}
Brazlândia Rosada	2690 ± 1480^{b}	440 ± 80^{h}	3130 ± 1470^{c}
Cambraia	1580 ± 310^{b}	350 ± 40^{k}	1930 ± 310^{c}
Cariru Vermelha	3810 ± 990^{a}	390 ± 40^{i}	4200 ± 980^{b}
Marmel	2230 ± 1180^{b}	320 ± 40^{l}	2550 ± 1190^{c}
Palmas	2080 ± 980^{b}	290 ± 30^{m}	2370 ± 990^{c}
Princesa	2220 ± 410^{b}	350 ± 10^{k}	2570 ± 420^{c}
Tomba Carro I	4130 ± 900^{a}	510 ± 20^{f}	4640 ± 900^{b}
UFVJM 01	2210 ± 1310^{b}	380 ± 40^{i}	2590 ± 1300^{c}
UFVJM 04	2250 ± 1490^{b}	350 ± 60^{k}	2600 ± 1490^{c}
UFVJM 06	2270 ± 1040^{b}	600 ±40^{d}	2870 ± 1030^{c}
UFVJM 14	2500 ± 1680^{b}	580 ± 190^{e}	3080 ± 1690^{c}
UFVJM 20	1680 ± 310^{b}	400 ± 80^{i}	2080 ± 510^{c}
UFVJM 21	3930 ± 1330^{a}	460 ± 60^{g}	4390 ± 1320^{b}
UFVJM 23	1900 ± 520^{b}	300 ± 10^{m}	2200 ± 520^{c}
UFVJM 28	4940 ± 1210^{a}	995 ± 20^{a}	5930 ± 1200^{a}
UFVJM 45	1560 ± 500^{b}	690 ± 40^{c}	2250± 500^{c}
UFVJM 46	2210 ± 660^{b}	840 ± 150^{b}	3050 ± 660^{c}
UFVJM 48	2220 ± 820^{b}	240 ± 30n	2460 ± 830^{c}
Méan	2520	450	2980
CV (%)	39.20	35.53	33.17

Means followed by the same lower case letter in the columns do not differ by the Scott-Knott test at $p < 0.05$.

for the yield of ethanol from the roots and branches obtained with the UFVJM 28 genotype, a productivity of 5930 L ha^{-1} in a six-month cycle was estimated (Table 6). In the best scenario, the estimated production for this genotype could reach 11860 L ha^{-1} year^{-1}. The productivity of sugarcane can vary from 40 to 70 tons per hectare, depending on the country. Some specific varieties are able to reach 150 tons of sugarcane per hectare under experimental conditions (Morais et al., 2015). Considering a mean of 82 L of bioethanol per ton of processed sugarcane (Boddey et al., 2008), a maximum of 3279 to 12300 L ha^{-1} year^{-1} could be produced. The average ethanol yield per hectare in Brazil is 6280 L ha^{-1} year^{-1} (Boddey et al., 2008). This ethanol productivity is lower than the highest values estimated in this study.

In addition to the carbohydrate content present in the roots and branches of the genotypes, the digestibility of starch or cellulose present in these biomasses is relevant to the choice of biomass or technology to be used in the saccharification process of polysaccharides. The digestibility experiment using dried and crushed roots of sweet potato genotypes (Table 7) indicated a digestibility between 58.2 and 91.2%. The highest digestibility values, between 79.9 and 91.2%, were observed for the Brazlândia Rosada, Princesa, UFVJM 14, UFVJM 28, and UFVJM 45 genotypes. Whereas the digestibility trial was undersized to allow differentiation between biomasses, the outstanding clones are extremely susceptible to enzymatic saccharification, an intrinsic step in the production of ethanol from starchy raw materials.

The digestibility assay of the cellulosic fraction of the dry matter from the branches of the sweet potato genotypes selected was based on the estimated yield of ethanol (Table 6) or the compositional characteristics (Table 4) provided before the pretreatment with dilute inorganic acid. After the acid pretreatment, the percentage of cellulose in the insoluble material recovered increased (Table 8) as a natural consequence of the removal of the hemicellulose fraction (Carvalho et al., 2015). After enzymatic digestion of the pretreated material, the cellulose content in the recovered insoluble material decreased by different degrees that depended on the genotype (Table 8). The insoluble residue recovered after enzymatic digestion of the branches from the Palmas genotype contained the smallest amount of cellulose (Table 8). The digestibility assay of each material, which was based on the amount of glucose released after enzymatic action, yielded higher values for Marmel, Palmas, and UFVJM 28 genotypes (Table 8), whose digestibility values were close to 40%. The UFVJM 46 genotype, despite having the highest yield of branches,

Table 7. Digested starch and enzymatic digestibility of starch in the roots from the sweet potato genotypes.

Genotype	Digested starch (% g/g)	Digestibility (%)
Batata Mandioca	34.45 ± 2.84[b]	69.76 ± 3.35[c]
Brazlândia Branca	39.20 ± 4.68[b]	71.60 ± 7.01[c]
Brazlândia Rosada	46.35 ± 4.50[a]	81.24 ± 3.97[b]
Cambraia	37.32 ± 2.77[b]	69.55 ± 5.02[d]
Cariru Vermelha	38.06 ± 3.47[b]	68.33 ± 2.60[d]
Marmel	33.62 ± 5.50[b]	75.73 ± 1.96[c]
Palmas	34.34 ± 4.83[b]	65.13 ± 7.47[d]
Princesa	48.40 ± 0.32[a]	91.25 ± 1.46[a]
Tomba Carro I	36.54 ± 2.26[b]	64.56 ± 3.79[d]
UFVJM 01	38.00 ± 2.83[b]	73.70 ± 3.20[c]
UFVJM 04	30.56 ± 2.97[c]	59.90 ± 4.72[e]
UFVJM 06	29.26 ± 4.91[c]	58.22 ± 2.47[e]
UFVJM 14	45.00 ± 3.20[a]	79.88 ± 6.24[b]
UFVJM 20	35.10 ± 2.18[b]	65.36 ± 4.63[d]
UFVJM 21	35.32 ± 3.94[b]	62.16 ± 4.00[e]
UFVJM 23	36.19 ± 4.02[b]	66.97 ± 7.51[d]
UFVJM 28	49.31 ± 6.79[a]	83.51 ± 6.90[b]
UFVJM 45	46.70 ± 3.55[a]	82.69 ± 2.48[b]
UFVJM 46	36.71 ± 2.03[b]	59.97 ± 3.99[e]
UFVJM 48	42.82 ± 5.33[a]	76.21 ± 6.22[c]
Mean (%)	38.66	73.81
CV (%)	9.47	6.81

Means followed by the same lower case letter in the columns do not differ by the Scott-Knott test at $p < 0.05$.

Table 8. Content of cellulose after acid pretreatment (CL_{PT}), cellulose content after enzymatic saccharification (CL_{SC}) and enzymatic digestibility (ED) from branches of selected sweet potato genotypes.

Genotype	CL_{PT} (%)	CL_{SC} (%)	ED (%)
Marmel	23.72 ± 0.28[b]	14.25 ± 0.01[b]	39.60 ± 3.20[a]
Palmas	19.64 ± 0.33[c]	2.09 ± 0.01[e]	42.41 ± 21.01[a]
UFVJM 14	26.98 ± 0.91[a]	13.79 ± 0.01[c]	28.63 ± 1.33[b]
UFVJM 28	27.09 ± 0.72[a]	4.80 ± 0.10[d]	41.06 ± 6.60[a]
UFVJM 46	25.00 ± 0.29[c]	20.31 ± 0.01[a]	14.24 ± 2.71[b]
UFVJM 48	21.00 ± 1.32[c]	14.18 ± 0.01[b]	18.36 ± 4.28[b]
Mean (%)	23.90	11.57	30.72
CV (%)	3.34	0.37	30.47

Means followed by the same lower case letter in the columns do not differ by the Scott-Knott test at $p < 0.05$.

along with the UFVJM 28 genotype, was recalcitrant to enzymatic attack and its digestibility was only 14.2%.

Conclusion

The genetic variability of the evaluated genotypes was marked by differences in the agricultural productivity and the carbohydrate contents. UFVJM 28 was shown to be the most productive sweet potato genotype in terms of starch, total soluble sugars, cellulose and hemicellulose quantities produced per planted area. The highest digestibilities for the starch contained in the roots and for the cellulose contained in the branches were also obtained for the UFVJM 28 genotype. These characteristics resulted in a higher estimated ethanol yield, 5930 L

ha^{-1}, in a 180-day production cycle, or 11860 L ha^{-1} $year^{-1}$ if two contiguous cycles were considered. The possibility of utilizing the branches of the sweet potatoes represented an increase of up to 20% in the estimated production of bioethanol. Disregarding the cost of producing the ethanol from sweet potatoes, which was not evaluated here, the genotype highlighted earlier seems to be as promising, or more promising, than corn or sugar cane when they began to be used as raw material for fuel ethanol production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

Financial support was furnished by the Fundação de Amparo de Pesquisa do Estado de Minas Gerais (FAPEMIG). An undergraduate research scholarship was granted by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPQ). D. L. Nelson was the recipient of a PVNS fellowship furnished by the Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES).

REFERENCES

Aditiya HB, Mahlia TMI, Chong WT, Nur H, Sebayang AH (2016). Second generation bioethanol production: A critical review. Renew Sust. Energ. Rev. 66:631-653.

Adu-Kwarteng E, Sakyi-Dawson EO, Ayernor GS, Truong V-D, Shih FF, Daigle K (2014). Variability of Sugars in Staple-Type Sweet Potato (*Ipomoea batatas*) Cultivars: The Effects of Harvest Time and Storage. Int. J. Food Prop. 17:410-420.

Andrade Júnior VC de, Viana DJS, Pinto NA, Ribeiro KG, Pereira RC, Neiva IP, Azevedo AM, Andrade PC de R (2012). Características produtivas e qualitativas de ramas e raízes de batata-doce. Hortic. Bras. 30:584-589.

Azevedo AM, Andrade Júnior VC, Fernendes JSC, Pedrosa CE, Oliveira CM (2015). Desempenho agronômico e parâmetros genéticos em genótipos de batata-doce. Hortic. Bras. 33:084-090.

Boddey RM, Soares LHB, Alves BJR, Urquiaga S (2008). Bio-Ethanol Production in Brazil. In: Pimentel, D, ed. Biofuels, Solar and Wind as Renewable Energy Systems. Springer Netherlands, Heidelberg, Germany. pp. 321-356.

Carvalho DM, Sevastyanova O, Penna LS, Silva BP da, Lindström ME, Colodette JL (2015). Assessment of chemical transformations in eucalyptus, sugarcane bagasse and straw during hydrothermal, dilute acid, and alkaline pretreatments. Ind. Crops Prod. 73:118-126.

Dangler JM, Locascio SJ, Halsey LH (1984). Sweet potato for biomass. *Biomass* 4:253-261.

Duvernay WH, Chinn MS, Yencho GC (2013). Hydrolysis and fermentation of sweet potatoes for production of fermentable sugars and ethanol. Ind. Crops Prod. 42:527-537.

FAO (2016). Food and Agriculture Organization of the United Nations - Statistics Division. Available in: http://www.fao.org/faostat/en/#data/QC. Accessed Sept 20, 2016.

Filgueira FAR (2008). Novo manual de olericultura: agrotecnologia moderna na produção e comercialização de hortaliças.' (Ed. UFV: Viçosa)

Masiero SS, Peretti A, Trierweiler LF, Trierweiler JO (2014). Simultaneous cold hydrolysis and fermentation of fresh sweet potato. Biomass Bioenergy 70:174-183.

McCready RM, Guggolz J, Silviera V, Owens HS (1950). Determination of Starch and Amylose in Vegetables. Anal. Chem. 22:1156-1158.

Miller GL (1959) Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal. Chem. 31:426-428.

Morais LK, Aguiar MS, Albuquerque e Silva P, Câmara TMM, Cursi DE, Júnior ARF, Chapola RG, Carneiro MS, Bespalhok Filho JC (2015) Breeding of Sugarcane. 'Industrial Crops'. (Eds VMV Cruz, DA Dierig) (Springer New York: New York, NY) pp. 29-42.

Oliveira MAS, Blank AF, Alves RP, Arrigoni-Blank MF, Maluf WR, Fernandes RPM (2017). Performance of sweet potato clones for bioethanol production in different cultivation periods. Hortic. Bras. 35:057-062.

Parmar A, Hensel O, Sturm B (2017). Post-harvest handling practices and associated food losses and limitations in the sweetpotato value chain of southern Ethiopia. NJAS Wagen. J. Life Sci. 80:65-74.

Ray RC, Tomlins KI (2010). Sweet potato: post-harvest aspects in food, feed and industry. Nova Science Publ: New York.

Saini JK, Saini R, Tewari L (2015). Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. 3 Biotech 5:337-353.

Santana WR, Martins LP, Silveira MA, Santos WF, Gonçalves RC, Souza FR, Resplandes GRS, Lima MM (2013). Identificação agronômica de genótipos de batata-doce em banco de germoplasma para fins industriais de etanol carburante. Tecnol. Ciên. Agropec. 7:31-34.

Shumbusha D, Tusiime G, Edema R, Gibson P, Adipala E, Mwanga ROM (2014). Inheritance of root dry matter content in sweet potato. Afr. Crop Sci. J. 22:69-78.

Suarez MH, Hermández AIM, Galdón BR, Rodríguez LH, Cabrera CEM, Mesa DR, Rodríguez-Rodríguez EM, Romero CD (2016). Application of multidimensional scaling technique to diferentiate sweet potato (*Ipomoea batatas* (L.) Lam) cultivars according to their chemical composition. J. Food Compost. Anal. 46:43-49.

Trinder P (1969) Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. J. Clin. Pathol. 22:158-161.

van der Weijde T, Torres AF, Dolstra O, Dechesne A, Visser RGF, Trindade LM (2016). Impact of different lignin fractions on saccharification efficiency in diverse species of the bioenergy crop Miscanthus. Bioenergy Res. 9:146-156.

Van Soest PJ (1967). Use of detergents in the analyses of fibrous feeds. 2. A rapid method for the determination of fiber and lignin. J. Assoc. Off. Anal. Chem. 46:829-835.

Waluyo B, Roosda AA, Istifadah N, Ruswandi D, Karuniawan A (2015). Identification of fifty sweetpotato (*Ipomoea batatas* (L.) Lam.) promising clones for bioethanol raw materials. Energy Procedia 65:22-28.

Widodo Y, Wahyuningsih S, Ueda A (2015). Sweet potato production for bio-ethanol and food related industry in Indonesia: Challenges for sustainability. Procedia Chem. 14:493-500.

Ziska LH, Runion GB, Tomecek M, Prior SA, Torbet HA, Sicher R (2009). An evaluation of cassava, sweet potato and field corn as potential carbohydrate sources for bioethanol production in Alabama and Maryland. Biomass Bioenergy 33:1503-1508.

African Journal of Biotechnology

Related Journals Published by Academic Journals

- *Biotechnology and Molecular Biology Reviews*
- *African Journal of Microbiology Research*
- *African Journal of Biochemistry Research*
- *African Journal of Environmental Science and Technology*
- *African Journal of Food Science*
- *African Journal of Plant Science*
- *Journal of Bioinformatics and Sequence Analysis*
- *International Journal of Biodiversity and Conservation*

academicJournals