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Full Length Research Paper

Ex-vivo evaluation of crab shell chitosan as absorption enhancer in ciprofloxacin tablet formulation

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This study was aimed at evaluating crab shell chitosan as absorption enhancer in ciprofloxacin tablet formulation using the *ex-vivo* model. Six batches of ciprofloxacin tablets containing varying concentrations of crab shell-derived chitosan ranging from 0 to 5% w/w at 1% w/w intervals were produced. Batch CTS-0 containing no chitosan served as the control. The crushing strength, friability, disintegration time, dissolution profile and permeation profile of all the batches were determined. Friability was not significantly affected but the crushing strength and disintegration time of tablets decreased with increase in concentration of chitosan. There was no significant difference in the cumulative percent drug released in 1 h but the cumulative percent drug permeated in 4 h increased with increase in the concentration of chitosan. It increased from 68% (when no chitosan was added) to 81.8% (when 5% w/w chitosan was incorporated). The polymer caused a faster onset of drug release but the eventual total drug released was not significantly influenced. It also improved the permeation of the released drug. This study correlates with *in-vivo* bioavailability study because the usual oral bioavailability of ciprofloxacin without absorption enhancer is 70%. Hence, crab shell chitosan at concentration of 5% w/w could increase the absorption of ciprofloxacin from 70 to 82%. The study suggests the use of the chitosan at this concentration to improve the absorption of ciprofloxacin.

Key words: Crab shell chitosan, ciprofloxacin, dissolution, permeation, absorption.

INTRODUCTION

Ciprofloxacin is a fluoroquinolone antibacterial with a wide spectrum of activity (Campoli-Richard et al., 1998). It inhibits bacterial growth and replication by interfering with the action of DNA gyrase (topoisomerase II) and topoisomerase IV. A peak plasma concentration of 2 - 3 µg/mL occurs within 2 h after an oral administration of

500 mg dose. It has an oral bioavailability of about 70% (Chambers, 2004). Drugs have been classified into four in the Biopharmaceutic Classification System (BCS) by Amidol et al. (1995). This classification is based on biopharmaceutical properties of solubility and permeability which are the two properties used in assessing

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Table 1. Tablet formula.

Ingredient	Batches					
	CTS-0	CTS-1	CTS-2	CTS-3	CTS-4	CTS-5
Ciprofloxacin (%)	80	80	80	80	80	80
Maize starch (%)	10	10	10	10	10	10
Lactose (%)	5	4	3	2	1	0
Acacia (%)	3	3	3	3	3	3
Chitosan (%)	0	1	2	3	4	5
Talc (%)	1.5	1.5	1.5	1.5	1.5	1.5
Mg stearate (%)	0.5	0.5	0.5	0.5	0.5	0.5
Tablet weight (mg)	625	625	625	625	625	625

bioavailability of orally administered drugs. Ciprofloxacin has unique characters that are intermediate between BCS classes II and III (Wu and Benet, 2005). This implies that the solubility is not as good as that of class III drugs and the permeability is not as good as that of class II drugs. Hence, the drug has both solubility and permeability limitations resulting to its incomplete absorption.

Chitosan has been employed in many drug delivery systems (Hu et al., 2013; Yin et al., 2009). It is a hydrophilic polymer with disintegrant property. Its effect on drug release from tablets is intermediate between the effect of corn starch and that of sodium starch glycolate (Ritthidej et al., 1994). It is also capable of causing temporary increase in the permeability of intestinal mucosa promoting drug permeation (Kos et al., 2008). Since the oral route requires that a drug dissolves in the gastrointestinal fluid and then penetrates the epithelial cells of the intestinal mucosa to get into systemic circulation (Ashford, 2007), the presence of chitosan in a formulation might exert an effect on both drug release from tablets and drug permeation across intestinal membrane leading to improved drug absorption.

Ciprofloxacin is the most widely used of the fluoroquinolones, though it has the lowest bioavailability (Vance-Bryan et al., 1990). Chitosan is deacetylated product of chitin, the second most abundant polysaccharide. Chitin is a major component of the shells of crustaceans which are abundantly available at all coastal regions as wastes from seafood (Olorunsola et al., 2015). Chitosan has the potential of positively influencing both drug release and permeation. This study evaluates crab shell chitosan as absorption enhancer in ciprofloxacin tablet formulation using the *ex-vivo* model. The chitosan is incorporated in the tablet formulation and the effect of the polymer on the drug release and intestinal permeability evaluated.

MATERIALS AND METHODS

The materials used were: ciprofloxacin powder (Hopkin & Williams, England), maize starch B.P. (BDH Chemicals, England), lactose (BDH Chemicals, England), acacia gum (BDH Chemicals, England),

talc (BDH Chemicals, England) and magnesium stearate (BDH Chemicals, England).

Crab shell collection and chitosan extraction

Shells of *Callinectes gladiator* were obtained from Oron, Akwa Ibom State, Nigeria. They were sun-dried for five days to remove moisture from the shells. The dried shells were crushed in a mortar and then powdered using laboratory blender (Christison, United Kingdom). Twenty five gramme sample of the powdered shell was weighed and transferred into a 250 ml capacity beaker. Chitin was obtained by deproteination with 100 ml of 4% *w/v* NaOH and demineralization with 135 ml of 1% *w/v* HCl; and chitosan was derived from the chitin by deacetylation with 100 ml of 50% *w/v* NaOH using the methods described by Olorunsola et al. (2015).

Preparation of granules

Six batches of granules were prepared using the wet granulation method based on Table 1. Each batch was prepared using 3% *w/w* acacia gum as binder and 10% *w/w* maize starch as disintegrant. Batch sizes of 50 tablets were prepared with each tablet containing 500 mg ciprofloxacin.

The weighed quantities of ciprofloxacin, maize starch B.P and lactose were dry-mixed for 5 min and then moistened with mucilage of acacia (binder). The wet mass was screened through a 2.0 mm mesh and dried in a hot air oven (Gallenkamp, Germany) at 60°C for 1 h. The dried granules were then screened again through a 1.0 mm mesh.

Preparation of tablets

The required quantities of chitosan were added to appropriate batches such that batches: CTS-0, CTS-1, CTS-2, CTS-3, CTS-4 and CTS-5 contained 0, 1, 2, 3, 4 and 5% *w/w* chitosan respectively. The chitosan was gently blended with the granules over a period of 3 min. Talc and magnesium stearate were also weighed and gently blended with the granules over a period of 3 min. The granules were then compressed at a pressure of 60 KN using a single punch tableting machine (Erweka, Germany).

Tablet evaluation

Uniformity of weight

Twenty (20) tablets from each of the batches were individually weighed using an analytical weighing balance (Mettler, Germany).

The mean weight and the standard error of the mean were calculated.

Crushing strength

The crushing strength of five tablets from each batch was determined with Mosanto hardness tester (Laboratory Tree Co., India). It was done by holding tablet between a fixed anvil and a moving jaw. The load was gradually increased until the tablet just fractured. The applied force was recorded and the mean crushing strength was calculated.

Friability

Ten (10) tablets were dusted, weighed together and then subjected to abrasion test using Roche friabilator (model TAR 10, Erweka, Germany) operated at 25 rpm for 4 min. The tablets were then dusted properly and reweighed collectively. The difference in weight was determined and the friability value was calculated. The procedure was carried out thrice.

Disintegration time

Six tablets from each batch were subjected to disintegration test in a freshly prepared 0.1 N HCl at 37°C using the BP disintegration apparatus (Erweka, Germany). The disintegration time was taken to be the time when no particle remained inside the basket of the disintegration apparatus.

In-vitro drug release

A tablet was placed in the dry basket of the U.S.P. dissolution apparatus (UNICO Shanghai Instrument, China) containing 900 ml of 0.1 N HCl thermostatically maintained at $37 \pm 0.5^\circ\text{C}$. The apparatus was set to a rotational speed of 100 rpm for 1 h. A 10 ml sample was taken at 10 min interval with subsequent replacement with equal volume of the dissolution medium. Each withdrawn sample was filtered and the absorbance was taken at 277 nm using UV spectrophotometer (UNICO Shanghai Instrument, China). Cumulative percent drug released was obtained and then plotted against time.

Isolation of the absorption tissue

The animal (pig), housed in a cross-ventilated room (temperature of $25 \pm 2.5^\circ\text{C}$) was sacrificed in accordance with internationally accepted laboratory animal use and the guidelines and rules for animal experimentation. Six portions of approximately same diameters and length of 15 cm were cut out from the small intestine of the freshly sacrificed pig and used immediately for the permeation study.

Ex-vivo permeation study

Drug permeation study was performed using the method described by Sharma et al. (2013). The segments of the small intestine were used as donor chambers. Each segment was tied at one end and filled with 5 ml of simulated intestinal fluid (pH 6.8). A tablet was introduced into each of the chambers and the segment was then tied at the other end. The donor chamber was immersed into a dissolution apparatus containing 900 ml of the simulated intestinal fluid (the receptor medium). The temperature was maintained at 37

$\pm 0.5^\circ\text{C}$ and the apparatus was set to operate for 5 h. Samples were withdrawn at 30 min intervals from the receptor medium with replacement using pure medium. The samples were diluted, filtered and the absorbance was taken at 277 nm using a UV spectrophotometer (UNICO Shanghai Instrument, China). A graph of cumulative percent drug permeated was plotted against time. The time for 50% drug permeation ($t_{50\%}$) was read from the plot. Value of steady state drug flux (J) and permeation coefficient (K_p) were calculated using Equations 1 and 2 respectively.

$$J = \frac{dQ}{dtA} \quad 1$$

where dQ is the change in the quantity of drug permeated (μg) through the membrane of surface area A (cm^2) within time dt (min). The value of dQ/dt was estimated from the slope of the straight line portion of the graph.

$$K_p = \frac{J}{C} \quad 2$$

Where, J is the drug flux and C is initial concentration of the drug in the donor compartment. The percent drug permeated in 4 h was also recorded.

Statistical analysis

Data obtained from tablet evaluation were expressed as mean values \pm standard error of the mean. Statistical analysis was done using one-way analysis of variance (ANOVA) followed by Turkey-Kramer multiple comparison test using GraphPad Instat-3 software. The differences in cumulative percent drug permeated in 4 h were explored using the Chi-square test. Significance of difference was taken at *p* - values less than 0.05.

RESULTS

The physical properties of the tablets are shown in Table 2. The crushing strength decreased with increase in concentration of chitosan. There was no significant difference in the friability values of the different batches. The disintegration time decreased with increase in the concentration of chitosan. However, the values were not significantly different. The batch containing 3% w/w chitosan had the highest (CS/FR)/DT value.

The dissolution profiles of the different tablet batches are shown in Figure 1. All the batches gave over 75% drug release within 1 h and there was no significant difference in the amount of drug released over this period.

The plot of cumulative percent drug permeated versus time is shown in Figure 2. The permeation coefficient, the time taken for 50% drug permeation and % drug permeated in 4 h are shown in Table 3. The permeation coefficient increased and the time for 50% drug permeation decreased with increase in the concentration of chitosan. The percent ciprofloxacin permeated per time increased with increase in the concentration of chitosan. The control (tablet without chitosan) gave 68.0%

Table 2. Physical properties of tablets.

Batch	Weight (mg)	Crushing strength (kgf)	Friability (%)	Disintegration time (min)	(CS/FR)/DT
CTS-0	628 ± 0.15	5.2 ± 0.75	0.36 ± 0.00	10.54 ± 0.12	1.37
CTS-1	631 ± 0.18	5.0 ± 0.63	0.34 ± 0.01	10.44 ± 0.21	1.40
CTS-2	626 ± 0.13	5.0 ± 0.89	0.33 ± 0.02	10.30 ± 0.18	1.47
CTS-3	628 ± 0.24	4.8 ± 0.56	0.30 ± 0.01	10.26 ± 0.26	1.55
CTS-4	636 ± 0.24	4.6 ± 0.56	0.31 ± 0.04	10.21 ± 0.23	1.45
CTS-5	630 ± 0.18	4.4 ± 1.20	0.33 ± 0.01	10.14 ± 0.24	1.44

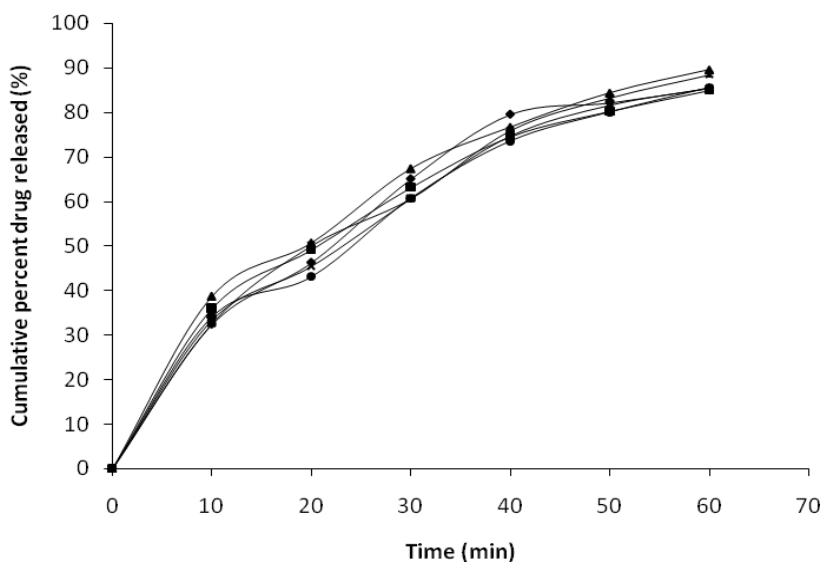


Figure 1. Plot of cumulative percent ciprofloxacin released versus time. Key: CTS-0 (♦), CTS-1 (■), CTS-2 (▲), CTS-3 (×), CTS-4(x), CTS-5 (●).

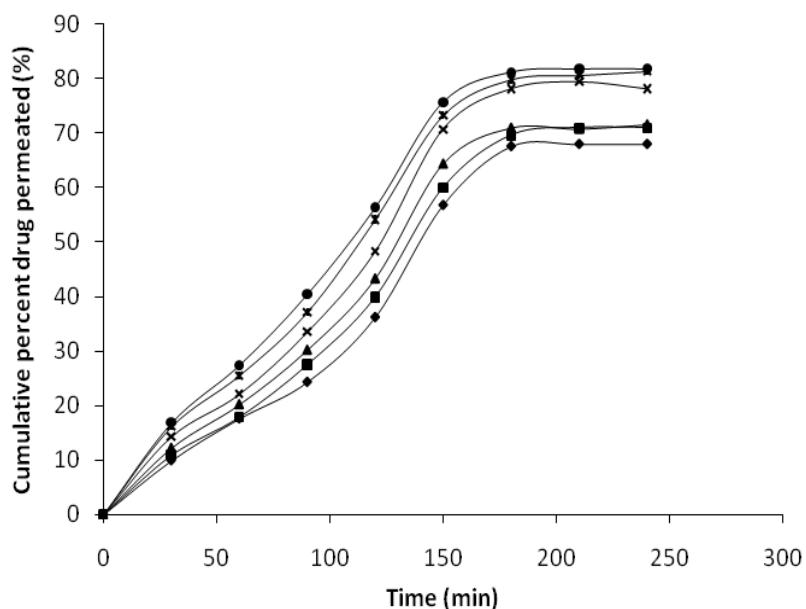


Figure 2. Plot of cumulative percent ciprofloxacin permeated versus time Key: CTS-0 (♦), CTS-1 (■), CTS-2 (▲), CTS-3 (×), CTS-4(x), CTS-5 (●).

Table 3. Permeation parameters.

Batch	Permeation coefficient (cm/s)	Time for 50% drug permeation (min)	Amount of drug permeated in 4 h (%)
CTS-0	29.22×10^{-6}	137.5	68.0
CTS-1	39.06×10^{-6}	135.2	71.2
CTS-2	46.50×10^{-6}	128.2	71.6
CTS-3	50.22×10^{-6}	122.1	78.2
CTS-4	55.32×10^{-6}	113.7	81.3
CTS-5	56.42×10^{-6}	104.8	81.8

ciprofloxacin drug permeation, CTS-1 (tablet with 1% ^w/w chitosan) gave 71.2% permeation and CTS-5 (tablet with 5% ^w/w chitosan) gave 81.8% permeation in 4 h.

DISCUSSION

Batch CTS-0 which contained no chitosan served as the control. Weight uniformity is an essential property of a tablet formulation since it guarantees the provision of a uniform dosing of medication. The variation expressed by the different batches of the tablets as shown in Table 2 indicates conformity to the standard for weight uniformity. The USP 31 - NF 26 (2008) states that not more than two of individual weight should deviate from mean weight by more than 5% and none should deviate by more than 10% for tablets of mean weight greater than 324 mg. All the tablet batches passed the test for uniformity of weights.

The normal range of crushing strength for conventional tablet is 4 - 7 kgf and the crushing strength of all the tablet batches fell within this range. The decrease in crushing strength of tablets with increase in concentration of chitosan is an indication that the presence of chitosan reduced tablet strength perhaps by decreasing the amount of plastic deformation occurring during compression (Uhumwangho et al., 2006). The test for friability of tablet measures the ability of the tablet to withstand abrasion during packaging, handling and shipping and a friability value of less than 1% is needed for a tablet to pass friability test (Alderborn, 2007). All the tablet batches passed the test and the values for the different batches were not significantly different. Hence, while chitosan decreases tablet strength in terms of hardness, it does not significantly affect tablet strength in terms of friability.

From the study, the disintegration times for all the batches conform to the standard value of 1-15 min for uncoated tablets. The decrease in the disintegration time of the tablets with increase in the concentration of chitosan is in consonance with the work of Ritthidej et al. (1994) where disintegrant property of chitosan was reported. Since tablet disintegration is the initial step of dissolution, chitosan promotes rapid onset of drug release. The crushing strength – friability – disintegration

time index [(CS/FR)/DT] provides a measure of the overall quality of tablets (Alebiowu and Itiola, 2003). The increase in this index with increase in the concentration of chitosan up to 3% ^w/w shows that this concentration produced tablets with the best quality. There was a decrease in the index with further increase in the concentration of chitosan beyond 3% ^w/w. It could be inferred that the decrease in the strength of tablet above this chitosan concentration did not lead to corresponding decrease in the disintegration time.

For a conventional tablet to pass dissolution test, at least 75% of the drug must be released within 1 h (USP 31 – NF 26, 2008). All the batches passed the test for dissolution and there was no significant difference in the amount of drug released in 1 h. The increase in percent ciprofloxacin permeated in the presence of chitosan can be linked to the ability of the polymer to increase permeability of intestinal mucosa (Yin et al., 2009). The possible mechanism employed is the opening of tight junction in the mucosa wall and also the widening of paracellular route (Sonia and Sharma, 2011). There was a continuous increase in the cumulative percent drug permeated with increase in the concentration of chitosan; with 5% ^w/w chitosan causing an increment from 68% (when no chitosan was added) to 81.8%. While the cumulative percent drug permeated in 4 h with respect to tablets containing 1 - 3% ^w/w chitosan were not significantly different from that of tablet without chitosan, the cumulative percent drug permeated with respect to tablets containing 4 and 5% ^w/w chitosan were significantly different.

This study correlates with *in-vivo* bioavailability study because the oral bioavailability of ciprofloxacin without permeation enhancer is 70% (Chambers, 2004). Hence, crab shell chitosan at concentration of 5% ^w/w could increase the bioavailability of ciprofloxacin from 70 to 82%. *In-vivo* bioavailability study can be carried out to obtain the pharmacokinetic parameters of these formulations.

Conclusion

The presence of chitosan improves the overall quality of ciprofloxacin tablet. The polymer brings about a faster

onset of drug release without significantly affecting the total drug released. It promotes the permeation of the released drug across biological membrane. This study suggests the use of crab shell chitosan at concentration of 5% ^w/_w of tablet to improve the absorption of ciprofloxacin. Further study in form of *in-vivo* evaluation is recommended to validate this conclusion.

Conflict of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Optimization of somatic embryogenesis procedure for commercial clones of *Theobroma cacao* L.

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The first objective of this study was to assess and optimize somatic embryo production in a genetically diverse range of cacao genotypes. The primary and secondary somatic embryogenesis response of eight promising cacao clones and a positive control was evaluated using modified versions of standard protocols. The second objective was to optimize the efficiency of primary somatic embryogenesis for a commercially important cacao clone, CCN 51, which has proven to be quite recalcitrant to standard protocols, relative to CCN 10, a clone also included in our analysis. The efficiency of the overall process was assessed by determining the number of somatic embryos produced per starting somatic tissue explant, as well as the quality of embryos (normal vs. abnormal) produced. Donor floral explants were subjected to five tissue culture steps, each 15-25 days in duration. Although all studied genotypes produced primary somatic embryos, most of them originated only from brown or brown-white callus. Overall, flower petals performed better than staminodes, and our best performing genotype yielded an average of 7-10 embryos produced in brown callus explants with embryogenic response during primary somatic embryogenesis procedures. In conclusions our analysis from a pilot in a small-scale are: 1) it is possible to achieve a high production of plants by somatic embryogenesis, although the efficiency is highly genotype-dependent; it is therefore necessary to optimize hormone balance and hormone type, as well as the explant type for each genotype, 2) through the use of secondary somatic embryogenesis, it is possible to increase somatic embryogenesis production at least ten-fold, and 3) the observed response variation between genotypes may reflect differences in endogenous and exogenously-supplied hormones. The importance of adapting the tissue culture protocol to the genotype is discussed.

Key words: Somatic embryogenesis, 2,4-D, cellular competence, propagation, abnormalities, ethylene, cacao.

INTRODUCTION

Theobroma cacao L. is a tropical understory tree originating from the Amazon and Orinoco valleys (Wood and Lass, 2008; Motamayor et al., 2008). Since the

Mayas domesticated the cocoa tree, cacao has become a major commodity crop cultivated in numerous tropical countries, where it represents a significant source of

income for small farmers (Franzen and Borgerhoff, 2007). Until the 1980s, Brazil had the highest cocoa bean production in South America and it was the second highest producer worldwide, with an average production of 400,000 tons per year (FAO, 2013). By the late 1980s, annual Brazilian production decreased to 291,868 tons (IBGE, 2015), primarily due to outbreaks of devastating cacao diseases, in particular witches' broom (*Moniliophthora perniciosa*), which caused massive economic loss and much social distress (Marelli, 2008). In response to this crisis, numerous government agencies and universities were enlisted to develop disease-resistant, high-yielding cacao clones (CEPLAC, 2009).

Once superior clones are developed, large-scale propagation will be required. Cacao is traditionally propagated using rooted cuttings or by grafting. The use of rooted cuttings is constrained by biology: a cacao tree has two types of branches: orthotropic (vertical growing) ones that generate trees of desirable architecture, but which are limited in number; the second type are plagiotropic (horizontal growing) ones. Although, more numerous, plagiotropic grafts produce trees of undesirable architecture that require long time and labor investments (Miller, 2009).

The solution for cacao, like other commercially important trees, is somatic embryogenesis (SE), which is a method for generating plant embryos asexually. Somatic embryogenesis relies on the ability of somatic plant cells to de-differentiate and be reprogrammed along an embryonic developmental pathway (reviewed by Fehér, 2015). Under specific tissue culture conditions, a single somatic cell (or groups of cells) can be converted into a single embryo that develops into a plant that is genetically identical to the donor plant from which the original cell was derived (Konieczny et al., 2012).

Importantly, SE affords opportunity for amplification, from a relatively small number of explants into hundreds or thousands of somatic embryos (SEs) per experiment. Although a half-century has passed since SE was first demonstrated in the model system *Daucus carota* (wild carrot) (Steward, 1958), the molecular mechanisms underlying the complex cellular reprogramming required to achieve totipotency are just beginning to be elucidated (Fehér, 2015; Mahdavi-Darvari et al., 2015). Luckily, early identification of critical parameters controlling SE induction, such as endogenous and/or exogenously supplied plant growth regulators (PGRs, for example, 2,4-dichlorophenoxyacetic acid (2,4-D)) have enabled this largely empirical science to evolve into the foundation of large-scale vegetative propagation for a large range of plant species.

Somatic embryogenesis induction and expression are

only possible if totipotent somatic plant cells can acquire the competence necessary to respond to embryogenic signals and initiate the embryogenesis process (Fehér, 2015); despite the broad success of SE methodology across the plant kingdom, within a single species, a high degree genotype-to-genotype variation in response has been observed, thereby necessitating protocol customization. Therefore, to be commercially viable in cacao, or any other species, somatic embryogenesis requires: 1) donor material with a high rate of competent cells to express the embryogenic pattern, 2) donor material relatively unlimited in supply and physically accessible, 3) genetic and epigenetic uniformity in the SEs generated, and 4) efficient conversion of SEs into plantlets.

In early cacao studies (for example, Sondahl et al., 1989, 1993), SEs were generated from a variety of somatic cell type explants, although yields were low. The first moderately successful cacao SE protocol was developed by Li et al. (1998); their exhaustive study highlights the importance of genotypic variation in SE response. Staminodes from 19 cacao genotypes were evaluated for their ability to undergo somatic embryogenesis; the diverse genotypes produced primary somatic embryos (PSEs) at very different rates. Explant response (callus growth) ranged from 1-100%, and the number of SEs per responsive explant ranged from 1-46. This basic cacao SE protocol was further optimized by Maximova et al. (2002), who developed a key method for secondary somatic embryogenesis (SSE) using cotyledon explants from PSEs.

In this study, eight diverse genotypes were tested, and from 4.8 to 24.7, secondary somatic embryos (SSEs) were generated per explant, within a 12-month SSE protocol. Yet another SE protocol (using flower petals) was developed by Lopez-Báez et al. (2001), using Murashige and Skoog 1962 (MS) salt-based induction medium that included testing of carbohydrate (sucrose, glucose and maltose), PGRs (2,4-D or 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and kinetin), in 12 diverse genotypes. Again, high genotype response variance was noted. Although the collective impression from cacao SE research indicates that an ideal, genotype-independent protocol has not yet been developed, several key efficiency determinants have been identified, including the concentration and type of sugar used in culture medium (Solano-Sanchez, 2008; Chanatasing-Vaca, 2004; Tan and Furtek, 2003). To enhance the efficiency of primary somatic embryogenesis (PSE) production and plant regeneration, we attempted to further optimize cacao SE protocols.

The overall objectives of the present study were therefore: 1) to evaluate a pilot scale the production

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efficiencies (number of embryos/explant) in both PSE and SSE procedures for eight commercial cacao clones and SCA 6 as a positive control, using a standard protocol with some modifications, and 2) we also attempted to improve the yield and quality (Increase the number of normal embryos/explant) of PSE generated from two cacao clones, Coleccion Castro Naranjal-51 (CCN 51) and Coleccion Castro Naranjal-10 (CCN 10), which are of commercial interest to Brazil. In these clones, we will use two types of somatic donor explants (petals and staminodes), and variation in glucose concentration and hormone balance.

MATERIALS AND METHODS

This study is divided into two parts. First is an evaluation of pilot scale production using a single protocol with some modifications in eight genetically diverse *T. cacao* clones (MCCS 14-056, POUND 7, TSH 1188, TSH 565, VB 1151, CCN 51, UF 613 and PS 1319) as well as Scavina 6 (SCA 6) as a control. SCA 6 is a clone that has historically performed well in a variety of somatic embryogenesis procedures (Solano-Sanchez, 2008; Chanatasing-Vaca, 2004; Maximova et al., 2002; Li et al., 1998). The second is an experiment aimed at improving a specific protocol for PSE in CCN 51 and CCN 10 clones. All the clones used in this work are from the Mars Center for Cocoa Science (MCCS) germplasm collection.

Evaluation of a pilot scale production of plantlets from nine commercial cacao clones by somatic embryogenesis

Primary somatic embryogenesis procedures

Immature cacao flowers (approximately 900 in total), 6-8 mm in length, from MCCS 14-056, POUND 7, TSH 1188, TSH 565, VB 1151, SCA 6, CCN 51, UF 613 and PS 1319 were collected in the morning in the field and kept in cold sterile distilled water (4°C), and transported to the laboratory, where they were submerged in a fungicide solution (per liter: 9 g Ridomil Gold Mz (Syngenta), 10 mL of carbendazim solution (0.1% in MeOH)) for 20 min. After rinsing three times with sterile water, samples were then surface-sterilized with 1% calcium hypochlorite for 25 min in a laminar flow hood, and again rinsed three times with sterile water. After sterilization, petals and staminodes were dissected. A total of 50 explants per Petri dish were cultured using the protocol for PSE published by Li et al. in 1998 (standard), with some modifications.

The PSE modified protocol follows four steps, while the standard protocol follows three steps. In the modified protocol, primary and secondary callus growth steps were done in the same way as in the original protocol. Primary Callus Growth (PGC) medium, which is comprised of 1X Driver and Kuniyuki Walnut Basal Salt (DKW) plus vitamins (Driver and Kuniyuki, 1984) supplemented with 2 mg L⁻¹ 2,4-D and 5 µg L⁻¹ thidiazuron (TDZ), 250 mg L⁻¹ L-glutamine, 200 mg L⁻¹ myo-inositol, and 20 g L⁻¹ glucose. The DKW vitamin stock solution yielded final concentrations of 1 mg L⁻¹ nicotinic acid and 2 mg L⁻¹ glycine, 0.1 g L⁻¹ myo-inositol, 2 mg L⁻¹ thiamine-HCl. The pH of the solution was adjusted to 5.8 and supplemented with 2 g L⁻¹ phytigel prior to autoclaving. After two weeks of culture at 27±2 °C in the dark, explants were transferred to Petri dishes containing secondary callus growth medium (SCG), comprised of 1X woody plant medium (WPM) salts (Lloyd and McCown, 1980) and 1X Gamborg's vitamin solution (Gamborg, 1966), 2 mg L⁻¹ 2,4-D and 50 µg L⁻¹ 6-benzylaminopurine (BAP), 20 g L⁻¹ glucose, plus 2 g L⁻¹ phytigel, pH 5.8. After a further two weeks of culture, calli were transferred to embryos development-4 medium (ED4) for another

two weeks and finally to embryos development-3 medium (ED3) for eight weeks. ED4 and ED3 media are comprised of 1X DKW salts and vitamins, supplemented with 40 g L⁻¹ or 30 g L⁻¹ sucrose, respectively, 2 g L⁻¹ phytigel and pH 5.8. ED3 and ED4 cultures were 8-10 weeks maintained in darkness at 27±2°C until embryos were about 1 cm in length but they are not reached their maturity. Embryo Development medium both ED4 and ED3 are a modification from ED medium used in Li et al. (1998) protocol. The modifications are in the carbohydrate source. In our protocol, we used 40 g L⁻¹ and 30 g L⁻¹ of sucrose in ED4 and ED3 medium, respectively, instead of 1 g L⁻¹ of glucose and 30 g L⁻¹ of sucrose as it is used in ED medium in the original one. The other components in the medium are the same that are used in the original protocol. Data were collected every 14 days between each transfer during the ED3 culture steps. At the end of the procedure (two to three months of ED3 culturing) the total and average numbers of embryos produced per explant were calculated for the statistical analysis. The PSEs that were obtained in this step we used as a source of explants for SSEs production in a pilot scale.

Secondary somatic embryogenesis procedures

Secondary somatic embryogenesis procedures were initiated using the original protocol established by Maximova et al. (2002) with some modifications. Basically, the Maximova's protocol follows the same steps as Li et al. (1998) protocol with the difference that there is not primary callus growth step, and the initial explants are cotyledons from PSEs instead of flowers parts (petal or staminodes). The embryos development step is the same for both protocols mentioned before. In our protocol the modifications are only in embryos development step where we have used ED4 and ED3 instead of ED medium. Cotyledons from PSEs with ~1 cm in length and light yellow in color were used as donor explants.

Approximately 1,080 PSEs were source of explant to the aim to produce 10,000 SSEs in this pilot scale production (roughly 120 PSEs per genotype). The cultures for pilot scale production were done in three batches per year (40 PSEs per each genotype were used to start the each batch of production). They were cut with a scalpel into small pieces (~30 pieces/cotyledon; each square piece measured approximately 1 mm² on a side), and introduced into SCG for two weeks to induce callus formation (two cotyledons per Petri dish); during this step two different cytokinins were tested: kinetin (300 µg L⁻¹) or BAP (50 µg L⁻¹). Half of the initial explants were treated with kinetin and the other half with BAP. Callus formed in SCG were transferred to ED4 medium for two weeks and then to ED3 medium for more 12 weeks, for embryo development step until SSEs formed and matured (as the same way in the PSE procedure mentioned before). It typically required 12-14 weeks of dark incubation at 27 ± 2°C for SEs emerge and reach their maturation. SEs was deemed mature when their leaves developed pink and purple pigmentation, their stems were white and a radicle has started to show up. These features reflect the ability of the embryos to store substances required for subsequent adaptation and germination.

Efficiency of embryogenesis calculation

Our objective was to determine the efficiency of embryogenesis (EE) values for nine cacao genotypes in both PSE and SSE protocols in pilot scale. Genotypes were compared in their relative responsiveness to SE procedures by determining the ratio of the number of SEs produced at the end of an experiment to the number of initial explants in each experiment (Maximova et al., 2002). This ratio, termed the EE or efficiency index, is a measure of the net efficiency of a given clone to the numerous, sequential culture steps and conditions.

For PSE procedures, 50 explants per Petri dishes were used in *EE* value calculations from a total of 1000 explants (500 petals and 500 staminodes) per each genotype (20 Petri dishes as experimental units) and the *EE* is calculated from the ratio: number of PSEs produced/number of donor explants. Whereas for SSE procedures, the *SSE EE* is calculated from: the number of SSEs produced/number of PSEs used as source of donor explants. Approximately, 120 Petri dishes were used in *SSE EE* value calculation from a total 120 PSEs as explants (two cotyledons per Petri dish) per each genotype (1,080 Petri dishes as experimental units). Data from PSE and SSE for these experiments were collected every two weeks for up to 28 weeks after culture initiation from petals or staminodes. At the end of primary and secondary embryo production (seven to eight months of culture), the total and average numbers of embryos produced per explant in PSE and SSE was calculated for statistical analyses.

Conversion and regeneration of plants

After incubation for about 12-14 weeks in ED3 medium, SSEs showed morphological characteristics of mature embryos, and were transferred to embryo development in light (EDL) medium for 12 weeks at $27 \pm 2^\circ\text{C}$ under a 16-h light/8-h darkness photoperiod regimen, in which the photosynthetically active radiation (PAR) used ranged from 50 – 190 $\mu\text{mol m}^{-2} \text{s}^{-1}$ during the light phase. EDL medium is the same Primary Embryo Conversion (PEC) medium from cacao tissue culture protocol book (Young et al., 2003) that we adapted to our protocol. EDL medium is comprised of 1 X DKW salts plus 1 X vitamins supplemented with 20 g L^{-1} glucose, 0.3 g L^{-1} KNO_3 , 45.65 mg L^{-1} L-lysine, 32.80 mg L^{-1} L-leucine, 51.05 mg L^{-1} L-tryptophan, 43.55 mg L^{-1} L-arginine, 18.76 mg L^{-1} glycine, 2 g L^{-1} phytigel, at pH 5.8. The modification in this medium was only in the carbon source, where we have used 20 g L^{-1} glucose unlike the PEC that use 20 g L^{-1} glucose and 10 g L^{-1} sucrose. Embryos were maintained in EDL medium until they developed into plantlets with 2-3 leaves, after which they were transferred to a modified version of EDL medium: half-strength of DKW basal salts, 1 X DKW vitamins, 0.3 g L^{-1} KNO_3 , 3.0 mg L^{-1} indole-3-butyric acid (IBA) and 1.8 g L^{-1} phytigel at pH 5.8 with the aim to develop roots. Data were collected every three to four months until the plantlets had 4-5 sets of permanent leaves and a well-developed root system. Plantlets from SSE take seven to eight months to get ready for acclimatization since cotyledons from PSSEs are introduced in SSE process.

Plant acclimatization

Plant acclimatization was achieved by incubating individual plantlets in plastic cone tubes (290 cm^3 in volume) covered with polyethylene bags in a greenhouse with environmental conditions of 70% of shading in the first two months, then 50% of shading for the remainder of the process, a temperature range of $29 \pm 5^\circ\text{C}$ in the day and $19 \pm 5^\circ\text{C}$ at night. Lighting was provided for 12 h per day. Data were collected every three months until the plantlets became acclimatized. Three-month-old plantlets with 4-6 leaves were washed with sterile distilled water to remove residual phytigel; their roots were then submerged in a 0.2 mg L^{-1} solution of Derosal fungicide followed by a two-minute immersion in IBA (6 g L^{-1}). Plants were then transferred to pots containing substrate comprised of: 1 part coconut fiber, 1 part *Holambra* (organic substrate) and $\frac{1}{4}$ part of perlite. During acclimatization, plants were watered every 30 days with 1X modified Hoagland solution (Hoagland and Arnon, 1950). The solution was modified in the micro-nutrients stock salt solution, where Manganese (II) Sulfate Monohydrate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), zinc sulfate Heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) and sodium molybdate dihydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) concentration were increased (from

1.690, 2.875 and 0.404 g L^{-1} to 6.760, 5.750 and 0.605 g L^{-1} , respectively). The plants were completely acclimatized with three months in the greenhouse.

Primary somatic embryogenesis protocol optimization in two commercial cacao genotypes

The PSE protocol developed by Li et al. (1998) used in the first section of this work was adjusted. The objective of this experiment was to develop an improved protocol for PSE in CCN 51 and CCN 10 clones by optimizing hormone balance and glucose concentrations in PCG medium. The experiments comprised a factorial design with five independent variables as follows: two explants (petals and staminodes) X two clones (CCN 51 and CCN 10) X five 2,4-D concentrations (0.25 - 2 mg L^{-1}) X five TDZ concentrations (0.003 - 0.009 mg L^{-1}) X five glucose concentrations (20 - 80 g L^{-1}). A total of 500 treatments with three replicates per treatment were done. Each replicate was comprised of 25 explants (25 petals or staminodes per Petri dish), and the experiment was repeated two times. Data were collected every 14 days (at the ED3 transfer steps). At the end of the embryos production (three to four months of culture in ED3), the total and average of embryos (normal and abnormal) produced per explant was calculated for the statistical analysis. The procedures and environmental conditions in this section are the same that were used in PSE procedures in the first section. The rest of PCG medium components were the same as the original protocol.

Statistical analysis

In the first part of this work, the differences among the means of the total number of PSEs and SSEs produced per explant were analyzed for nine genotypes. We compared the means for the number of total PSEs produced per embryogenic explant (only calli with embryos formation) from staminodes versus petals, and total SSEs produced per PSEs as initial explant as a function of cytokinin type. The *EE* was calculated from the mean of the counting the number of PSEs produced per initial explant (per Petri dish as an experimental unit), and the mean of the total SSEs produced per PSEs as initial explant (using one PSE (cotyledons) per Petri dish as an experimental unit) from three batches of production. *EE* (as an efficiency index) is a decimal value where the value embryos number with relation of total explants used for all the experiment with either calli formation or not. Data were analyzed using descriptive statistics, calculating means and variances. The interval of confidence was calculated with respect to the mean with 5% of error.

In the second part, data were processed using deviance analysis for completely randomized design with five independent variables. The dependent variables observed were the numbers of normal and abnormal embryos counting in a fixed interval of time. A generalized Poisson model was adapted to link function to estimate the number of normal embryos produced in function at the best treatment in combinations of: hormone balances, carbohydrate concentration and the types of explants used for each genotype. Both, first and second section analyses were performed with the aid of R 3.2.5 software.

RESULTS

Evaluation of a pilot scale production of plantlets from nine commercial cacao clones by somatic embryogenesis

After eight weeks in culture, both petal and staminodes

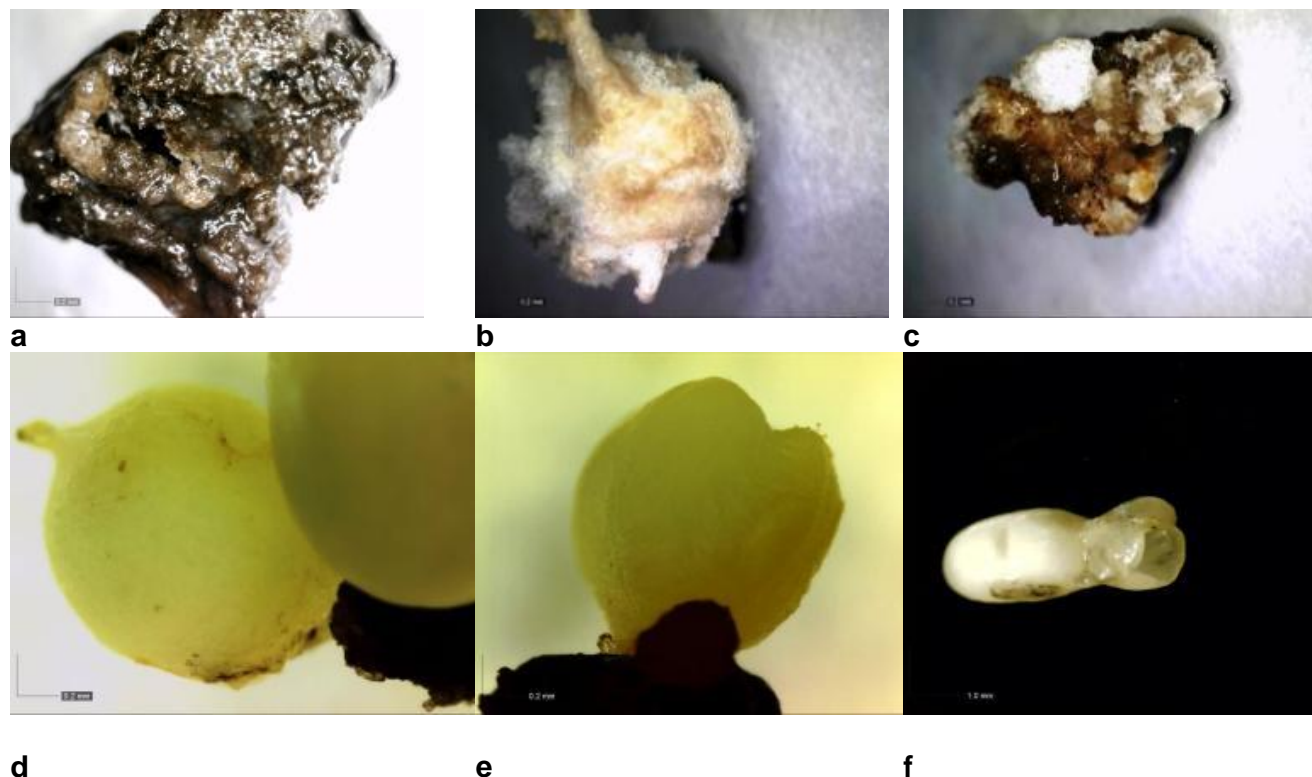


Figure 1. Three callus types in *T. cacao* somatic embryogenesis (a) Brown callus, (b) White callus, (c) Mixed Brown/White callus. SSE stage: globular (d) heart (e) and torpedo (f).

explants formed callus in most genotypes. During the ED3 culture steps, explants developed into three distinct types of callus: brown, white and mixed (brown and white). Over time, brown callus becomes coated with a dark brown material (oxidized phenolic compounds) that covers the entire explant surface (Figure 1a); whereas white callus has a compact, uniform dry appearance (Figure 1b); mixed callus has both brown and white areas (Figure 1c).

Each callus type (Figure 1a to c) was subsequently evaluated for embryogenic response. Once callus had formed, PSEs began to appear, and developed through three distinct developmental stages: globular, heart, and torpedo (Figure 1d-f). All genotypes produced PSEs, the highest number of which developed from brown or brown-white callus, for both petal bases and staminodes. Petals had the best overall performance compared with staminodes; the average of number of callus exhibiting and embryogenic response ranging from 20-70% (data not shown). MCCS-14-056 had a better response in petal bases, with an average of 7-10 embryos produced per brown callus with embryogenic response. For staminodes, Pound 7 was the most productive, with an average of six embryos produced per each brown-white callus with embryogenic response. As expected from published studies (Maximova et al., 2002), the positive control SCA 6 gave good results in petals, producing six embryos per

each brown-white callus with embryogenic response. It should be noted that all embryos produced from brown-white callus originated from the brown portion of the mixed callus. The overall results are shown in Figure 2; values are given as mean \pm interval of confidence with 5% of error.

Secondary somatic embryogenesis response

To evaluate the production of embryos in the SSE procedure, we assessed callus formation and SE production using PSE-derived explants. Specifically, we first counted the three different callus types in secondary embryogenesis under culture conditions that used two different cytokinins (BAP and kinetin). BAP induced a higher overall response in callus formation for most (77 %) clones (data not shown). Only TSH 1188 and UF 613 showed a (slightly) higher response with kinetin compared to BAP (data not shown). For SSE, we calculate production of embryos per responsible explant (brown or brown-white calli). All clones had a high proportion of brown calli, irrespective of the cytokinin used. For clones TSH 1188 and TSH 565, however, only small amounts of white and mixed callus formed. Analysis of SSE production as a function of callus type and cytokinin revealed that large numbers of embryos

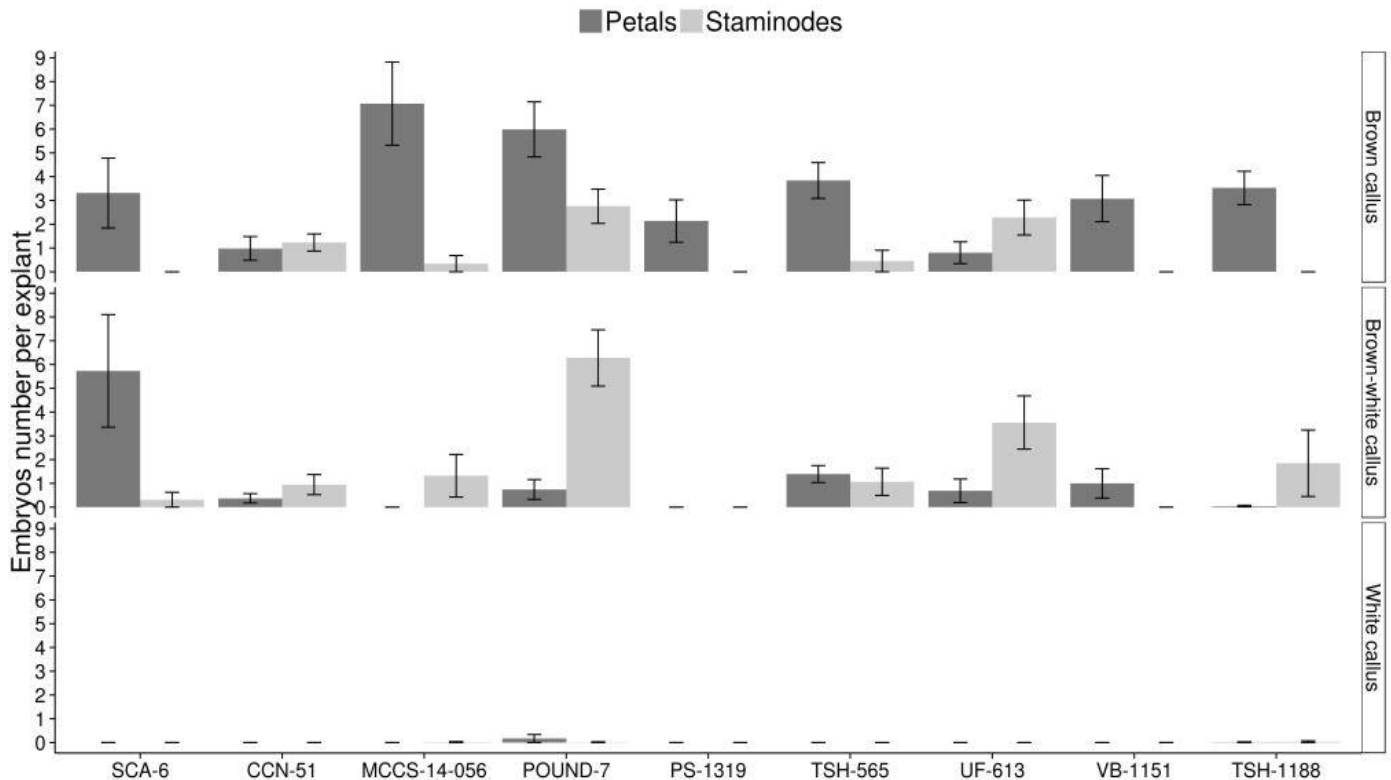


Figure 2. Average number of PSEs produced by each callus type and explant type that had response to the treatment (petals and staminodes) for the nine studied genotypes. Values are given as mean \pm interval of confidence with 5% of error.

can be produced on brown callus, in all genotypes. TSH 1188 and UF 613 showed better response with kinetin; whereas SCA-6 had the highest embryo production in BAP-containing medium (Figure 3).

Embryo efficiency in primary and secondary somatic embryogenesis

As shown in Figures 4 and 5, SSE production was higher than PSE production for all clones studied. An average of almost 10-fold enhancement of SE production was achieved using SSE compared with PSE procedures (Figure 5a, b); thus, each PSE with two cotyledons cut into pieces has the capacity to produce between 5-58 SSEs (Figure 4b). In contrast, petals or staminodes can produce 1-5 embryos per embryogenic explant (Figure 4a). TSH 1188 and UF 613 generated the largest numbers of embryos, with *EE* values of 12 and 58, respectively in SCG medium supplemented with kinetin (Figure 4b). The remaining clones responded better to BAP, with an average *EE* of 19 embryos per PSEs (Figure 4b). Secondary embryos with good maturation/morphology were able to convert efficiently into plantlets (Figure 5c, d) that developed 4-6 leaves prior to acclimatization.

Plant acclimatization

A total of 4,912 in vitro-generated plants were greenhouse-acclimatized using protocols developed at MCCS, from 9,000 SSE-derived plantlets. Clones MCCS 14-056 and Pound-7 had the highest acclimatization success rates (99.6 - 94.4% respectively); whereas other genotypes were lower: TSH 565, VB 1151, SCA 6 and PS 1319 had 35.8, 31.0, 11.2 and 8.3% acclimatization success rates, respectively. Table 1 shows the percentages of acclimatized plants with respect to total production per year from nine cacao genotypes. In total, 4,088 (45.43%) plants were lost, and in mean 54.57% plants were successfully acclimatized.

Primary somatic embryogenesis protocol optimization in two commercial cacao genotypes

Two commercial clones (CCN 10 and CCN 51) were used to study the effect of genotype, hormone balance and glucose concentration on PSE production. For both genotypes, PCG medium supplemented with glucose (80 g L⁻¹) had the best response in most treatments with various hormone combinations using petals as explant source. The other treatments using staminodes as

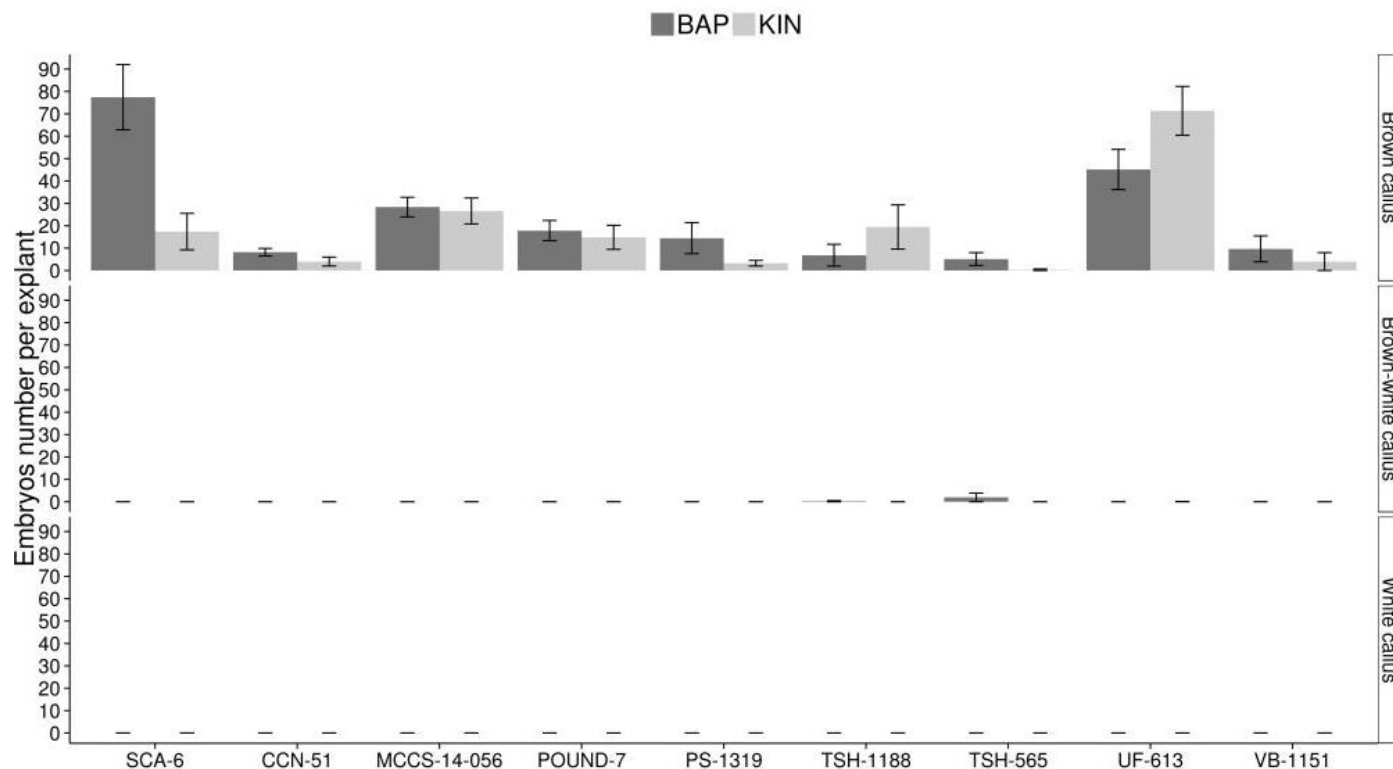


Figure 3. Average number SSEs formed from cotyledons as a function of cytokinin type. Values are given as mean \pm interval of confidence with 5% of error.

explants and glucose concentration inferior to 80 g L^{-1} were discarded in the statistical analysis, because there was not embryogenic response. Figure 6 is a graphic representation of embryogenic efficiencies of these experiments. Values (SEs per explant) for CCN 10 ranged from ≥ 10 (dark red) to ≤ 3.0 (dark blue); whereas CCN 51 values ranged from ≥ 3.0 (dark red) to ≤ 1.0 (dark blue). Production of SEs for CCN 10 was optimal (10 SEs per explant) with 0.25 mg L^{-1} 2,4-D plus $3.0 \mu\text{g L}^{-1}$ TDZ; whereas CCN 51 was (3 SEs per explant) with 2.0 mg L^{-1} 2,4-D with $3 \mu\text{g L}^{-1}$ TDZ; only 12.5% of SEs were normal for CCN 10, and 60% for CCN 51 (Supplementary Tables 1 and 2). When 2.0 mg L^{-1} 2,4-D and $5.0 \mu\text{g L}^{-1}$ TDZ were used in PCG medium, using an original protocol by Li et al. (1998), in CCN, 51's staminodes had higher production of embryos than petals, although both explant types had low indices (10 PSE/100 callus) (Figure 2).

Influence of hormone ratios in abnormal and normal morphologies

The final range of morphology (proportions of normal and abnormal) of the SEs produced (for both genotypes) was influenced by individual hormone treatments (Supplementary Tables 1 and 2). Figure 7 shows the

proportions of normal SEs, ranging from high (with values 1.0) to low (value of 0). For CCN 10, the highest proportion of normal SEs (five to seven normal SE/callus, respectively) was achieved with 2.0 mg L^{-1} 2,4-D in combination with 7.0 or $9.0 \mu\text{g L}^{-1}$ TDZ and 0.5 mg L^{-1} 2,4-D with $9.0 \mu\text{g L}^{-1}$ TDZ (Figure 7a). CCN51 had the best proportion (value 1.0) of normal SE using 0.25 mg L^{-1} 2,4-D with 4.0 or $9.0 \mu\text{g L}^{-1}$ TDZ, (1-3 SE per callus) where one petal had the capacity to form callus (Figure 7b). We had two objectives for the experiments summarized in Figure 6. The first was to identify specific, optimal PGR levels/balance necessary to achieve efficient SE production in the genotypes under study. The second was to determine if we could discern specific effective PGR ranges from the comprehensive analysis of the matrix of 25 conditions tested for each genotype. Although the first aim was achieved for both genotypes, the patterns generated in Figure 7 were unexpectedly complex; with numerous discontinuities of values (more continuous gradients were expected). Possible explanations for this include a sampling size that is not large enough to overcome experimental and/or biological variation. Such variation could also reflect variation of endogenous auxin levels in the explants, perhaps due to flower position within a tree (Tan and Furtek, 2003), or to environmental/seasonal fluctuation over the course of the extended flower collection times required for this large-

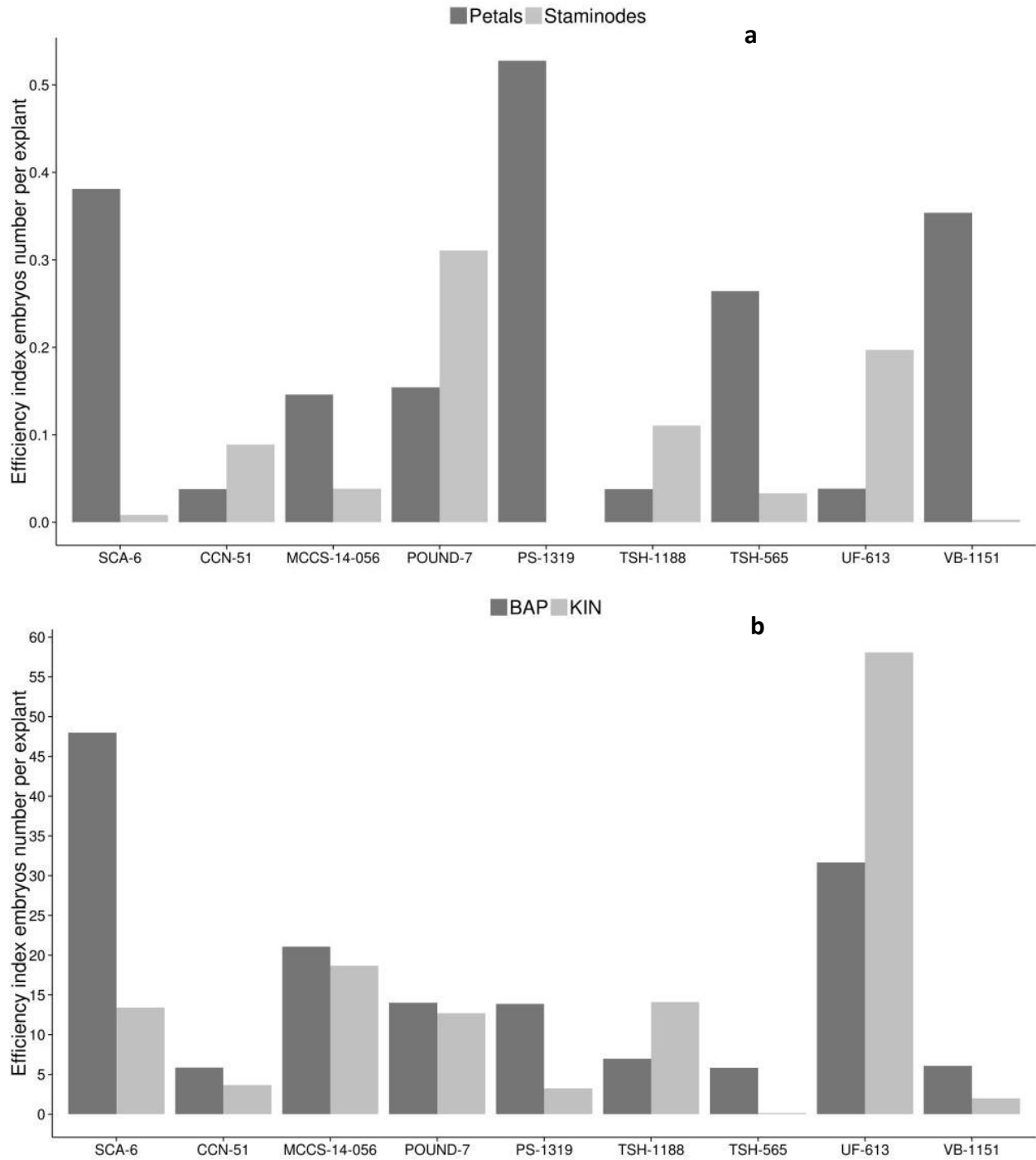


Figure 4. (a) Embryogenic efficiency index of primary somatic embryogenesis as a function of explant type (b) Embryogenic Efficiency Index of secondary somatic embryogenesis as a function of cytokinin type.

scale experiment.

DISCUSSION

Development of an efficient tissue culture-based cocoa

propagation system is crucial for the much-needed large-scale dissemination of elite *T. cacao* genotypes; however, existing protocols show high genotype-to-genotype variation in SE production rates (Maximova et al., 2002; Li et al., 1998; Lopez-Báez et al., 1993). This technical hurdle must be overcome through development

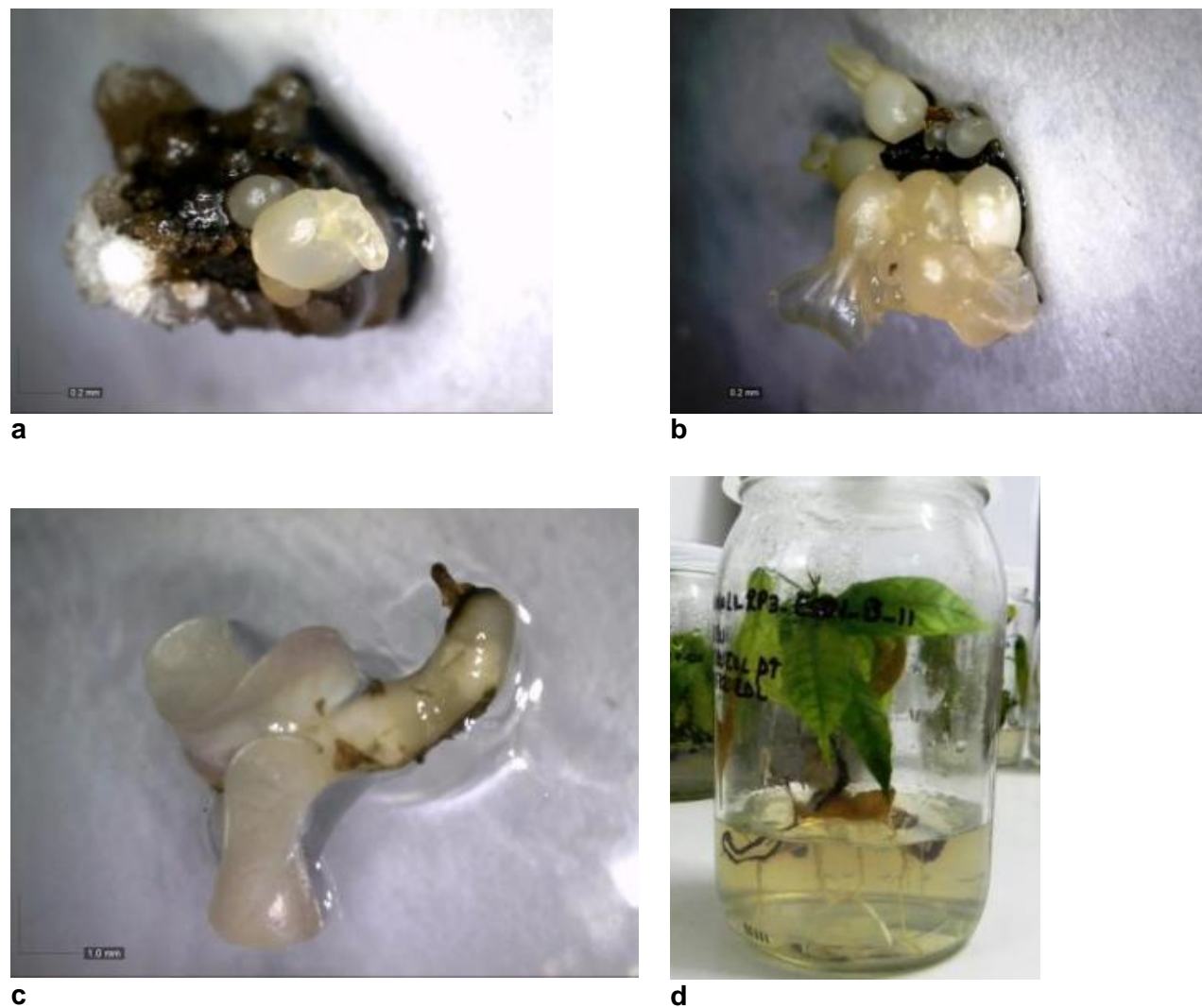
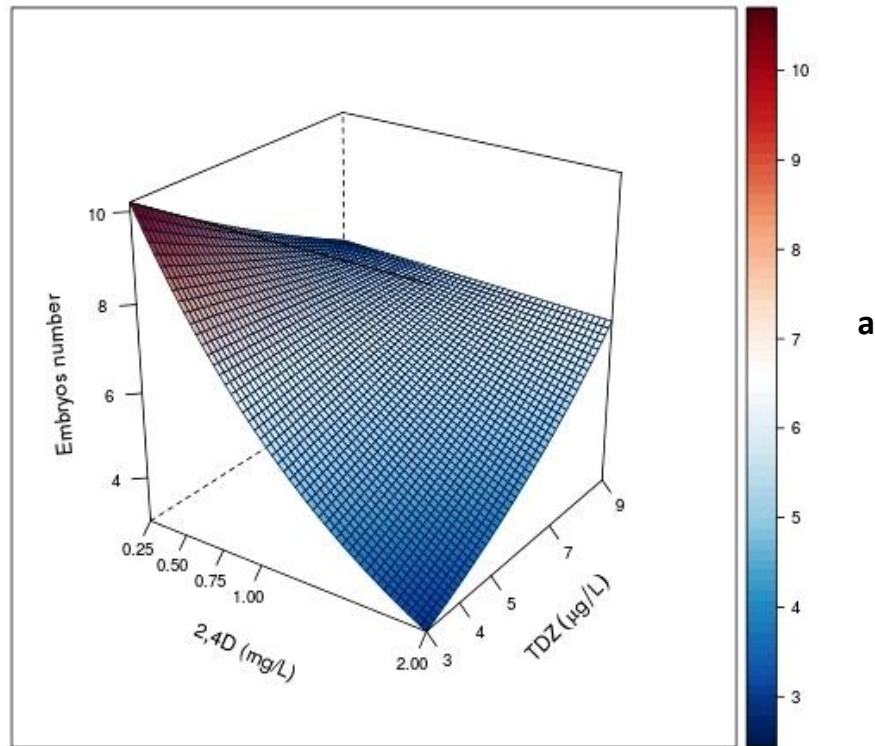


Figure 5. Somatic embryogenesis production response of brown callus in PSE and SSE: **(a)** Primary brown callus with PSEs and **(b)** secondary brown callus with SSEs. Germination and plant conversion of the SSEs. **(c)** Mature embryo and **(d)** young plantlets in EDL medium.

Table 1. Percentage of plants for each genotype produced in the propagation process in 14 months, the reference total (4,912) is the total number of plants that survived the production process. All the genotypes were cultured in parallel over a 36-week culture period. The production of SSE started with an average of 40 PSEs per clone as the source of explants in three batches.

Genotype	Total plants acclimated	Total plants produced	Percentage of survival (%)
CCN 51	398	500	79.6
MCCS 14056	1494	1500	99.6
POUND 7	755	800	94.4
PS 1319	58	700	8.3
SCA 6	223	2000	11.2
TSH 1188	282	700	40.3
TSH 565	143	400	35.8
UF 613	1435	2000	71.8
VB 1151	124	400	31.0
Total	4,912	9,000	\bar{X} 54,57

CCN 10



CCN 51

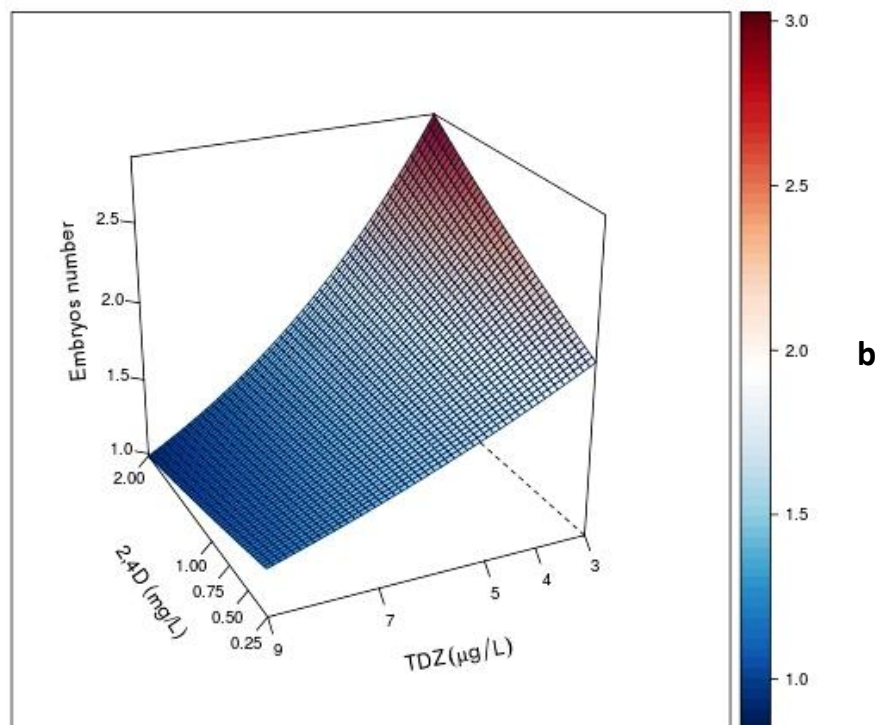
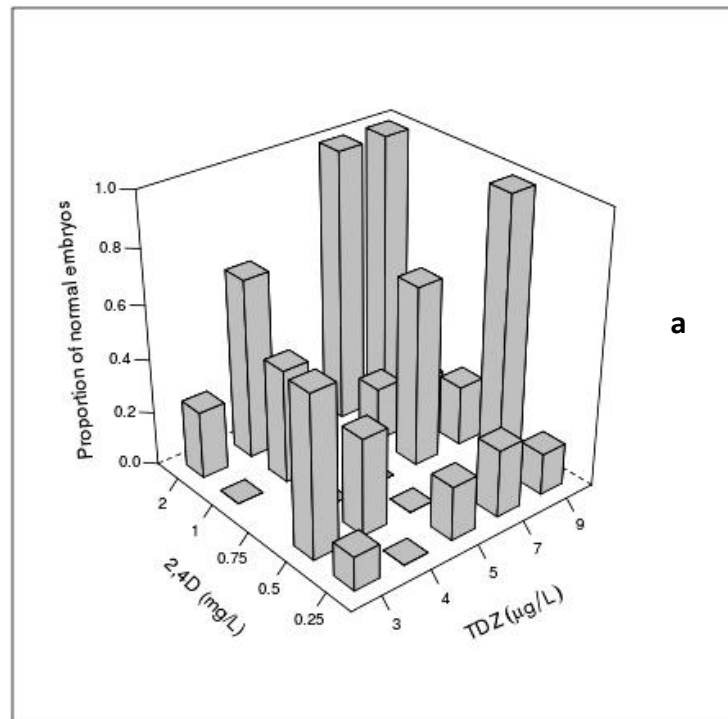


Figure 6. Total number of embryos produced as a function of hormone balance in petal explants using PCG medium with glucose 80 g L^{-1} . (a) PSE production in CCN 10 (b) PSE production in CCN 51.

CCN 10



CCN 51

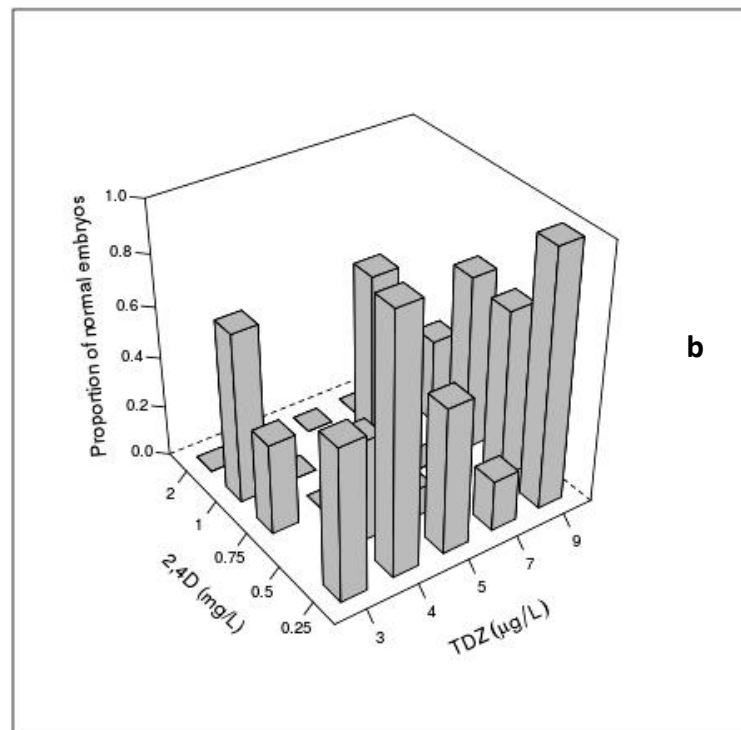


Figure 7. Proportion of normal SE embryos obtained as a function of hormone balance in petal explants using PCG medium with glucose 80 g L⁻¹. (a) PSE proportion of normal embryos in CCN 10. (b) PSE proportion of normal embryos in CCN 51.

of optimized general procedures, which was the goal of our study. For all genotypes examined in our experiments, SSE protocols (using PSE explants) proved to be more productive than PSE protocols (using floral explants). This difference could be attributed to the fact that, within a given explant, cotyledonary cells have more competition than the presumably smaller populations of competent cells in petals or staminodes.

Alternatively, a higher proportions of brown callus in the PSE-derived cultures might explain higher SE generation in SSE protocols, possibly due to larger populations of competent cells. The observed genotypic differences in SE productivity response likely reflect genetic variation in concentration/type of endogenously produced compounds (for example, polyamines, ethylene, phenolic compounds, auxin etc.), or to synergy of endogenously-expressed PGRs with medium-supplied PGRs (Niemenak et al., 2012; Lu et al., 2011; Li et al., 1998; Hatanaka et al., 1995). Additional variation in donor flower bud size, and/or the physiological stage might also underlie the observed performance variation (Batista et al., 2002).

Two general classes of SE procedures have been developed: direct and indirect. SE production via callus intermediate is a type of indirect somatic embryogenesis that has been achieved in numerous species (Ikeuchi et al., 2013). Embryogenic callus is typically produced from embryos or other immature tissue, such as flowers, leaves or microspores (Ahmed et al., 2013; Maximova et al., 2002). Three distinct callus types have been reported (Hoffmann et al., 1990). The first is light yellow in color, with a compact globular or nodular structure comprised of highly compacted small isodiametric cells. The second is a straw-colored, irregular callus consisting of loosely arranged cells with a semi-translucent appearance. The third type is also irregular in structure, and consists of extremely loose callus tissue containing giant translucent cells that are long, tubular and non-morphogenic. The second type, which is made up of aggregates of cytoplasmic-rich cells has been proposed to contain the so-called embryogenic units, the numbers of which reflect the embryogenic potential of this callus (Bajaj, 1995; Franz and Schel, 1991).

The callus types described above are similar to those that we observed in *T. cacao* in our study: the first type is white callus, which is compact with a light yellow-white color; white callus never produces embryos. The second type is brown callus, an embryogenic callus that is similar to that described by Bajaj (1995); globular-stage embryos begin to arise at eight weeks of culture from brown callus, which contains small nodular clusters with lower proliferation rates. The third callus type that we encountered is brown-white callus, characterized by high cell proliferation, but which also contains sections of white callus; SEs only develops from the brown portions of mixed callus.

To optimize SE procedures for cacao, it is necessary to elucidate the biological processes underlying SE

production. Numerous abiotic stressors have been demonstrated to impact somatic embryogenesis induction and subsequent development (Fehér, 2015; Jin et al., 2014; Karami and Saidi, 2010); such stressors include high sucrose concentrations, polyethylene glycol, abscisic acid, dehydration, water stress, heavy metal ions, pH, heat or cold shock treatments, hypoxia, antibiotics, ultraviolet radiation and mechanical treatment. In our experiments, SE response in *T. cacao* also appears to be influenced by the concentration of glucose in the induction medium (PCG); one explanation for this is that high sugar concentrations might induce osmotic stress, which in turn could enhance reprogramming cells to an embryogenic pathway (Fehér, 2015).

To achieve proper embryo development during SE procedures, auxin-related compounds must be omitted from tissue culture medium at some point to permit establishment of cell/tissue polarity; this is because, once dedifferentiation and totipotency have been achieved, polar auxin transport is a key step in meristem formation during subsequent embryo development (Nawy et al., 2008; Jiménez, 2005). In zygotic embryogenesis, polarity of cell division in the body axis is already apparent from the asymmetric division of the zygote (into a small apical and a large basal cell). This pivotal event is also presumed to occur in the somatic pathway of embryogenesis (Jiménez, 2005).

When 2,4-D is added to the SE induction medium, it increases indole acetic acid (IAA) concentration in the donor tissue and initiates reprogramming of genes that is mediated by DNA methylation and/or chromatin remodeling (Pasternak, 2002). In our study, depending on the concentration of 2,4-D added to the PCG medium, the percentage of abnormal SEs varied; this phenomenon might be related to endogenous IAA levels, which would be expected to impact SE morphogenesis (Leljak-Levanic et al., 2015; Abrahamsson et al., 2012).

In conclusion, our first set of experiments demonstrate that SE production is influenced by genotype and source of donor explant, PGR type and balance and sugar concentration; the nine genetically diverse genotypes tested showed different rates of PSE and SSE production. Flower petals, not staminodes, had the best response in PSE production for most of the tested clones; overall though, production of SSEs proved to be the more productive approach. Propagation of cacao by somatic embryogenesis in large-scale is possible, but first is necessary to know the embryogenic response in genotypes with commercial interest applying an efficient protocol. In consequence, it will be important to do the adjustments of the protocol for new clones without preliminary information.

Primary somatic embryogenesis is one of the principal problems to produce cacao plants in large-scale. Different studies in production of PSEs using petal and staminodes show low percentage of success including our study, even if it is varied the type and concentration of PGR.

Either the low or lack of production of PSEs per initial explant can increase the costs in large-scale production requiring high number of initial explant to start the cultures. It is important to look for new source of explants to make the PSE process more efficient. Secondary somatic embryogenesis in our work shows better results than PSE using solid medium. Next experiments might focus in production in large-scale using liquid medium and bioreactors bigger than 5L for SSE mass production because it might increase the efficiency. In the literature, there are few publications in this theme using bioreactors with not more than 1 L of capacity with good results but it is necessary to improve this process for large-scale (Niemenak et al., 2008).

For the experiments that focused on CCN 51 and CCN 10, SE production was highest using a high concentration of glucose in the induction medium (80 g L⁻¹). As discussed above, high glucose in the medium might function as a biological stressor that synergizes with 2,4-D and/or TDZ, to enhance the embryogenic response in SE procedures. In addition to the quantity of SEs produced, SE quality (normality vs. abnormality) also appears to be impacted by the balance of 2,4-D and TDZ in the PCG medium. CCN 51 produced more normal SEs with 0.25 mg L⁻¹ 2,4-D with 4.0 or 9.0 µg L⁻¹ TDZ; whereas CCN 10 was more normal SEs productive with 2.0 mg L⁻¹ 2,4-D in combination with 7.0 or 9.0 µg L⁻¹ TDZ.

Importantly, SE morphogenesis, like embryogenic induction, appears to be influenced by the balance of applied and endogenously-produced PGRs (Jiménez, 2005). Thus, treatments that produce high numbers of SEs per explant have a low proportion of normal SEs; whereas treatments that produce low numbers of SEs per explant have high proportion of normal embryos. Although we identified optimized culture conditions for two genotypes (CCN 10 and CCN 51), and starting conditions for many other clones, our analysis suggests that somatic embryogenesis is a complex, multifactorial process that may be compounded by the fact that the floral explants used were produced from outdoor, greenhouse-grown trees. Thus, environmental as well as phenological variation may contribute to the response complexity uncovered in our analyses (for example, Figure 7).

Any variation in endogenous auxin levels would likely impact embryogenic capacity, and further compound underlying genotype-to-genotype variation in response. The relationship between phenology and somatic embryogenesis response has been studied, and suggests that both flowering and fruiting levels can seasonally impact somatic embryogenesis (Issali et al., 2009).

Conflict of interests

The authors have not declared any conflict of interest.

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Abbreviations

2,4-D, 2,4-Dichlorophenoxyacetic acid; **2,4,5-T**, 2,4,5-trichlorophenoxyacetic acid; **BAP**, 6-benzylaminopurine; **TDZ**, thidiazuron; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **SE**, somatic embryogenesis; **SEs**, somatic embryos; **PGRs**, plant growth regulators; **PSE**, primary somatic embryogenesis; **PSEs**, primary somatic embryos; **SSE**, secondary somatic embryogenesis; **SSEs**, secondary somatic embryos; **PGC**, primary callus growth; **SCG**, secondary callus growth; **ED4**, embryos development-4; **ED3**, embryos development-3; **ED**, embryo development; **EE**, efficiency of embryogenesis; **EDL**, embryo development in light; **PEC**, primary embryo conversion.

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Supplementary Table 1. CCN 10. Total number embryos produced as a function of hormone balance (relation 2,4D/TDZ) in petals explant using PCG medium with glucose 80 g L.

Concentration TDZ ($\mu\text{g L}^{-1}$)	Concentration 2,4-D ($\mu\text{g L}^{-1}$)	Rate 2,4D/TDZ	Average of embryos per explant	Average of normal embryos per explant	Normal embryos (%)
3	250	83	10.0	1.2	13
3	500	167	8.5	5.1	60
3	750	250	7.0	0.0	0
3	1200	833	5.0	0.0	0
3	2000	667	3.0	1.0	25
4	250	63	9.5	0.0	0
4	500	125	8.0	3.0	36
4	750	188	7.0	0.0	0
4	1200	300	5.5	2.3	42
4	2000	500	3.5	2.3	67
5	250	50	9.0	1.8	20
5	500	100	8.0	0.0	0
5	750	150	7.0	0.0	0
5	1200	240	6.0	0.0	0
5	2000	400	4.0	0.0	0
7	250	36	8.0	2.0	25
7	500	71	7.5	0.0	0
7	750	107	7.0	4.5	67
7	1200	171	6.5	1.3	20
7	2000	286	5.0	5.0	100
9	250	28	7.0	1.0	16
9	500	56	7.0	7.0	100
9	750	83	7.0	1.5	22
9	1200	133	7.0	1.1	16
9	2000	222	7.0	7.0	100

Supplementary Table 2. CCN 51. Total number embryos produced as a function of hormone balance (relation 2,4D/TDZ) in petals explant using PCG medium with glucose 80 g L.

Concentration TDZ ($\mu\text{g L}^{-1}$)	Concentration 2,4-D ($\mu\text{g L}^{-1}$)	Rate 2,4D/TDZ	Average of embryos per explant	Average of normal embryos per explant	Normal embryos (%)
3	250	83	2.0	1.2	60
3	500	167	2.0	0.0	0
3	750	250	2.0	0.7	36
3	1200	400	2.5	1.6	67
3	2000	667	3.0	0.0	0
4	250	63	2.0	2.0	100
4	500	125	2.0	0.8	40
4	750	188	2.0	0.0	0
4	1200	300	2.0	0.0	0
4	2000	500	2.5	0.0	0
5	250	50	2.0	1.0	57
5	500	100	2.0	0.2	11
5	750	150	2.0	1.6	83
5	1200	240	2.0	0.0	0
5	2000	400	2.0	0.0	0
7	250	36	1.5	0.0	0
7	500	71	1.5	0.3	20

Supplementary Table 2. Contd.

7	750	107	1.5	0.0	0
7	1200	171	1.5	0.0	0
7	2000	286	1.5	0.0	0
9	250	28	1.0	1.0	100
9	500	56	1.0	0.6	67
9	750	83	1.0	0.7	69
9	1200	133	1.0	0.3	33
9	2000	222	1.0	0.0	0

Total explants per Petri dish 25.

Full Length Research Paper

Physiological responses by *Billbergia zebrina* (Bromeliaceae) when grown under controlled microenvironmental conditions

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Sucrose, the most commonly used carbon source in conventional *in vitro* culture, and limited air exchange in the culture containers are factors that affect the growth of *in vitro*-cultured plants. They may induce physiological disorders and decrease the survival rate of plants after transfer to *ex vitro* conditions. The aim of the present study was to analyze the effects of gas exchange and sucrose concentration on *Billbergia zebrina* plantlets during *in vitro* propagation. *In vitro*-established *B. zebrina* plantlets were transferred to culture media containing 0, 15, 30, 45, or 60 g L⁻¹ sucrose. Two different culture-container sealing systems were compared: lids with a filter (permitting gas exchange) and lids with no filter (blocking fluent gas exchange). Carbohydrate and chlorophyll (Chl a+b) concentrations were analyzed in plantlets at 45-days of culture. The addition of sucrose to the medium reduced the Chl a+b concentration in the plantlets. On the other hand, additional sucrose had a positive effect on the carbohydrate stock formation of the plantlets. The results showed that a photoautotrophic system (air exchange and a sugar-free medium) improves the *in vitro* propagation of *B. zebrina* without creating physiological disorders.

Key words: Bromeliad, *In vitro* plant, photoautotrophic growth, physiological disorders, sucrose.

INTRODUCTION

Plant micropropagation is the most common method used for the large-scale cloning of several horticultural crops, such as bulbous plants, fruit trees, and ornamentals. Bromeliads that are grown as flowering, potted ornamentals have a high commercial value and

account for a large part of the flower industry worldwide (Zhang et al., 2012). *Billbergia zebrina* (Herbert) Lindley is an epiphytic tank bromeliad native to the Atlantic Rainforest of Brazil. This plant has commercial value as an ornamental due to the quality its leaves and

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inflorescence (Vesco et al., 2011).

The application of plant tissue culture techniques to *in vitro* propagation of bromeliads has been reported previously (Huang et al., 2011; Martins et al., 2014; Resende et al., 2016; Viehmannova et al., 2016). Most of these studies are related to the use of plant growth regulators as major modulators of *in vitro* morphogenetic responses. In bromeliads, multiplication and rooting are strongly controlled by external plant growth regulators (Van Dijck et al., 1988).

In vitro culture conditions are thought to be stressful to plants (Desjardins et al., 2009) and may influence plant morphogenesis (Shin et al., 2013). Conventional *in vitro* propagation has been shown to induce plant anatomical and physiological disorders (Mohamed and Alsdon, 2010; Iarema et al., 2012), and it may interfere with the growth and survival rates of plants after transfer to *ex vitro* conditions (Fuentes et al., 2005; Shin et al., 2014).

In vitro plant disorders are directly or indirectly related to the heterotrophic conditions in a conventional micropropagation system, where sugar in the culture medium is the main cause of these disorders (Hazarika, 2006). Conventional *in vitro* propagation is mostly carried out using small, closed glass culture containers and the media most often contains sucrose as the major carbon source (Xiao et al., 2011). This external sugar supply is adequate for growth and organogenesis support (Hazarika, 2003). For bromeliads, 3% sucrose in the medium is recommended (Pérez et al., 2013; Martins et al., 2014; Resende et al., 2016). Sucrose effects on the physiology of *in vitro* plants have been previously documented (Iarema et al., 2012; Shin et al., 2014). Reduced photosynthetic ability (Shin et al., 2013), and plant survival and growth rates during later acclimatization periods are closely related to previous sucrose treatments (Mohamed and Alsdon, 2010; Shin et al., 2014). However, supplementing sugars positively affects carbohydrate stock formation by *in vitro* propagated plants (Ferreira et al., 2011) and a high carbohydrate stock improves plant performance during the acclimatization phase (Fuentes et al., 2005).

Limited gas exchange, caused by the type of culture containers used, affects the development of *in vitro* grown plants (Martins et al., 2015a). The *in vitro* environment is characterized by high relative humidity, potential ethylene build up, stagnant air, and a fluctuating CO₂ concentration caused by day and night cycles (Martins et al., 2015b). The CO₂ concentration has a considerable impact on photosynthesis and the growth of *in vitro* plants. It also affects plant metabolism (Iarema et al., 2012; Shin et al., 2013). Gas exchange improves in ventilated culture containers and this helps *in vitro* plantlets to grow photomixotrophically or even photoautotrophically, which results in improved plant quality and less propagule loss during the acclimatization process (Zobayed et al., 2000; Shin et al., 2014). Previous studies have indicated that the *in vitro*

photoautotrophic growth (sugar-free with gas exchange) of many plant species can be significantly improved by increasing the CO₂ concentration in the culture vessel and reducing the relative humidity (Xiao et al., 2011). Recently, we reported the effect of *in vitro* conditions on the growth and anatomy of *B. zebrina* plantlets (Martins et al., 2015b). We found that conventional *in vitro* culture induced anatomical plantlet leaf disorders and that these disorders had a negative effect on the acclimatization period. However, it is not clear how microenvironmental conditions influence the physiology of *in vitro* propagated bromeliads.

The aim of this study was to analyze the effects of gas exchange and sucrose concentration on the physiology of *B. zebrina* plantlets during *in vitro* propagation.

MATERIALS AND METHODS

Plant materials and culture conditions

B. zebrina plantlets, which had previously been established *in vitro* using seeds, were transferred to 250 mL glass containers containing 50 mL stationary, liquid Murashige and Skoog medium (Murashige and Skoog, 1962), supplemented with 30 g L⁻¹ sucrose and 3 mg L⁻¹ 6-benzylaminopurine (Martins et al., 2015a). After 30 days growth, side shoots were sub-cultivated for 45 days in 250 mL glass containers containing 50 mL stationary, liquid MS medium with no plant growth regulator, but supplemented with 30 g L⁻¹ sucrose. The medium pH was set at 5.8, before autoclaving at 120°C for 20 min. After sterile inoculation, the plant cultures were kept in a growth room at 26 ± 2°C with a 16-h photoperiod (8:00 to 00:00 h) under fluorescent lamps (Philips Master TL5 HO, 49W/840) that provided 90 μmol m⁻² s⁻¹ of PAR light.

Sucrose and gas exchange during *in vitro* propagation

B. zebrina side shoots that were approximately 4.0 cm in length were taken from the plantlets propagated in the previous *in vitro* stage. They were individualized (5 to 8 side shoots per seedling) with the aid of a scalpel and transferred to 280-mL polypropylene containers (ECO2 NV®, Geraardsbergen, Belgium) containing 50 mL MS medium solidified with 7 g L⁻¹ agar and supplemented with 0, 15, 30, 45, or 60 g L⁻¹ sucrose. Each container received five shoots. The pH was adjusted to 5.8 before autoclaving at 120°C for 20 min. Two different sealing systems were used: Container lids with an XXL filter (permitted gas exchange - minimum of 63 air exchanges per day) and the same container lids covered with two layers of polyvinyl chloride (PVC) transparent film (blocking gas exchange by the XXL filter exchange - 4.19 gas replacements per day). After sterile inoculation, the cultured plants were kept for 45 days in a growth room at 26 ± 2°C with a 16-h photoperiod (8:00 to 00:00 h) and under fluorescent lamps (Philips Master TL5 HO, 49W/840) that provided 230 μmol m⁻² s⁻¹ of PAR light.

Chlorophyll extraction and analyses

The aerial parts of five plants per treatment were divided into five independent samples. After weighing, the chlorophyll was extracted from the fresh plant samples (leaf discs) by incubating them in the dark for 72-h in dimethyl-formamide. The absorbance at 647 and 663 nm was measured by a spectrophotometer (UV-1800, Shimadzu, Japan). Final determination of the chlorophyll

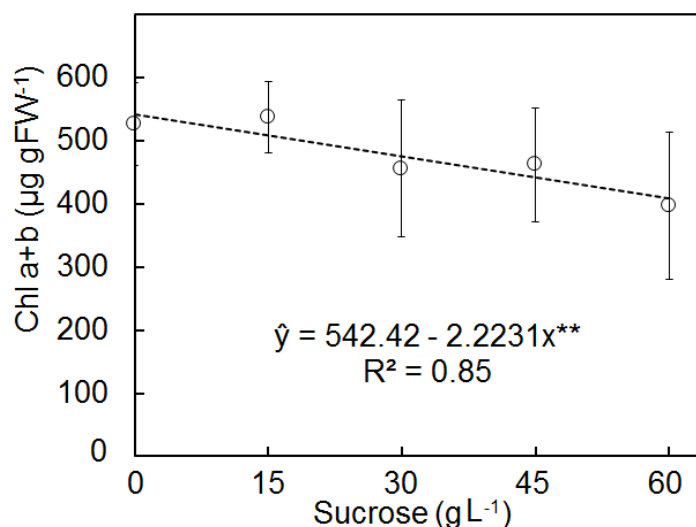


Figure 1. Chlorophyll a+b concentration of leaves taken from *B. zebra* plantlets exposed to different sucrose concentrations.

concentration was based on Wellburn (1994) and expressed as $\mu\text{g gFW}^{-1}$ (fresh weight) of tissue.

Extraction and analyses of plant metabolites

Fifteen plants per treatment were collected at 6:00 h. After mixing the plant material, three samples were taken. The leaves and roots were collected and given an identification mark with the aid of a scalpel. All the samples were immediately frozen in liquid nitrogen. The plant material (five plant mix) was lyophilized and crushed before taking a known amount of plant material for the metabolite analyses. An ENZYTEC system (Enzytec, Scil Diagnostics GmbH, Dormstadt, Germany), along with a spectrophotometer (DU-65; Beckman, Fullerton, CA, USA), set at 340 nm, were used to determine the malic acid, glucose, fructose, and sucrose concentrations in the leaves and roots. The protocol described by Ceusters et al. (2008) was followed. Starch concentration was determined as glucose equivalents after digestion with amyloglucosidase according to the protocol described by Enzytec, Scil Diagnostics. Analyses were performed on three independent biological samples. The glucose, fructose, and sucrose concentrations in the culture media were quantified by collecting three independent samples per treatment from the different containers and performing the above mentioned analyses. The concentration of plant metabolites was expressed as $\mu\text{mol gDW}^{-1}$ (dry weight) in the leaves and roots. Carbohydrate concentration in the culture media was expressed as $\mu\text{mol gFW}^{-1}$ (fresh weight).

Water loss in the containers

Five containers from each treatment were sampled randomly to evaluate water loss. They were weighed at 0 and 45 days, and the differences in the weights were used to determine water loss (%).

Statistical analysis

The experiment had a completely randomized design in a factorial arrangement (five sucrose concentrations \times two sealing systems). The data obtained were submitted to two-way analysis of variance

Table 1. Carbon metabolite concentrations ($\mu\text{mol gDW}^{-1}$) in *B. zebra* shoots at the start of incubation on different sucrose concentrations and different culture container aeration Samples were taken at 6:00 am.

Metabolites	Concentration ($\mu\text{mol gDW}^{-1}$)
Malic acid	112 \pm 13
Glucose	890 \pm 14
Fructose	386 \pm 34
Sucrose	nd*
Starch	573 \pm 223

*Not detectable.

(ANOVA), the averages of the factor sealing systems were compared using Tukey's test, and the sucrose concentrations were subjected to regression analysis.

RESULTS

Chlorophyll a+b (Chl a+b) levels decreased as the sucrose concentration rose, and this was independent of the sealing system (Figure 1). However, when air exchange was possible (filter containers), plant Chl a+b concentrations ($517.79 \mu\text{g gFW}^{-1}$) were higher than for the plants grown in containers without filters ($433.65 \mu\text{g gfw}^{-1}$).

The initial levels of the different metabolites in *B. zebra* shoots at the start of the treatments are shown in Table 1. The monosaccharide (glucose and fructose) concentrations were high in the shoots, but sucrose was not detected. Starch, expressed in glucose equivalents, was present at similar levels as the glucose and fructose. *B. zebra* is a CAM plant, and hence malic acid was also

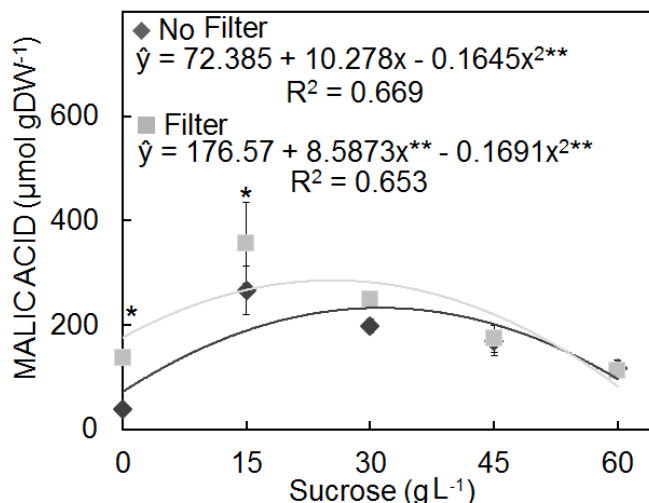


Figure 2. Malic acid concentration of leaves taken from *B. zebrina* plantlets exposed to different sucrose concentrations and to one of two different ventilation treatments. For each sucrose concentration, means followed by an asterisk differ from each other at the $P \leq 0.05$ level according to Tukey's test.

detected in the leaves.

Malic acid concentration had a positive quadratic relationship with increasing sucrose concentrations for both container sealing systems at 45-days growth. Plants cultivated in filter containers on the sugar-free medium or with 15 g L^{-1} sucrose had higher malic acid levels than plants subjected to the same sucrose concentrations, but grown in containers without filters (Figure 2).

The glucose and fructose concentrations of the leaves had positive linear, but negative quadratic relationships with increasing sucrose concentration, respectively, when they were cultivated in no filter and filter containers. Plants grown in a container with no gas exchange and on a medium with 60 g L^{-1} sucrose had the highest glucose concentration (Figure 3A). Conversely, the fructose concentration was highest in plants cultivated on the sugar-free medium and in an aerated container (Figure 3C). The monosaccharide concentrations were generally lower in the roots than in the leaves. The glucose levels in the roots showed a positive linear relationship with sucrose levels, which was independent of the container sealing system (Figure 3B). However, the fructose concentration was higher in plants grown in aerated containers and there was an improvement in the carbohydrate concentration when the sucrose concentrations rose (Figure 3D).

The sucrose concentration was higher in the roots than in the leaves for all sucrose levels. Sucrose levels for both plant tissues had linear relationships with increasing sucrose concentration in the medium (Figure 4A and B). Starch concentrations were higher in the leaves than in the roots after 45-days *in vitro* growth. The starch increase was positively related to rises in the sucrose

concentration. The highest starch concentration was recorded in the leaves of plants grown in containers with aeration and a high sucrose concentration in the medium. However, the starch concentration in the roots was low for all plants grown in containers with no aeration (Figure 4C and D).

The carbohydrate concentrations in all the nutrient media were analyzed. Glucose and fructose presented similar course in function of the sucrose level at the start of the culture time. They presented quadratic and linear models with increasing sucrose concentrations when they were cultivated in the presence or absence of a filter, respectively (Figure 5A and B). Sucrose in the medium showed a linear relationship with sucrose added in the medium at the beginning of the culture time, showing a higher accumulation when the air exchange potential was high (Figure 5C). All sugars analyzed in the media were higher in aerated containers than the ones with no aeration. Because sucrose uptake is dependent on the sucrose level of the media more sucrose has been hydrolysed in the leaves resulting in more free glucose and fructose.

All treatments were also performed in containers without plants and the carbohydrate concentrations measured at 45-days under the same growth conditions. As shown in the Table 2, there were differences in the carbohydrate concentrations between container sealing systems. It was correlated to dehydration of medium. Water loss occurred with both sealing systems, and this was independent of sucrose concentration. However, medium dehydration was higher in the aerated containers (Figure 6).

The morphology of *B. zebrina* plants was also

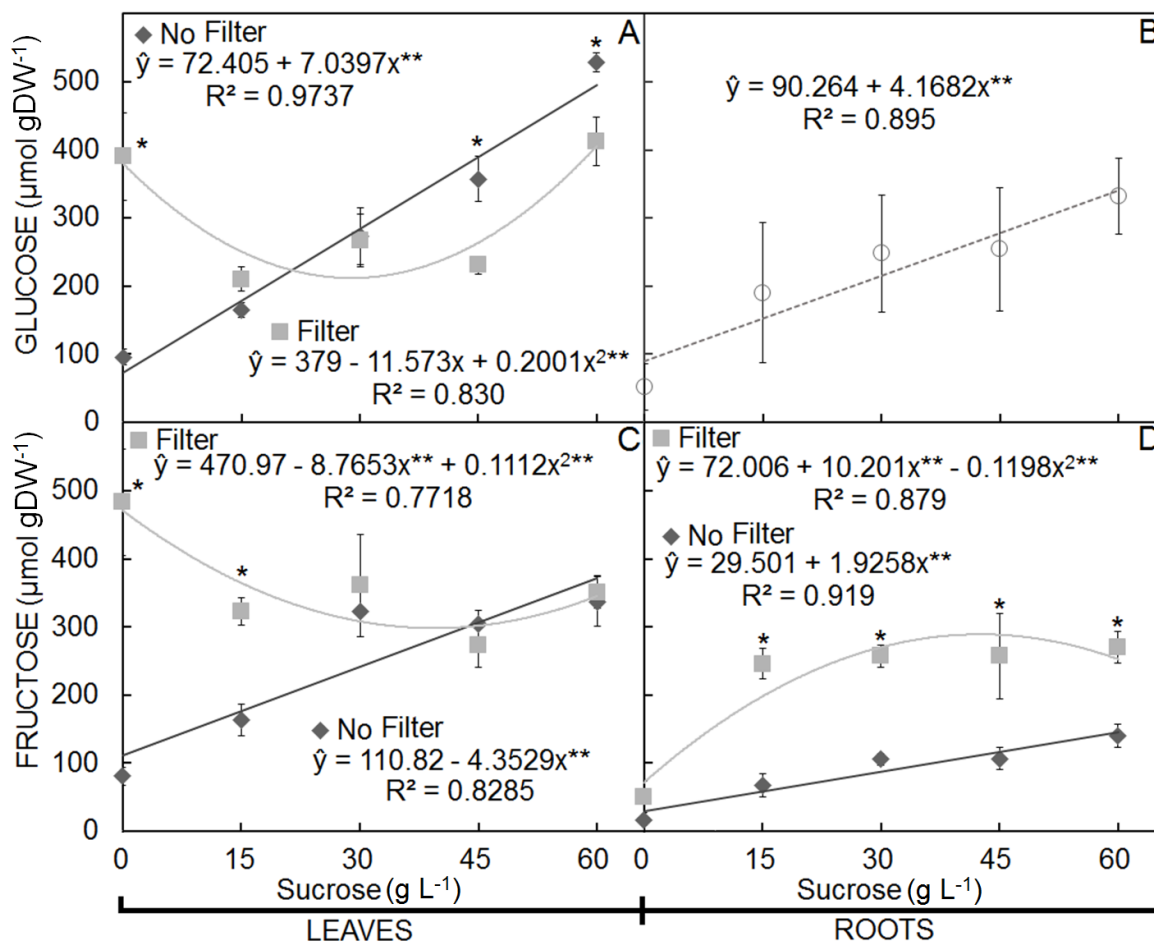


Figure 3. Glucose (A-B) and fructose (C-D) concentrations in leaves and roots at the end of the dark period (6:00am) for *B. zebrina* plants cultivated in media containing different sucrose concentrations and subjected to one of two different ventilation treatments. For each sucrose concentration, means followed by an asterisk differ from each other at the $P \leq 0.05$ level according to Tukey's test.

influenced by sucrose concentration and the gas exchange system. Growth was more vigorous under photoautotrophic conditions (sugar-free media and gas exchange). In contrast, plants cultivated with sucrose concentrations higher than 30 g L^{-1} had a low growth rate (data not shown) and showed leaf chlorosis, followed by necrosis (Figure 7).

DISCUSSION

B. zebrina plants showed different physiological responses to the different sealing systems and sucrose concentrations. *In vitro* plants may have poor chlorophyll concentrations, as occurred in the *B. zebrina* plants. This is because of the exogenous supply of sucrose, which does not promote the normal development of a photosynthetic apparatus. Although such plants may appear normal, their photosynthetic apparatus may not be active (Hazarika, 2006). A reduction in chlorophyll

causes a fall in light absorption and therefore provides less ATP and NADPH for the dark reactions (Sivanesan et al., 2008). In this respect, chlorophyll is a good first and easy-to-measure indicator of photosynthetic potential and apparatus status (Alvarez et al., 2012; Sáez et al., 2012). Lower energy inputs correlate well with the biochemical processes related to the quantity and activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Koch, 1996). The insufficient supply of ribulose-1,5-bisphosphate may also be related to increased plant susceptibility to feedback inhibition, which is possibly associated with an excessive accumulation of hexoses and starch (Le et al., 2001). This may explain the results for the *B. zebrina* plants, which had low chlorophyll levels (Figure 1), but high carbohydrate concentrations (Figures 3 and 4).

B. zebrina plants produced malic acid in all treatments, including shoots cultivated in the liquid media. Modulation of the CAM pathway occurs in the bromeliad *Guzmania monostachia*, depending on the water supply level

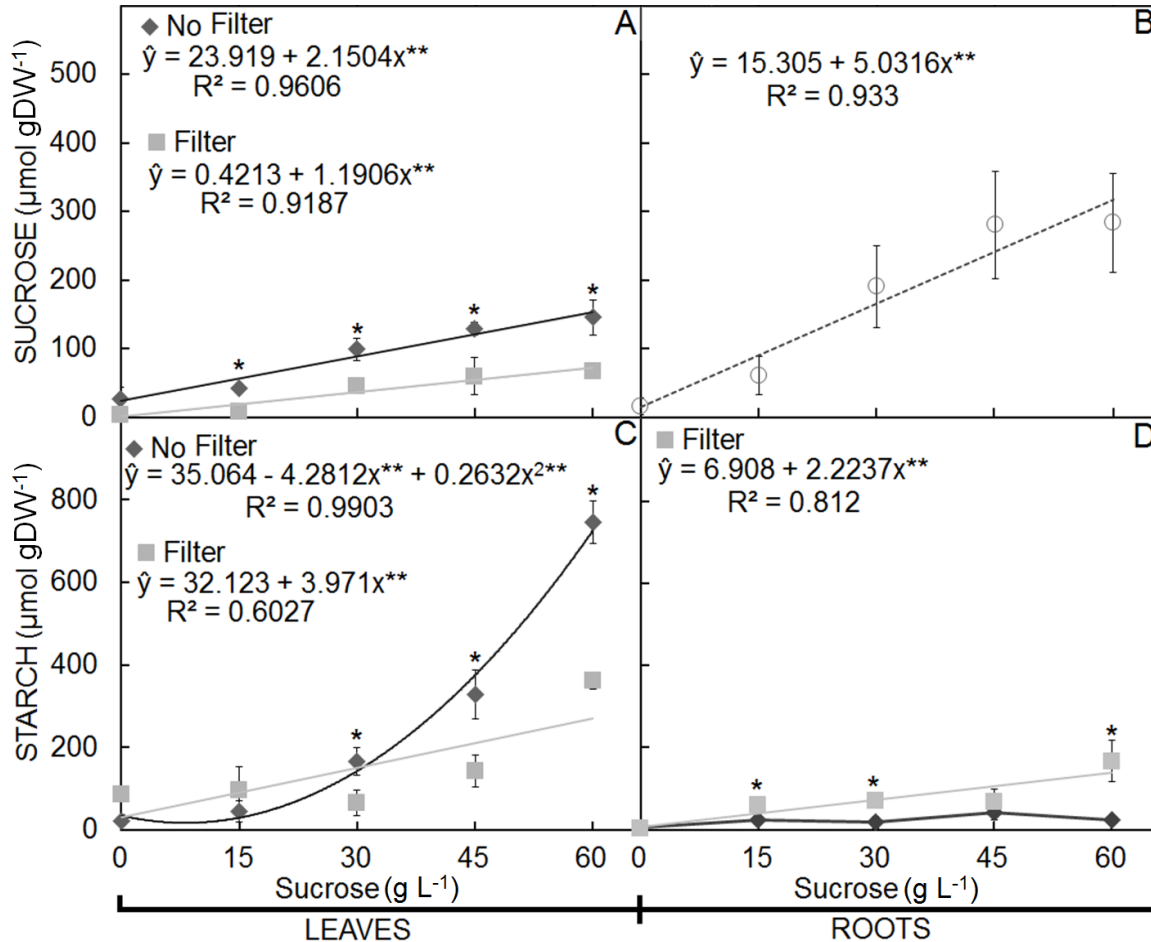


Figure 4. Sucrose (A-B) and starch (C-D) concentrations in leaves and roots at the end of the dark period (6:00am) for *B. zebrina* plants cultivated in media containing different sucrose concentrations and subjected to one of two different ventilation treatments. For each sucrose concentration, means followed by an asterisk differ from each other at the $P \leq 0.05$ level according to Tukey's test.

(Pereira et al., 2013). The decrease in malic acid concentration observed in plants cultivated in media where the sucrose concentrations were higher than 30 g L^{-1} was probably due to the osmotic stress caused by the media. Sucrose concentrations higher than 30 g L^{-1} in a medium may induce osmotic stress when cultivating *in vitro* plants (Cui et al., 2010). Photosynthesis is very sensitive to stress (Walters, 2005), and malic acid concentration has been shown to fall in leaves subjected to a long period of water stress, for example in the CAM plant *Aechmea* 'Maya' (Ceusters et al., 2009). These authors suggested that the roots might be involved in the metabolic response to water limitation. In this study, the lowest malic acid concentration ($39.35 \mu\text{mol gDW}^{-1}$) was found in plants grown in the sugar-free medium (no osmotic stress), but this may be related to insufficient availability of CO_2 . An increased CO_2 concentration improves the rate of malic acid formation and accumulation in the cytoplasm by PEPc (Zobayed et al., 2000). The increase in malic acid concentration in

Doritaenopsis occurred when additional CO_2 was available during *in vitro* photoautotrophic culture (Shin et al., 2013). During CAM activity, the glycolytic breakdown products of storage polysaccharides (e.g., starch) or soluble sugars (e.g., glucose, fructose, and sucrose) can be used for the production of the CO_2 acceptor phosphoenolpyruvate (PEP) during the dark period (Ceusters and Borland, 2011). This explains the results for the plants grown in the sugar-free medium and in the no ventilation containers, since those plants did not have enough carbohydrate stock to regenerate the PEPc enzyme.

Sucrose concentrations in the *in vitro* media created higher monosaccharide and sucrose stocks in the leaves and roots (Figures 3 and 4). Sucrose addition to the plant tissue cultures reduced the water potential of the media and increased the leaf tissue glucose, fructose, sucrose, and starch concentrations in a dose dependent manner. Monosaccharides are effective osmotic agents in plants because osmotic stress in roots can increase

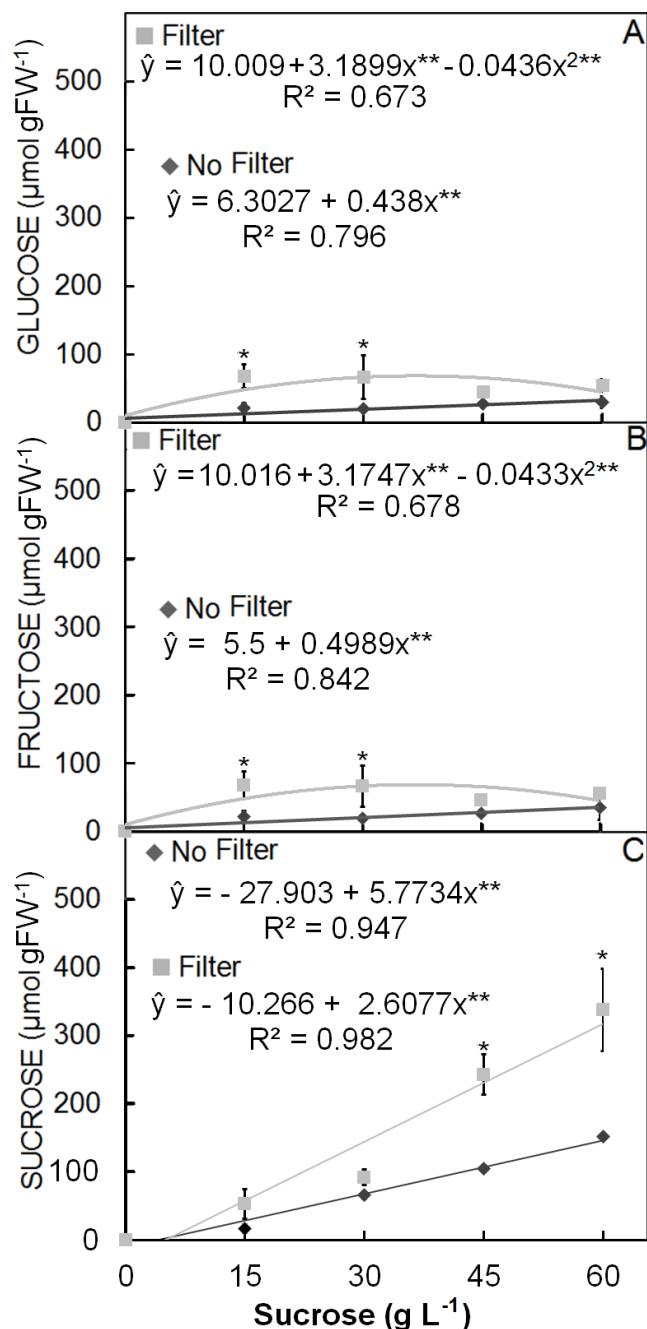


Figure 5. Carbohydrate concentrations in the culture media at 45-days in containers with *B. zebrina* plants. For each sucrose concentration, means followed by an asterisk differ from each other at the $P \leq 0.05$ level according to Tukey's test.

monosaccharide accumulation (Xie et al., 2009). Sugars, especially glucose, fructose, and sucrose, play an important role in stress alleviation through the regulation of plant osmotic potential (Polanco et al., 2014). Under photoautotrophic conditions, the plants had high monosaccharide concentrations in the leaves (Figure 3) and the accumulation of different carbohydrates

showed that the photosynthetic apparatus was performing well. The presence of organic reserves and a functional photosynthetic apparatus in the *in vitro* plants should improve acclimatization performance (Shin et al., 2013, 2014).

Sugars are crucial for building compounds in plants and are a key source of energy that can be used to induce biochemical processes (Piotrowska et al., 2010). *B. zebrina* plants cultivated under the sugar-free and no ventilation conditions had the lowest carbohydrate stocks due to the limited photosynthesis rate. Carbohydrates are required by plant cells as carbon resources, and supply energy for growth and biosynthetic processes (Ferreira et al., 2011). Increasing the sucrose levels in the media produced plants with higher starch concentrations (Figure 4C and D). Plants cultured with high sucrose concentrations during *in vitro* growth may have larger and higher numbers of starch granules in their chloroplasts (Capellades et al., 1991). The main reserves of carbon, and therefore energy, are sucrose and starch. When the export rate of sucrose is lower than the sucrose synthesis rate, an accumulation of hexoses and triose-phosphates occurs, and as a result, starch synthesis begins in the chloroplasts during the light period (Dennis and Blakely, 2000).

The use of containers with unlimited air exchange produced plants with lower starch concentrations. High osmotic stress, induced by dissolved sugars in the media, can limit starch formation. In general, under osmotic and drought stress, soluble sugars tend to increase, while starch concentration decreases (Chaves, 1991). Starch does not directly act as an osmoprotectant because it is not soluble in water. *B. zebrina* plants decreased the water osmotic potential in their roots by increasing the soluble sugar content. Lower starch and higher soluble sugar contents could have facilitated water flux from the medium into the plants.

At 45-days growth, the *B. zebrina* plants had not consumed all the added sucrose in the media, even in those without air exchange. Other plant species have also shown a low sugar reserve consumption in the same culture medium (Palonen and Junttila, 1999). After plants are transferred to the culture medium, they may hydrolyze sucrose into glucose and fructose using invertase that has been released into the culture medium (Karhu, 1997). This extracellular enzymatic system, which is associated with sucrose hydrolysis, is initiated by the presence of plant tissues, but can remain active even when plants are not present, which increases the fructose and glucose concentrations in the medium (Tremblay and Tremblay, 1995). Sucrose was hydrolyzed to glucose and fructose, which nearly doubled the osmolality of the medium (Bishnoi et al., 2000), and in this study, sucrose hydrolysis increased the osmolality of the media containing additional sucrose. This effect was probably even higher when combined with water loss from the media in containers with a filter, which would reduce the

Table 2. Carbohydrate concentrations in the culture media at 45-days in containers without plants.

Sucrose (g L ⁻¹)	Carbohydrate concentrations in the culture media without plants					
	Glucose (μmol/gfw)		Fructose (μmol/gfw)		Sucrose (μmol/gfw)	
	No filter	Filter	No filter	Filter	No filter	Filter
0	0.00±0.0 ^{a*}	0.00±0.0 ^{a*}	0.00±0.0 ^{a*}	0.00±0.0 ^{a*}	0.00±0.0 ^{a*}	0.00±0.0 ^{a*}
15	1.37±0.4 ^a	3.12±0.8 ^a	1.33±0.4 ^a	2.94±0.7 ^a	50.41±8.1 ^a	74.72±19.9 ^a
30	2.70±0.7 ^b	8.37±1.9 ^a	2.55±0.7 ^b	7.86±1.8 ^a	78.67±12.9 ^b	187.57±39.6 ^a
45	3.32±0.4 ^b	10.74±2.8 ^a	3.09±0.3 ^b	10.16±2.5 ^a	144.81±13.9 ^b	232.55±62.7 ^a
60	7.48±0.1 ^b	13.32±2.6 ^a	7.07±0.1 ^b	12.53±2.4 ^a	159.70±2.5 ^b	279.57±50.7 ^a

Averages followed by the same letter in the row for each carbohydrate concentration, do not differ according to the Tukey's test, at 5%.

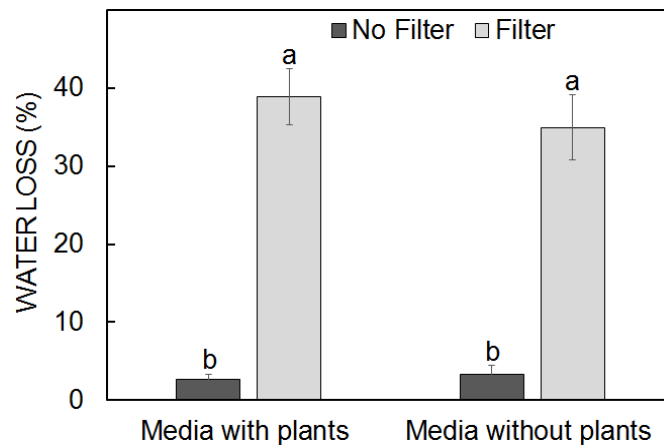


Figure 6. Water loss in containers with and without plants due to the sealing system after 45 days of growth. For each plant culture system, means followed by different letters differ from each at the $P \leq 0.05$ level according to Tukey's test.

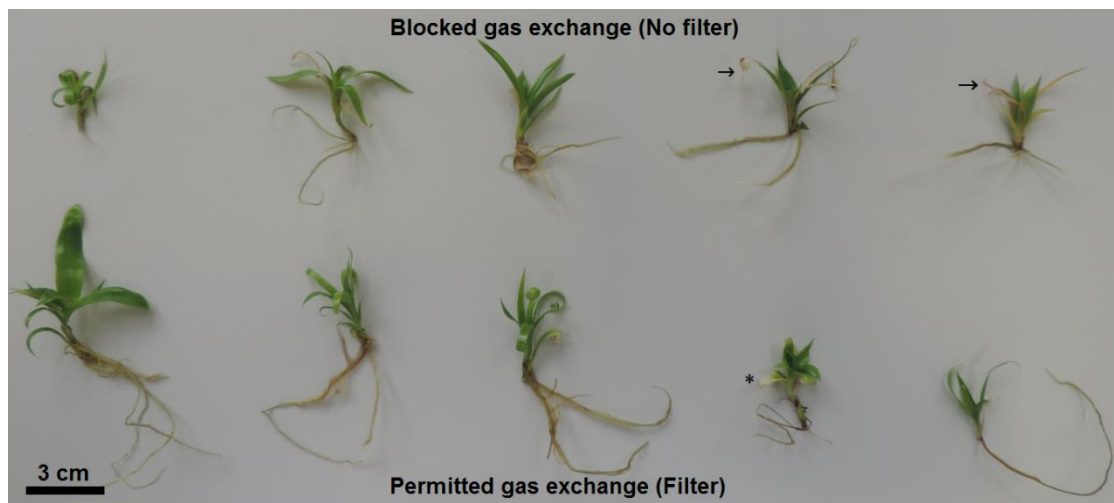


Figure 7. Visual aspects of *B. zebrina* plants after 45 days of *in vitro* growth. The plantlets had been exposed to different media sucrose concentrations (0, 15, 30, 45, or 60 g L⁻¹ sucrose, from left to right) and one of two ventilation treatments. Leaf chlorosis and necrosis are shown by an asterisk and an arrow, respectively.

solvent content.

Plants under environmental stress, e.g. osmotic stress, may show membrane lipid peroxidation, which increases cell membrane permeability and extravasation of cell-soluble substances (Zeng et al., 2006). Our results showed that plants grown under ventilated conditions and in media with 45 or 60 g L⁻¹ sucrose showed leaf senescence and a reduced growth rate. The increased chlorosis and necrosis may indicate that the plants were under osmotic stress.

Conclusion

In vitro culture conditions influenced the physiology of *B. zebrina* plantlets during micropropagation. Sucrose in the culture medium led to reduced chlorophyll concentrations and increased starch concentrations. Adding sucrose osmotically stressed *B. zebrina*. Ventilating the culture containers increased the stress responses because water loss was greater and it also led to increased media sugar concentration. Plants grown under photoautotrophic conditions (air exchange and sugar-free) did not show any physiological disorders and photoassimilate production was normal.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Fruit production and quality of guava 'Paluma' as a function of humic substances and soil mulching

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The uses of humic substances and organic residues for soil mulching have been more common during last years. This way, an experiment was carried out to evaluate the fruit quality and yield of guava cv. Paluma as a function of humic substances and organic soil mulching in Brazil. The experiment was performed from January 2012 to June 2013 (first trial) and from July to November 2013 (second trial) using a randomized blocks with treatments distributed in a factorial arrangement (5 × 2 × 2) of five humic substances doses (0, 10, 20, 30 and 40 mL of Humitec plant⁻¹), two organic mulching use (with and without organic mulching) and two consecutive harvests, with four replications of two plants each. Humic substances enhance fruit production more efficiently in guava plants grown without soil mulching. Guava fruit production increases from the first to the second harvest. Fruit quality for titratable acidity, soluble solids, vitamin C and soluble solids/titratable acidity ratio is adequate independently of soil mulching use. Humic substances improve fruit quality of guava cv. Paluma. Under soil and climate conditions, and considering the first two production cycles of guava, it is possible to recommend about 20 mL L⁻¹ of humic substances for production of high quality guava fruits.

Key words: Humic acids, post-harvest, *Psidium guajava*.

INTRODUCTION

Fruit production is an important horticultural activity that increases employment availability, makes possible the family establishment in the field, promotes a better income distribution within productive cycle besides it acts

as promising expectations of Brazilian and foreign markets. Among the economically important fruit crops, guava (*Psidium guajava* L.) presents high viability for farmers, especially for expansion of production areas in

Brazil (Amorim et al., 2015a).

The increasing consumption of fresh and processed fruits, including guava, is a worldwide tendency that stimulates the production of quality fruits (Amorim et al., 2015b). In Northeastern Brazil, guava crop is economically important especially in irrigated areas of Bahia and Pernambuco states where water potential, soil and climate conditions are favorable for growing guavas commercially using horticultural technologies for water and nutrient management (IBGE, 2014; Rodrigues et al., 2015).

Paluma is one of the high quality guava cultivars available for Brazilian guava farmers, and one of the most widespread throughout Brazil. Guava detaches among other tropical fruits for its attractive features of fruits such as color, pleasant smell and taste, mineral composition, high lycopene content and possibilities of consumption, attributes that guarantee preference for different consumer markets worldwide, especially because guava fruit has been consumed as fresh or processed fruit (Ramos et al., 2010; Amorim et al., 2015a).

Despite the social and economic importance of Brazilian guava, there is little information on soil fertilizing management for production of fruit quality fruits and high yields. This scientific shortage is worse for small and medium farmers, the majority in Northeastern Brazil (Silva et al., 2009). Accordingly, Serrano et al. (2007) reported that it is necessary to use more intensive management systems for irrigation and fertilizing management of guava trees (Natale et al., 2011; Amorim et al., 2015b).

Concern about agricultural sustainability is evident in recent years worldwide. This way, the soil quality maintenance is one of the essential factors for a sustainable production system, which demands an adequate soil management as main component. Nowadays guava farmers have also invested for simultaneous fertilization of mineral and organic fertilizers, emphasizing the use of humic substances in crop management (Souza et al., 2014; Silva et al., 2015).

The agricultural techniques for water loss reduction and, consequently, increase water use efficiency aim to reduce losses by evaporation using soil mulching that reduces water losses through air evaporation, decreases soil temperature and maintains soil moisture for longer time in addition to providing nutrients after decomposition of plant material (Silva et al., 2013; Bakshi et al., 2015). Soil mulching has been used for production of fruits and vegetables in Brazil and worldwide providing viability to the production system, especially where water availability is limited (Sarkar and Singh, 2007; Silva et al., 2013).

Hence, the present study aimed to evaluate the fruit

quality and yield of guava cv. Paluma as a function of humic substances and organic soil mulching in Brazil.

MATERIALS AND METHODS

Plant material and growth conditions

Guava (*P. guajava*) plants cv. Paluma propagated by cuttings were used in this study. The study was carried out from January 2012 to June 2013 (first trial) and from July to November 2013 (second trial) on the Macaquinhos experimental farm of Federal University of Paraíba, Remigio County, Paraíba State, Brazil (Northeastern Brazil).

During the execution of the experiment, the climatic data were collected by a meteorological station installed inside the experimental farm (Table 1), while physical and chemical characteristics of the soil before the experiment are in Table 2. The soil is a Xerorthent (Ultisol - American classification Soil Taxonomy).

One year old guava plants, spaced with 3 m between the rows and 3 m between the plants, were daily micro-sprinkle irrigated with one emitter per plant, for a flow of 60 L h⁻¹, following the daily evaporation recorded in a class A tank corrected through Kc value (0.75) defined by Ferreira et al. (2010) for guava. All agronomic practices performed in the experiment for pruning, control of pests, diseases and weed, followed the instructions of Natale et al. (2009).

During the execution of the experiment, all the plants were fertilized with 1.166 kg ha⁻¹ of urea (45% of N) and 1.166 kg ha⁻¹ of potassium chloride (60% K₂O) monthly; and 467 kg ha⁻¹ of fosmag (18% of P₂O₅) bimonthly according to the instructions by Natale et al. (2009).

The humic substances used in the experiment were extracted from leonardite, and the source adopted was Humitec (Tradecorp™), whose complete composition included humic extract (16.5%), organic carbon (11.2%), humic acids (13.2%) and fulvic acids (3.3%).

Treatments and experimental design

The experiment was randomized blocks with treatments distributed in a factorial arrangement (5 × 2 × 2) of five humic substances doses (0, 10, 20, 30 and 40 mL of Humitec plant⁻¹), two organic mulching use (with and without organic mulching) and two consecutive harvests, with four replications of two plants each.

The humic substances doses were defined following the recommendations of the producer, that is, 30 mL diluted in 6 L of water applied every 60 days after pruning, reaching 18 applications for each experiment. Organic mulching was composed of dehydrated *Brachiaria decumbens* with a layer of 5.0 cm.

Variables recorded and statistical analyses

During the fruit harvest time, that is, August 2012 to January 2013 and April to November 2013, 10 fruits per parcel were manually harvested (still firm) at intermediate green color (yellow-green color) placed in plastic boxes and taken to the Laboratory for post-harvest fruit quality analyses. This harvest parameter for fruit selection was recommended by Hojo et al. (2007) for commercial farms.

The fruit analyses of the guava fruits followed the instructions of

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Table 1. Average precipitation, air temperature and air humidity during the experiments.

Month	2012			2013		
	P ¹ (mm)	AT ² (°C)	AH ³ (%)	P ¹ (mm)	AT ² (°C)	AH ³ (%)
January	141	26.5	66	38	26.7	59
February	112	24.4	56	66	24.9	45
March	8	23.9	72	41	24.9	68
April	36	23.8	76	135	24.7	70
May	75	23.2	79	58	24.2	76
June	195	22.3	86	155	22.4	80
July	106	22.5	92	118	22.0	82
August	17	21.15	86	82	21.5	86
September	4	24.4	78	30	24.7	78
October	7	27.2	63	13	27.4	62
November	0	27.4	54	21	28.7	54
December	0	27.3	56	43	28.2	56

¹Precipitation, ²air temperature, ³air humidity.

Table 2. Chemical and physical characteristics of the soil (0-20 and 20-40 cm soil depth) where the experiment was carried out.

Soil characteristic	0-20 cm	20-40 cm
pH (in water)	5.02	5.03
	cmol_c dm⁻³	
Ca ²⁺	0.98	0.24
Mg ²⁺	0.50	0.76
Al ³⁺	0.08	0.22
H ⁺ + Al ³⁺	2.68	1.98
CEC	4.33	3.11
P (mg dm ⁻³)	5.31	1.69
K (mg dm ⁻³)	68.01	50.78
	%	
Organic matter	0.73	0.40
Al ³⁺ saturation	1.88	7.61
Basis saturation	38.11	36.33
	g kg⁻¹	
Clay	788	732
Silt	106	130
Sand	106	138
Soil density (kg dm ⁻³)	1.62	1.60
Particle density (kg dm ⁻³)	2.67	2.69
Micro porosity (m ³ m ⁻³)	0.14	0.15
Macro porosity (m ³ m ⁻³)	0.25	0.25
Available water (%)	7.87	7.81

P, K: Mehlich 1; H⁺ + Al³⁺: calcium acetate (extractor) 0.5 M pH 7; Al³⁺, Ca²⁺, Mg²⁺: KCl 1 M extractor; CEC: cationic exchangeable capacity.

brand precision balance (0.01 g precision) and expressed in g; ii) for the titratable acidity (TA), 20 g of macerated fruit pulp was taken from yellow passion fruits and brought to a final volume of 100 mL by adding distilled water. A 20 mL sample was taken from the mixture; and three to four drops of phthalein were used as an indicator. This suspension was titrated with 0.1 N sodium hydroxide (NaOH). The results were expressed as a percentage; iii) the soluble solids (SS), expressed as °Brix, were measured using an AbbeTM refractometer (Bausch and Lomb, Rochester, NY, USA); iv) The vitamin C content was defined with 5 g of fruit pulp taken from acerola and brought to a final volume of 100 mL by adding distilled water plus 1 mL of 1% amid solution. A 20 mL sample was taken from the mixture and titrated with 1 N iodine. The results were expressed in mg 100 g⁻¹ of fresh fruit; v) the pulp pH was measured using a MarconiTM pH meter; vi) after chemical analyses, the relation between the soluble solids and the titratable acidity (SS/TA ratio) was calculated; and vii) the fruit yield were measured as ton ha⁻¹.

Statistical analysis

Statistical analyses included analysis of variance (ANOVA), a mean separation of mulching using the Tukey test and regression analysis of the humic substances doses of two consecutive harvests. All calculations were performed using the Sigmaplot software, and the terms were considered significant at P ≤ 0.01.

RESULTS AND DISCUSSION

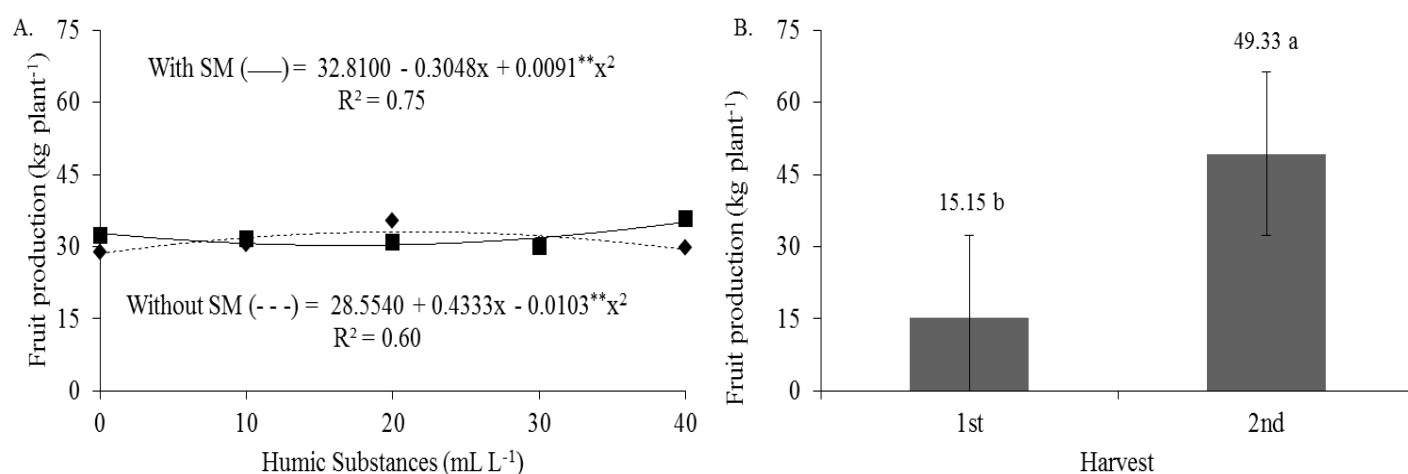
As shown in Table 3, fruit production (kg plant⁻¹) was affected by humic substances x soil mulching interaction. For fruit quality variables, the humic substances x soil mulching x harvest interaction was significant for fruit mass, soluble solids and titratable acidity. Vitamin C and pH average values were affected by humic substances x harvest interaction (Table 3).

For plants grown without soil mulching, humic substances doses increase from 0 to 21 mL L⁻¹ enhanced guava fruit production from 28.5 to 33.1 kg plant⁻¹, followed by a consecutive decay, while plants grown with

Table 3. Variance analysis for fruit production (FP) and fruit quality [fruit mass (FM), pH, soluble solids (SS), vitamin C (Vit. C), titratable acidity (TA) and SS/TA ratio] of guava as a function of humic substances and soil mulching.

Variance analysis	FP	FM	pH	SS	Vit. C	TA	SS/TA
HS	51572792.95 ^{ns}	201.36 ^{ns}	0.06 ^{ns}	3.06*	59.60*	0.003 ^{ns}	81.87 ^{ns}
SM	30482274.05 ^{ns}	1387.19*	0.15*	0.32 ^{ns}	3.67 ^{ns}	0.005 ^{ns}	57.95 ^{ns}
Trial (T)	233605.94**	189245.59**	6.45**	12.70**	1594.27**	0.20**	4120.02**
HS × SM	117385974.45**	806.07*	0.01 ^{ns}	12.31**	24.21 ^{ns}	0.004 ^{ns}	203.32**
HS × T	19692028.58 ^{ns}	248.1 ^{ns}	0.12**	2.01 ^{ns}	140.88**	0.009*	17.72 ^{ns}
SM × T	14499342.05 ^{ns}	1098.97*	0.04 ^{ns}	6.09*	56.56 ^{ns}	0.001 ^{ns}	56.83 ^{ns}
HS × SM × T	446745845.58 ^{ns}	828.91*	0.01 ^{ns}	4.32**	32.27 ^{ns}	0.011**	73.98 ^{ns}
Residual	23368245.25	255.91	0.03	1.01	21.05	0.003	38.60
CV (%)	14.99	16.41	4.36	13.76	28.73	19.24	22.28

HS: Humic substances; SM: soil mulching; **significant at P<0.01 probability error; *significant at P<0.05 probability error; ns: non-significant; CV: coefficient of variation.

**Figure 1.** Fruit production of guava plants as a function of humic substances and soil mulching (SM) (A) in two consecutive harvests (B).

soil mulching had fruit production decrease until 16.8 mL L⁻¹, followed by an increase until it reached the maximum fruit production of 35.2 kg plant⁻¹ at the highest humic substances dose (Figure 1A). Guava fruit production increased significantly from the first (15.15 kg plant⁻¹) to the second (48.33 kg plant⁻¹) harvest showing superiority of 225.6% (Figure 1B).

The benefits of humic substances on guava fruit production occurred in congruency to the adequate management practices of the orchard for pests control, diseases control, pruning, water supply and fertilizer management, especially using a slow releasing fertilizer, more efficient on nutrient supply. In addition, Souza et al. (2014) reported enhancement of fruit production of guava cv. Paluma with increasing doses of by-product of guava processing industry from a 1089.65 to 1327.19 kg plant⁻¹ in six cumulative harvests.

At the first harvest, humic substances doses increasing without soil mulching enhanced fruit mass until it reached

a calculated peak at 19.7 mL L⁻¹, corresponding to 135.1 g, while for plants with soil mulching, fruit mass decreased from 142.6 to 129.1 g (Figure 2A). At the second harvest, humic substances doses increase without soil mulching promoted a fruit mass linear increase from 173.7 to 196.4 g, while for plants with soil mulching, fruit mass was enhanced until the stemmed average of 15.6 mL L⁻¹, corresponding to the individual fruit mass of 197.0 g. This data distribution indicate fruit mass increase of 13.1 and 3.5% as a function of humic substances with and without soil mulching, respectively (Figure 2B). The fruit mass reduction for higher humic substances doses occurred due to the higher number of fruits per plant (data not presented), a characteristic known in the scientific literature for guavas (Ramos et al., 2010).

Fruit pH enhanced with humic substances doses increase in the first harvest, reaching 3.54 at 20.8 mL L⁻¹, but an inverse data distribution was registered in the

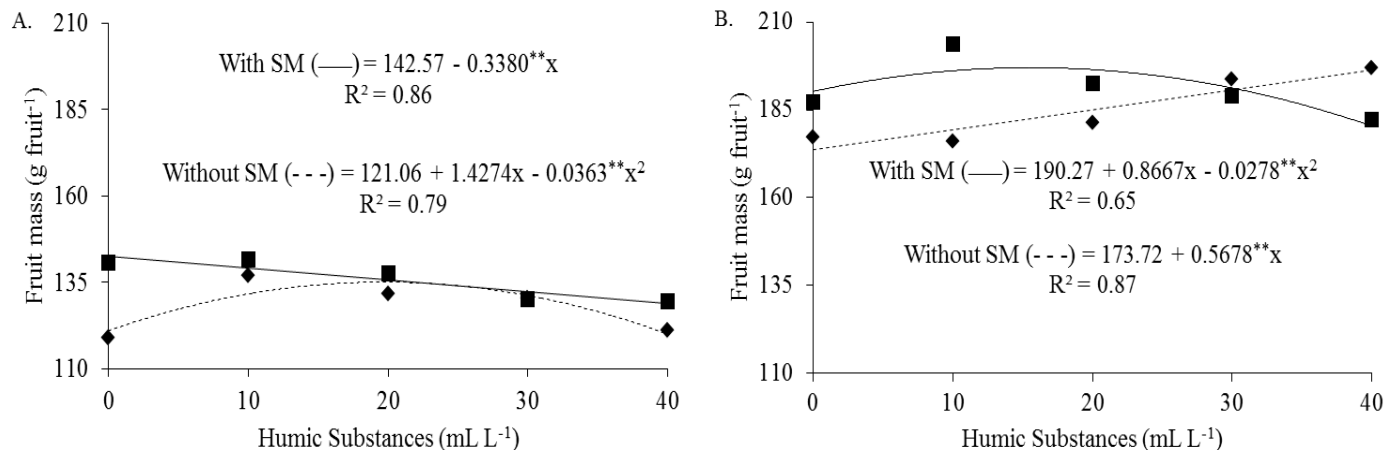


Figure 2. Fruit mass (A and B) of guava as a function of humic substances and soil mulching (SM) in two consecutive harvests.

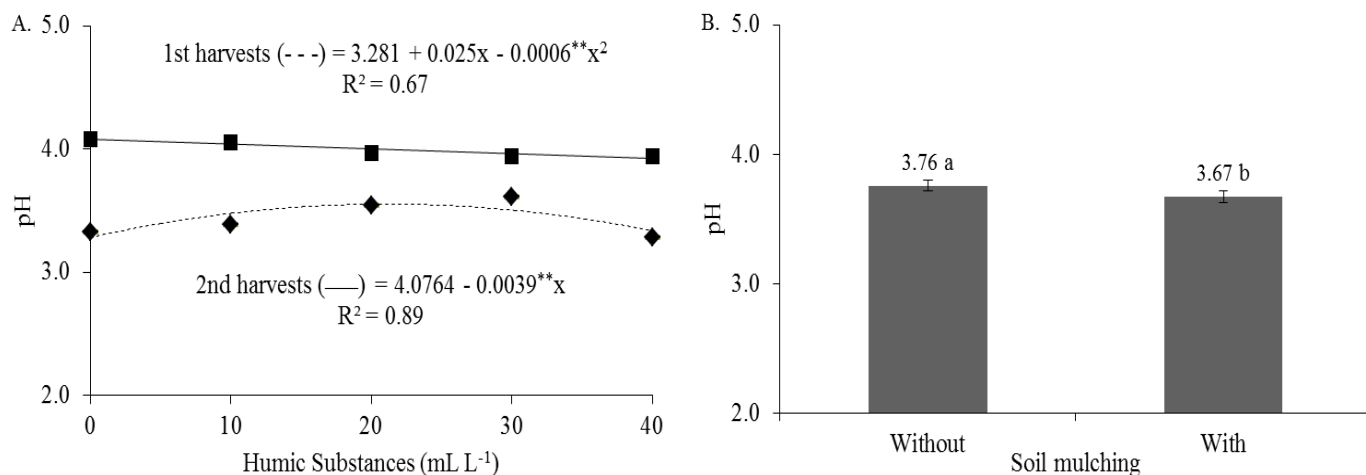


Figure 3. Fruit pH of guava as a function of humic substances and soil mulching (SM) (A) in two consecutive harvests (B).

second harvest, that is, a fruit length linear decrease of 0.0039 mm per unitary enhancement of humic substances (Figure 3A). Beyond pH average reduction, the lower pH value of the second harvest was 10.7% higher than that recorded in the first harvest. As shown in Figure 3B, there was reduction in fruit pH from 3.76 to 3.67, caused by soil mulching. According to Ramos et al. (2010), fruit pH higher than 3.5 needs addition of organic acids for fruit processing. High pH shows possible deterioration of industrialized products because the maximum limit is 4.2 for better product conservation. According to Mariano et al. (2011), fruit pH is used to evaluate acid characteristic and shelf life of fruits. Campos et al. (2007) reported that less acid fruits are more recommended for consumption as fresh fruit, while more acid fruits are required for food industry.

In the first harvest, fruits produced without soil mulching presented a soluble solids linear increase of 0.0244 per

unitary enhancement of humic substances reaching 8.1 °Brix, which corresponds to an enhancement of 12.5% in relation to those fruits produced without humic substances (Figure 4A). Soluble solids of fruits produced with soil mulching decreased with humic substances doses increase until the lower average value of 5.56 °Brix, recorded at 20.76 mL L⁻¹ (Figure 4A). In the second harvest, soluble solids values was enhanced with humic substances doses increase until reaching a peak at 8.6 and 7.5 °Brix referring to 17.8 and 25.4 mL L⁻¹, without and with soil mulching, respectively. These results are above 9.33 °Brix reported by Ramos et al. (2011), and lower than 10.08 at 10.48 °Brix reported by Ramos et al. (2010).

In the first harvest, plants grown with soil mulching presented a titratable acidity linear increase of 0.0004% per unitary enhancement of humic substances reaching 0.27% (Figure 4C), while for fruits produced without soil

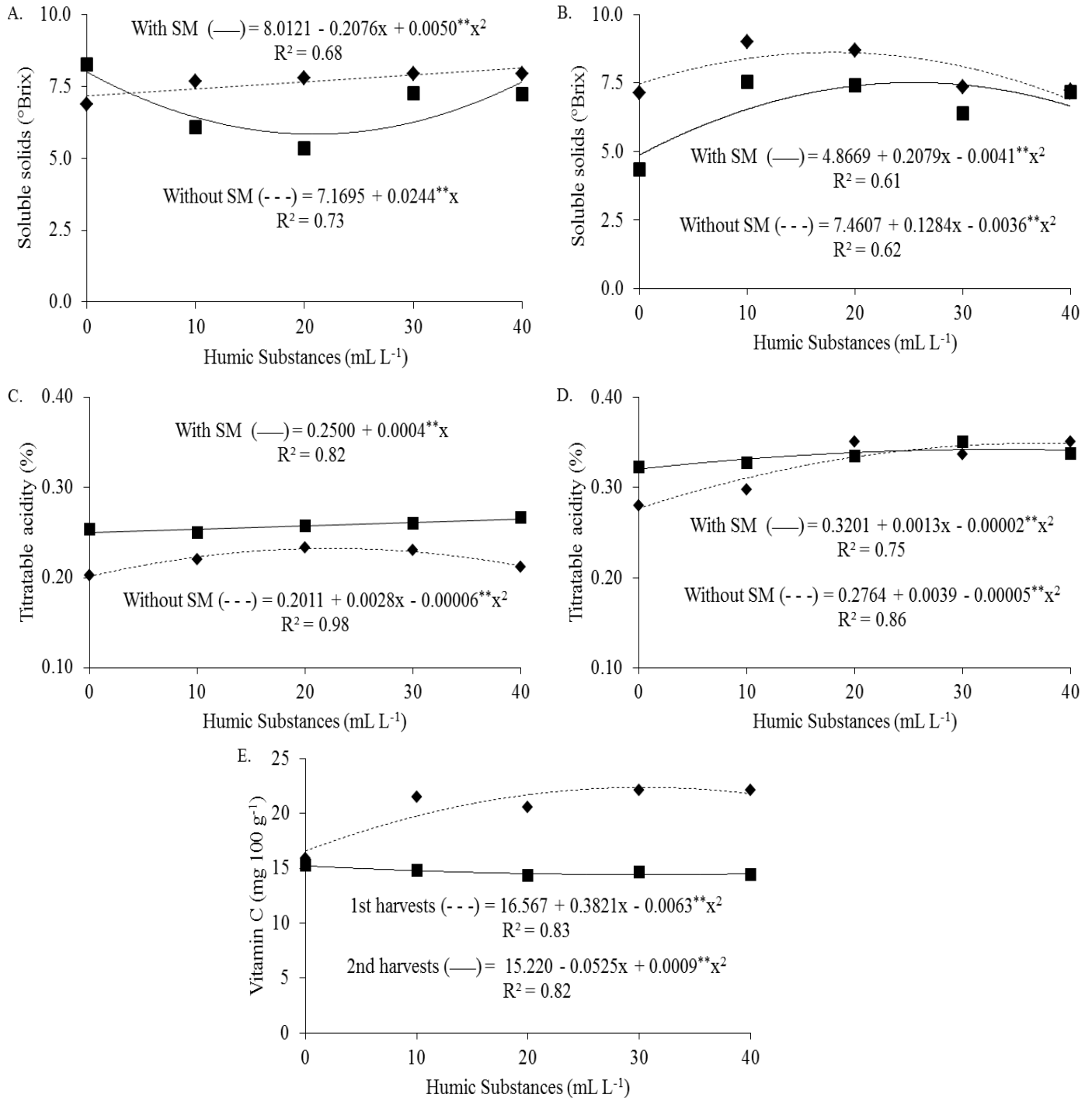


Figure 4. Fruit soluble solids (A and B), titratable acidity (C and D) and vitamin C (E) of guava as a function of humic substances and soil mulching (SM) in two consecutive harvests.

mulching, titratable acidity increased until 0.23% at 23.3 mL L⁻¹. In the second harvest, titratable acidity of fruits produced without and with soil mulching increased until 0.35 and 0.34%, with regards to calculated doses of 39.0 and 32.5 mL L⁻¹, respectively (Figure 4D). The 0.23 at 0.35% range recorded in both harvests is higher than

0.15 at 0.19% range reported by Soares et al. (2007) for white guavas; lower than 0.32 at 0.99% range of Singh and Pal (2008) who studied 'Lucknow-49', 'Allahabad Safeda' and 'Color Apple' guava cultivars in India.

Vitamin C fruit concentrations in the first harvest increased until it reached the calculated peak of 22.36

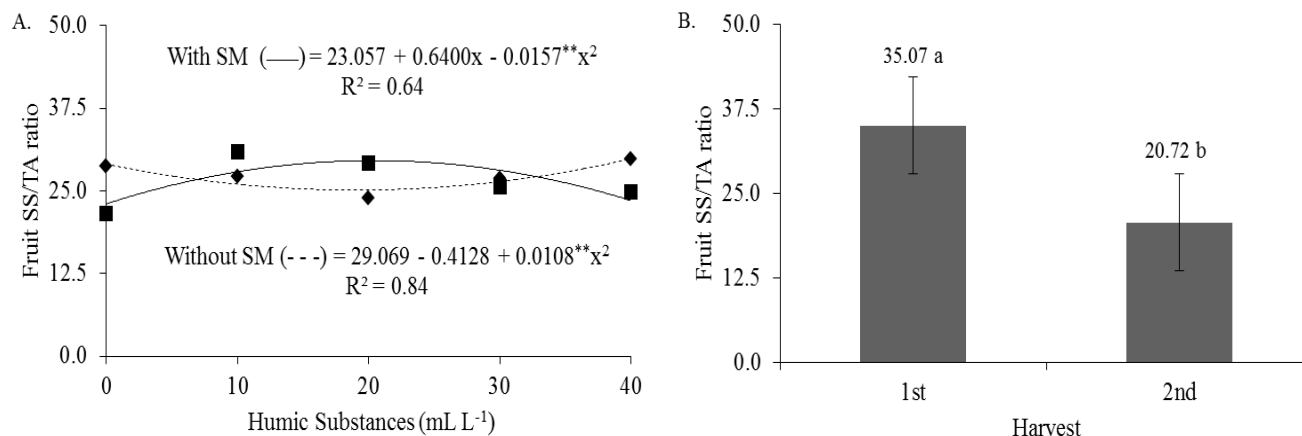


Figure 5. Fruit SS/TA ratio of guava as a function of humic substances and soil mulching (SM) (A) in two consecutive harvests (B).

mg 100 g⁻¹, recorded at 30.33 mL L⁻¹ (Figure 4E). In the second harvest, vitamin C averages reduced to 14.4 mg 100 g⁻¹, with regards to 29.2 mL L⁻¹. These values are lower than 66.67 at 93.50 mg 100 g⁻¹ range reported by Brackmann et al. (2012) for guava fruits cv. Paluma.

Average values of soluble solids/titratable acidity (SS/TA) ratio presented different data distribution as a function of soil mulching use (Figure 5A). For treatments without soil mulching, SS/TA ratio decreased to 25.12 at 19.1 mL L⁻¹, followed by a consecutive increasing with humic substances dose enhancement (Figure 5A). As shown in Figure 5B, average SS/TA ratio decreased from 35.07 in the first harvest to 20.72 in the second harvest, corresponding to a reduction of about 40.9%. The lower average shown in Figure 5B is higher than 26.04 presented by Ramos et al. (2011) for guava cv. Paluma. It is important to infer that SS/TA ratio is a characteristic for fruit sweetness evaluation due to the balance of acids and sugars, which is more representative than the measurement of these parameters alone. Therefore, when SS/TA ratio is high, it means that the fruit has a good taste and adequate maturation stage, as this ratio increases when there is a decrease in acidity and high content of soluble solids derived in maturity (Batista et al., 2013).

The reduction in SS/TA ratio from the first to the second harvest (Figure 5B) may have occurred due to rainfall and lower temperatures from April to July 2013. According to Souza et al. (2010), independent of maturation stage, rainfall reduces pH and SS/TA ratio as a function of more water content of fruits.

Conclusions

Humic substances enhance fruit production more efficiently in guava plants grown without soil mulching. Guava fruit production increases from the first to the

second harvest. Fruit quality for titratable acidity, soluble solids, vitamin C and soluble solids/titratable acidity ratio is adequate independent of soil mulching use. Humic substances improve fruit quality of guava cv. Paluma. Under soil and climate conditions, and considering the first two production cycles of guava, it is possible to recommend about 20 mL L⁻¹ of humic substances for production of high quality guava fruits.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Growth and physiological aspects of bell pepper (*Capsicum annuum*) under saline stress and exogenous application of proline

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This study aimed to evaluate growth and physiological aspects of 'All Big' bell pepper, under saline stress and exogenous application of proline on the leaves. The research was conducted in pots adapted as drainage lysimeters under greenhouse conditions, using sandy-loam eutrophic Regolithic Neosol, in the municipality of Campina Grande-PB, Brazil. The experiment was set in randomized blocks, in order to test two levels of irrigation water electrical conductivity - EC_w (0.6 and 3.0 dS m⁻¹) associated with four proline concentrations (0, 10, 20 and 30 mmol L⁻¹). Plants were grown in 10-L pots for 50 days after transplantation. This period corresponds to the transition of vegetative and reproductive stages, in which plants were evaluated for growth, gas exchanges and chlorophyll a fluorescence. The increase in irrigation water salinity reduced growth, gas exchanges and the efficiency of photosystem II in bell pepper plants. Proline concentrations from 12.8 to 16.8 mmol L⁻¹ incremented the activity of gas exchanges, reducing the effect of saline stress on bell pepper plants. Proline concentrations under study did not influence chlorophyll a fluorescence of 'All Big' bell pepper plants.

Key words: Saline water, gas exchanges, chlorophyll a fluorescence, proline.

INTRODUCTION

Bell pepper (*Capsicum annuum* L.) is one of the main Solanaceous crop cultivated in the world. In Brazil, the species is among the ten most important vegetables in the market, for being an attractive crop, with short period

for the beginning of production, which promotes rapid return of investments, thus being widely explored by small- and medium-sized farmers, especially in the Northeast region of the country, where it is mainly

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consumed fresh (Leonardo et al., 2007; Campos and Cavalcante, 2009; Nascimento et al., 2011).

In Northeast Brazil, more precisely in the semiarid region, bell pepper is an important crop and its cultivation is located in irrigated areas, where family farming is practiced. However, due to the scarcity of water, caused by the low rainfalls and high atmospheric demand, which restrict the availability and use of good-quality water in agriculture, it is necessary to use waters of lower quality for irrigation, such as saline water (Cavalcante et al., 2011). Nevertheless, the use of saline water affects the development of sensitive crops, such as bell pepper, which tolerates, without significant yield losses, contents of salts in the soil between 1.3 and 3.0 dS m⁻¹ in terms of electrical conductivity of the soil saturation extract or between 0.8 and 2.0 dS m⁻¹ in terms of irrigation water salinity (Ayers and Westcot, 1999).

In general, the effects of salinity on plants are attributed to the osmotic effect and to the specific action of ion concentration. In bell pepper, countless disorders have been observed due to the excess of salts in the soil, among which: disorders in the permeability of cell membranes, alterations in stomatal conductance, photosynthesis and ionic balance, which lead to reduction in plant development, regardless of the cationic nature of the salts (Aktas et al., 2006; Leonardo et al., 2008). In sweet pepper, salinity causes deficiency in chlorophyll content, proline accumulation, increase antioxidant enzyme activity such as catalase both in roots and leaves (Chookhampaeng, 2011), in addition reduces shoot and root length, dry weight and leaf area (Ziaf et al., 2009). Al-Jasim et al. (2012) observed application of proline (0, 1, 5, 10 mM), sprayed exogenously, on seedlings of bell pepper seedlings caused decrease in almost all growth parameters of the non-stressed plant.

Thus, there appears the need to search for strategies regarding the management of saline waters in bell pepper cultivation, aiming to maintain the homeostasis when subjected to conditions of osmotic and ionic stress. Among the studies aiming at the establishment of plants under saline stress, the management of osmotic adjustment presents itself as the most promising strategy, since this physiological mechanism is the most effective for maintaining cell turgor, through the accumulation of compatible solutes (proline, glycine betaine, trehalose, sucrose, polyamines, mannitol, pinitol, etc.) in the vacuole or in the cytosol, promoting the maintenance of water balance inside the plant, under deficit conditions, caused by saline stress (Okuma et al., 2004; Ashraf et al., 2011; Lacerda et al., 2012; Marijuan and Bosch, 2013; Monteiro et al., 2014). The amino acid proline is the one of the most studied solute, due to its response, sensitivity and effectiveness under stress conditions (Trovato et al., 2008; Verbruggen and Hermans, 2008; Ashraf et al., 2011; Khan et al., 2015). Lacerda et al. (2012), evaluating exogenous application of proline in yellow melon plants under saline stress, reported that

proline application at concentrations of up to 12 mmol L⁻¹ was efficient at reducing the effects of stress caused by irrigation water salinity, promoting increase of 2.5 t ha⁻¹ in the production of plants under stress. This study aimed to evaluate growth, gas exchanges, and chlorophyll a fluorescence and physiological aspects of 'All Big' bell pepper, as a function of saline stress and exogenous application of proline on the leaves.

MATERIALS AND METHODS

The experiment was carried out during May and August 2015 in a greenhouse, at the Center of Technology and Natural Resources of the Federal University of Campina Grande (CTRN/UFCG), in the municipality of Campina Grande-PB, Brazil, in the mesoregion of 'Agreste Paraibano', situated at the geographic coordinates of 7°15'18" S, 35°52'28" W and mean altitude of 550 m.

The treatments were distributed in a randomized block design, in 2 x 4 factorial scheme, with four replicates each consisting of one plant, corresponding to two levels of electrical conductivity of the irrigation water – EC_w (0.6 and 3.0 dS m⁻¹) associated with three proline concentrations through foliar application (10, 20 and 30 mmol L⁻¹) and a control treatment without application of proline. Water with EC_w of 3.0 dS m⁻¹ was prepared using the salts of sodium chloride (NaCl), calcium chloride (CaCl₂·2H₂O) and magnesium chloride (MgCl₂·6H₂O) in order to have a equivalent proportion of 7:2:1 of Na:Ca:Mg, similar to observed in most water used in irrigation in the northeast region of Brazil (Medeiros et al., 2003). The amount of each salt was determined based on the relationship between EC_w and the concentration of salts (10*mmol_c L⁻¹ = 1 dS m⁻¹).

The bell pepper hybrid used in the experiment was 'All Big', which belongs to the group known as 'cascadura'; this material has upright growth, small size, firm and thick pulp with sweet flavor, high yield and cycle of around 120 days (Araújo et al., 2009). In addition, the hybrid is tolerant to blight (*Phytophthora capsici*) and tomato mosaic virus (ToMV).

The seedlings of 'All Big' bell pepper were produced on expanded polystyrene trays with 128 cells, using the commercial substrate Plantmax[®], and were transplanted to pots when they produced the second pair of definitive leaves.

Plants were grown in 10-L plastic pots filled with a layer of 0.3 kg of crushed stone (number zero), which covered the bottom of the pots, and 14 kg of a eutrophic Regolithic Neosol of sandy loam texture (layer of 0-20 cm), from the rural area of the municipality of Esperança-PB, properly pounded to break up clods, with the following physico-chemical characteristics: Sand = 656.6 g kg⁻¹; Silt = 175 g kg⁻¹; Clay = 168.4 g kg⁻¹; Total porosity = 53.64 m³m⁻³; Available water = 18.42 dag kg⁻¹; Apparent density = 1.27 kg dm⁻³; pH_(1:2.5) = 6.24; EC_{se} = 2.5 dS m⁻¹; OM = 10.79 dag kg⁻¹; P = 48.0 mg kg⁻¹; K⁺ = 0.28 cmol_ckg⁻¹; Na⁺ = 1.82 cmol_ckg⁻¹; Ca⁺² = 7.41 cmol_ckg⁻¹; Mg⁺² = 5.23 cmol_ckg⁻¹; Al⁺³ = 0.0 cmol_ckg⁻¹ and H⁺ = 3.07 cmol_ckg⁻¹. The analyses were performed at the Laboratory of irrigation and salinity of the UFCG, according to the methodologies proposed by Claessen (1997).

After filling the pots, soil water content was brought close to field capacity and, during the experiment, the moisture content in soil was maintained near to field capacity through daily irrigations, which consisted in the application of water corresponding to the treatment in each pot. The volume of water applied in each irrigation was estimated by the water balance in the previous irrigation, that is, water volume applied minus volume drained in the previous irrigation, plus a leaching fraction of 0.15, in order to avoid the excessive accumulation of salts in the soil, according to Ayers and Westcot (1999).

Fertilizations with nitrogen (N), phosphorus (P) and potassium (K) were performed based on the recommendations of Novais et al. (1991), through top-dressing, along with the irrigation water, of 100, 150 and 300 mg kg⁻¹ of soil of N, K₂O and P₂O₅, respectively, in three equal applications, at intervals of fifteen days, and the first application was performed 10 days after transplantation (DAT). Urea, monoammonium phosphate and potassium chloride were used as sources of N, P and K, respectively. Foliar application of proline was performed weekly from 15 DAT on, using a spray bottle in order to obtain the complete wetting of the plants, with a volume that ranged from 10 to 40 mL plant⁻¹, according to the development stage of the plant.

Gas exchanges were determined using the portable device "LCPro+" (ADC BioScientific Ltda.) for photosynthesis measurement, operating with control of temperature at 25°C, irradiation of 1200 μmol photons m⁻² s⁻¹ and air flow of 200 mL min⁻¹, and CO₂ coming from the environment at a height of 3 m from the soil surface. The following variables were analyzed: CO₂ assimilation rate (*A*) (μmol m⁻² s⁻¹), transpiration (*E*) (mol of H₂O m⁻² s⁻¹), stomatal conductance (*g_s*) (mol of H₂O m⁻² s⁻¹) and internal CO₂ concentration (*C_i*) (μmol m⁻² s⁻¹) on the third leaf from the apex. Based on these data, the intrinsic water use efficiency (*WUE*) (*A/E*) [(μmol m⁻² s⁻¹) (mol H₂O m⁻² s⁻¹)⁻¹] and instantaneous carboxylation efficiency (*A/C_i*) (*EiCi*) (Silva et al., 2014) were quantified. These data were obtained in the transition of vegetative and reproductive stages, at 40 DAT.

In the same period, chlorophyll *a* fluorescence was determined using a pulse-modulated Fluorometer (Model OS5p - Opti Science). The Fv/Fm protocol was used in order to determine the variables of fluorescence induction: Initial fluorescence (*F₀*), Maximum fluorescence (*F_m*), Variable fluorescence (*F_v* = *F_m*-*F₀*) and Maximum quantum efficiency of photosystem II (*F_v*/*F_m*) (Sá et al., 2015). Such protocol was performed after leaf adaptation to the dark, and at sunrise, using a clip of the device, in order to guarantee that all primary acceptors are oxidized, that is, the reaction centers are open.

Also using the pulse-modulated fluorometer, two hours after fluorescence evaluations with adaptation to the dark, the evaluations under conditions of light were performed using the Yield protocol, by applying a source of actinic lighting with multi-flash saturation pulse, attached to a clip for the determination of photosynthetically active radiation (PAR-Clip) in order to determine the variables: Initial fluorescence before the saturation pulse (*F'*), maximum fluorescence after adaptation to saturating light (*F_m'*), electron transport rate (ETR) and quantum efficiency of photosystem II (*Y_{II}*).

The effects of the treatments on the growth of 'All Big' bell pepper were evaluated through the determination of plant height (PH), measured with a graduated ruler, stem diameter (SD), measured using a digital caliper and the number of leaves, through the count of mature leaves, at 50 DAT.

The collected data were subjected to analysis of variance by F test and, when significant, regression analysis was performed for the quantitative factor i.e. proline concentrations, while the Tukey's test at 0.05 probability level was applied for comparison of means of irrigation water salinity, using the statistical program SISVAR-ESAL (Ferreira, 2011).

RESULTS AND DISCUSSION

The plant height, stem diameter and number of leaves of bell pepper was reduced (*p*<0.05) by 16.4, 9.3 and 15.4%, respectively (Figure 1A, C and E) under irrigation water salinity of 3.0 dS m⁻¹ in comparison to that of 0.6 dS m⁻¹ at 50 DAT. Increment in irrigation water salinity increased soil salinity to levels above the threshold of the

crop, causing physiological and nutritional alterations in the bell pepper plants, due to toxicity by specific ions; for example, the reduction in the permeability of cell membranes, photosynthesis and ionic balance, as observed by Leonardo et al. (2008) and Aktas et al. (2006), in bell pepper cultivation under saline water irrigation.

There was no effect of proline concentrations on the variable number of leaves (*p* > 0.05). The variables plant height and stem diameter tended to decrease as the proline concentrations increased from 0 to 30 mmol L⁻¹, regardless of the salinity condition (Figure 1B and D). The exogenous application of proline possibly induces the osmotic adjustment, even in the absence of water restrictions in the soil, thus causing an expenditure of energy, which compromises plant growth (Taiz and Zaiger, 2013).

There was no influence of application of proline in different concentrations on the net CO₂ assimilation rate (*A*) of bell pepper plants cultivated in the control treatment (EC_w=0.6 dS m⁻¹), with a mean value of *A* of 21.98 μmol m⁻² s⁻¹. However, plants subjected to saline stress responded quadratically to the increment in proline concentrations, with maximum *A* (22.75 μmol m⁻² s⁻¹) at the concentration of 15.7 mmol L⁻¹ (Figure 2A).

The results observed for CO₂ assimilation rate corroborate those of *C_i*, for which there was also no effect of proline concentrations in the control treatment. However, under high-salinity conditions, plants subjected to foliar applications from 0 to 30 mmol L⁻¹, where the lowest values of *A* were observed, showed the highest *C_i* values (Figure 2B). This indicates that the lower photosynthetic activity in this treatment is not related to limitations performed by the stomatal activity under the CO₂ inflow, but by the low activity of the ribulose 1,5-bisphosphate carboxylase (RuBisCo), inefficiently acting in the carboxylation of CO₂ (Machado et al., 2010; Lacerda et al., 2012).

The previously cited assumptions are confirmed by the results of instantaneous carboxylation efficiency (*EiCi*) (Figure 2C). There was low *EiCi* in plants at the highest and lowest studied concentrations of proline (0 and 30 mmol L⁻¹) under conditions of high salinity (Figure 2C). This denotes the low carboxylation efficiency of the RuBisCo enzyme, which may be related to the action of factors of non-stomatal nature, such as the low availability of ATP and NADPH from the electron transport chain of the photosystem II (PSII) (Silva et al., 2014; Sá et al., 2015).

The best photosynthetic activity observed at the intermediate proline concentrations may be related to the maintenance of cell turgor, through the accumulation of this solute (vacuole or cytosol), thus promoting the maintenance of water balance inside the plant, under deficit conditions, caused by saline stress (Okuma et al., 2004; Ashraf et al., 2011; Lacerda et al., 2012; Marijuan and Bosch, 2013; Monteiro et al., 2014; Khan et al.,

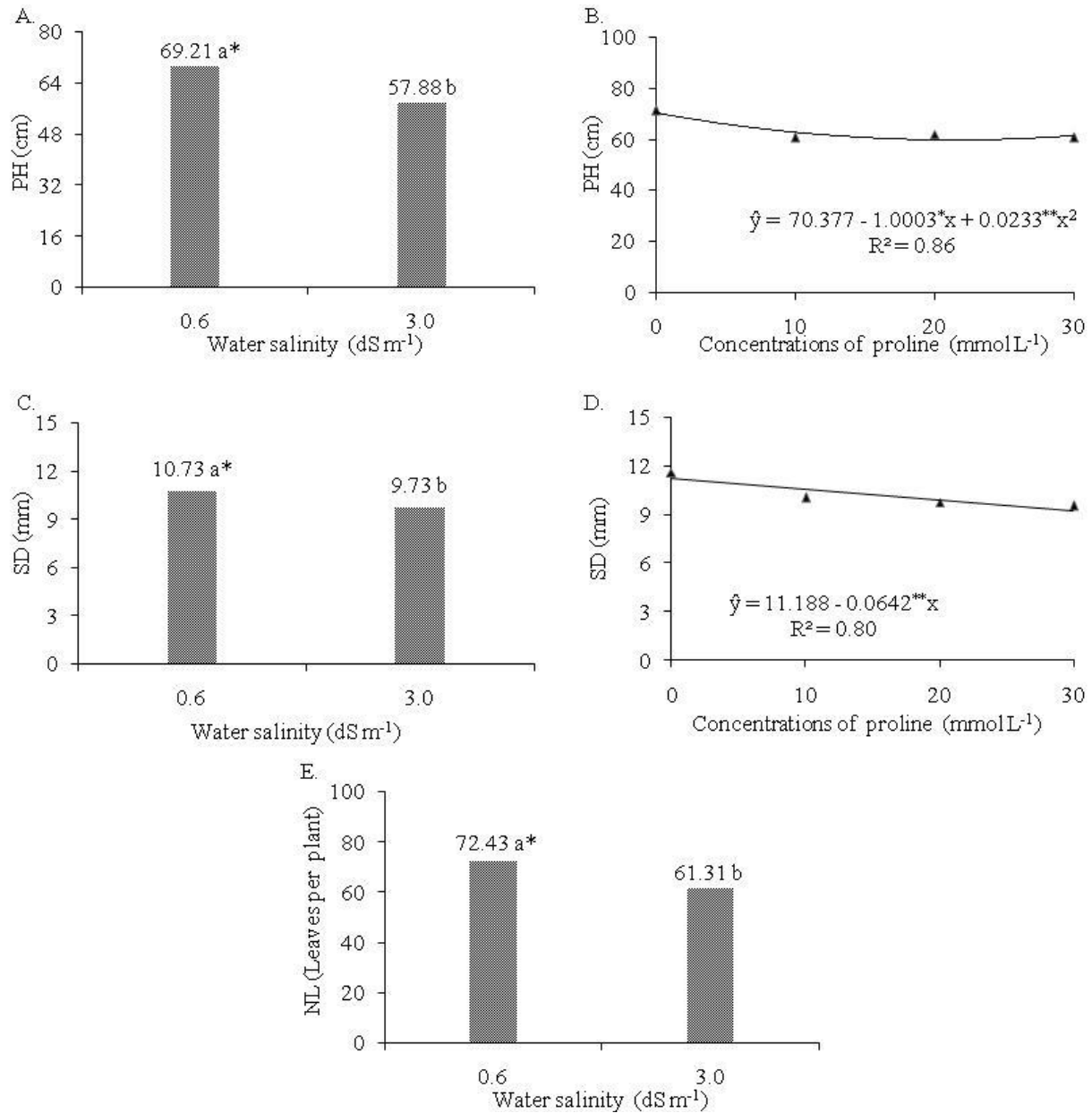


Figure 1. Plant height (PH) (A and B), stem diameter (SD) (C and D) and number of leaves (NL) (E) of 'All Big' bell pepper plants under different levels of water salinity and exogenous application of proline on the leaves. (*) and (**) significant at 0.05 and 0.01 probability, respectively; (NS) not significant; Means followed by different letters indicate difference between treatments by Tukey's test at $p < 0.05$.

2015). Believed to that the highest proline concentration (30 mmol L⁻¹) reduced the internal water potential of bell pepper plants to very low levels and, when associated with irrigation using water of higher EC_w, stimulated greater absorption of water and consequently greater absorption of toxic ions by the plant (Aktas et al., 2006).

Exogenous application of proline on the leaves reduced stomatal conductance and, consequently, affected the transpiration of bell pepper plants irrigated with water of low salinity (Figure 1D and E). It should be pointed out

that the reduction in stomatal conductance in plants cultivated under low salinity reached lower levels, compared with plants under high salinity. These results denote the efficiency of exogenous application of proline in the stomatal regulation of bell pepper plants, promoting higher transpiration without any damage to photosynthetic activity and with gains in water use efficiency.

Stomatal conductance and transpiration of bell pepper plants, when irrigated with water of high salinity, responded quadratically, with maximum values of

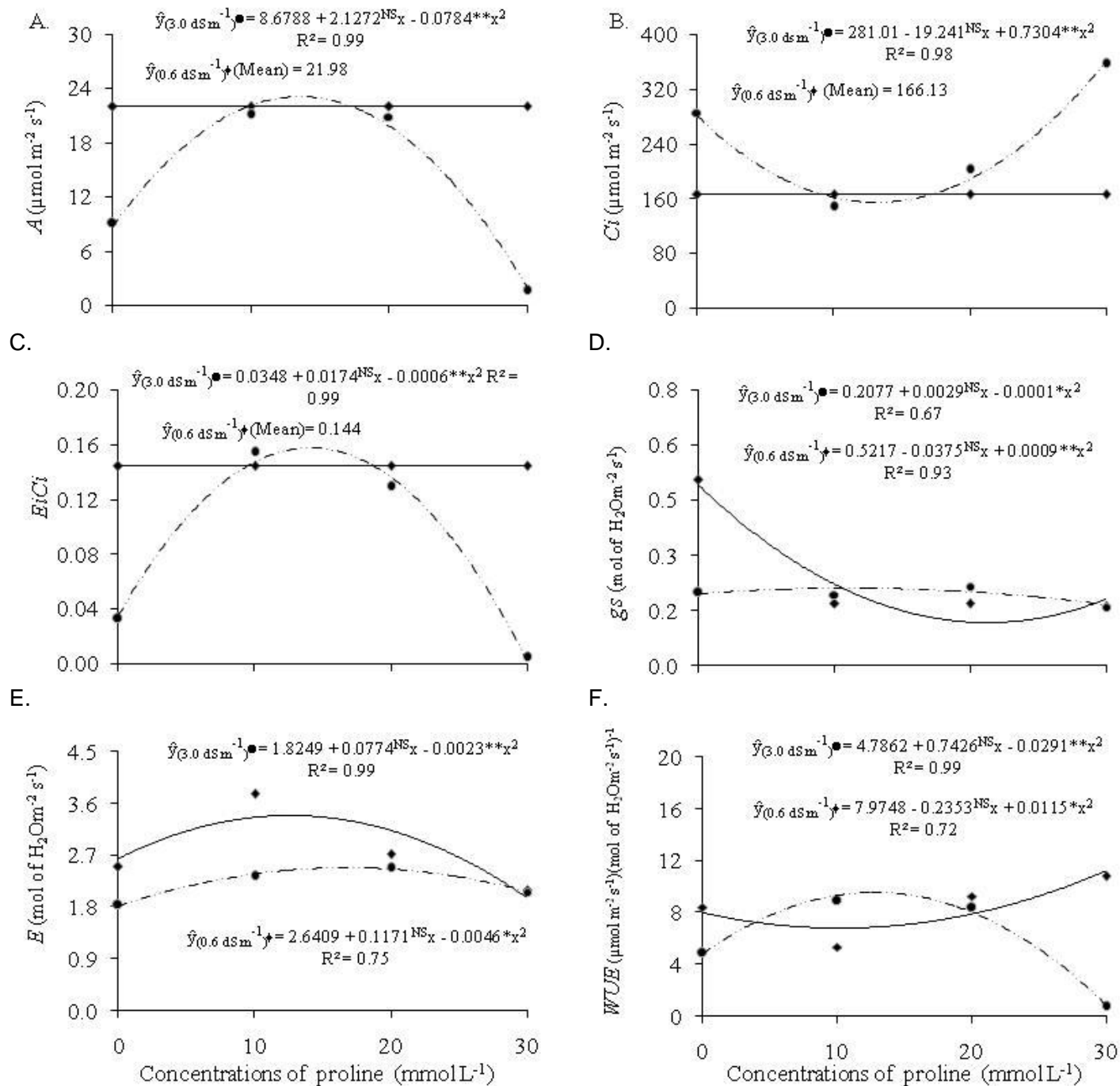


Figure 2. CO₂ assimilation rate (*A*, μmol m⁻² s⁻¹) (A), internal CO₂ concentration (*C_i*, μmol m⁻² s⁻¹) (B), instantaneous carboxylation efficiency (*E_i/C_i*) (C), stomatal conductance (*g_s*) (D), transpiration (*E*) (E), water use efficiency (*WUE*) (F) of 'All Big' bell pepper plants under levels of water salinity and concentrations of proline applied on the leaves. (*) and (**) significant at 0.05 and 0.01 probability, respectively; (NS) not significant.

approximately 0.23 and 2.48 mol of H₂O m⁻² s⁻¹, at the estimated proline concentrations of 14.5 and 16.8 mmol L⁻¹ (0.21 and 1.82 mol of H₂O m⁻² s⁻¹), respectively (Figure 2D and E). It is observed that, despite the small variation in stomatal conductance, there was a significant

increase in the transpiration rate of the plants, when subjected to the concentrations of proline. These responses may be related to the osmotic adjustment promoted by the exogenous application of proline on the leaves, so that transpiration guaranteed the occurrence

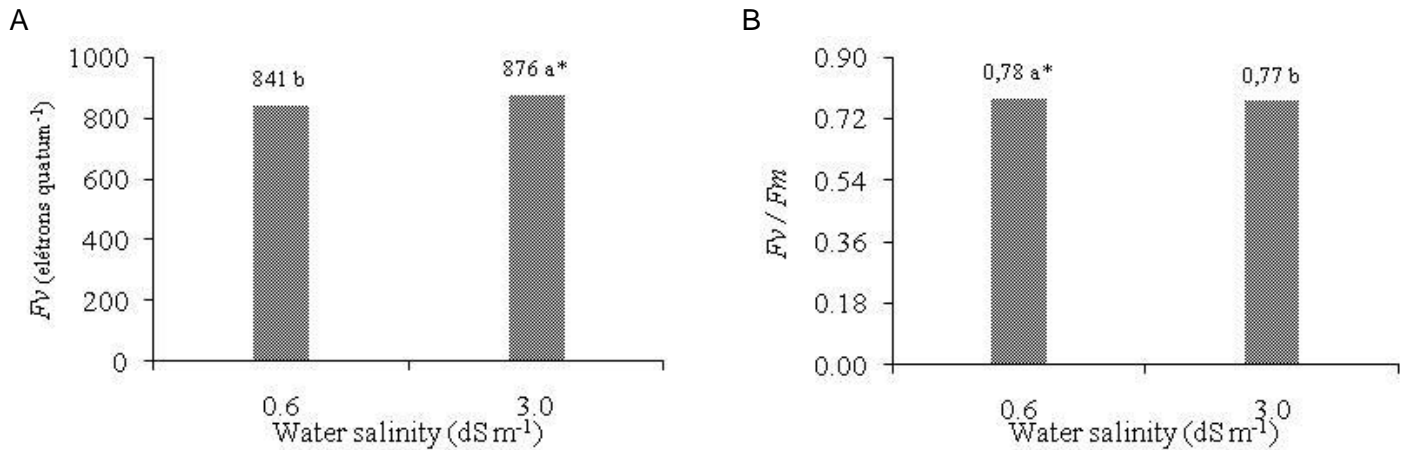


Figure 3. Variable fluorescence (F_v) (A) and quantum efficiency of photosystem II (F_v/F_m) (B) of 'All Big' bell pepper plants under levels of water salinity and concentrations of proline applied on the leaves. Means followed by different letters indicate significant difference between treatments by Tukey's test, $p < 0.05$.

of absorption and transport of water inside the plant, maintaining its water relationships active, through stomatal regulation (Shimazaki et al., 2007; Taiz and Zaiger, 2013). Thus, the increase in transpiration is an indication of the efficiency of exogenous application of proline in the osmotic regulation of bell pepper plants, especially because there was an increase in the water use efficiency of these plants, with increment in proline concentration until the estimated value of 12.8 mmol L⁻¹ (Figure 2F).

As to chlorophyll fluorescence, there was significant influence ($p < 0.05$) of the levels of irrigation water salinity on variable fluorescence and quantum efficiency of PSII; in comparison to low salinity water, there was an increase of 4.0% in F_v and reduction of 1.3% in F_v/F_m , when subjected to irrigation with water of high salinity (3.0 dS m⁻¹) (Figures 3A and B). The increase in variable fluorescence is possibly a strategy of the species to mitigate the effects of saline stress on the photosynthetic activity, increasing photochemical activity in order to meet the necessity of ATP and NADPH in the biochemical stage of photosynthesis and, consequently, maintain the activity of gas exchanges at satisfactory levels, considering the reductions observed in these variables with the increase in irrigation water salinity. This result indicates that saline stress is acting on the photochemical activity of bell pepper plants in the beginning of the reproductive stage at 40 DAT, because the reduction in the quantum efficiency of PSII is an indication of photo inhibitory damages, which result in the loss of efficiency in the transfer of energy from the photosystem II (P_{680}) to the photosystem (P_{700}) and, consequently, reduction in the synthesis of ATP and NADPH (Baker and Rosenqvst, 2004; Silva et al., 2014; Sá et al., 2015). However, the observed damages are still incipient, considering that there was no influence of the treatments on the electron transport rate (ETR).

Conclusions

Irrigation with high salinity water (EC=3.0 dS m⁻¹) reduce growth, gas exchanges and efficiency of the photosystem II in 'All Big' bell pepper plants.

Exogenous applications of proline in concentrations from 12.8 to 16.8 mmol L⁻¹ increase the activity of gas exchanges, reducing the effect of saline stress on bell pepper plants.

Proline concentrations influence positively the variable fluorescence and reduce quantum efficiency of photosystem II of chlorophyll *a* of 'All Big' bell pepper plants.

Conflict of interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Protective effects of some fruit juices with low-fat diet on rat testis damaged by carbon tetrachloride: A genetic and histological study

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Oxidative stress, free radical, lipid peroxidation and antioxidant have become a common expression with most disease and methods for protection. Carbon tetrachloride (CCl₄) is an industrial solvent which has destructive effects on a cell while most fruit juices have antioxidant effects. The aim of this study was to investigate the protective role of fruit juice on testis after toxic effect with CCl₄ through oxidative stress with basal diet and low-fat diet. Seventy-five male albino rats were used for this study in which the juices of three fresh fruit, yellow apples (*Malus domestica*, L.), red grapes containing seeds (*Vitis vinifera*, L.) and pomegranates (*Punica granatum*, L.) were used as therapeutic agents. Histological sections of testis indicated that low-fat diet has obvious effects than basal diet in both the low-fat diet with CCl₄ "LdC" con++", LdC with grape juice 2 ml "grpL2", LdC with pomegranate juice 2 ml "pomL2" and hyper effect in LdC with pomegranate juice 4 ml "pomL4" while it was equal in effect with basal diet in the other treatments. Low-fat diet gave significant effects (about 75% recovery in con++, LdC with Apple juice 2 ml and 4 ml "appL2, appL4", LdC with grape juice 4 ml "grpL4" and pomL2) while 25% began to recover as shown in basal diet with pomegranate juice 4 ml "pomB4" and grpL2. Treatment of rats with pomegranate juice ameliorated the toxic effects of CCl₄ with low-fat diet on Semi-random RAPD-DNA profile. Low-fat diet with fruit juice had positive effect against toxicity induced by CCl₄ in testes of rats on the level of histological and DNA-RAPD studies.

Key words: CCl₄, rat testis, yellow apples, red grapes, pomegranates, low-fat diet, oxidative stress.

INTRODUCTION

By alteration of protein and nucleic acid structure, oxidative stress has a destruction effect on cells.

Moreover, oxidative stress increases the intracellular free calcium, destruction of cells by lipid peroxidation thereby

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damaging the membrane ion transport and permeability (Reckmage et al., 1989). Because of its association with some of the abnormal physiological processes, lipid peroxidation has attracted much attention in recent years (Hartley et al., 1999; Reckmage et al., 1989). Due to the abundance of highly unsaturated fatty acids and the presence of potential reactive oxygen species generating systems, Testicular micro-environment characterize by low oxygen tensions, but this tissue remains vulnerable to oxidative stress. Testicular micro-environment generated from the mitochondria and a variety of enzymes including the xanthine and NADPH-oxidases (Banfi et al., 2001; Kumagai et al., 2002) and the cytochrome P450s (Zangar et al., 2004).

Carbon tetrachloride (CCl_4) is an industrial solvent that cause damages in experimental animals especially in kidney, lungs and testicular (Abraham et al., 1999). CCl_4 is xenobiotic which can be metabolized by hepatic microsomal cytochrome P450 to trichloromethyl free radical which reacts with sulfhydryl groups and antioxidant enzymes. CCl_4 can induce chemical liver injury and over production of trichloromethyl free radicals which initiate a membrane lipid peroxidation that is viewed as a complicated biochemical reaction. These biochemical reactions involving metal ions, free radicals, oxygen and a host of other factors in the biological system and leading to various pathological changes (Bast, 1993; Cemek et al., 2010; Szymonik-Lesiuk, et al., 2003).

CCl_4 -induced reproductive toxicity in male rats, free radicals of CCl_4 bind with polyunsaturated fatty acid of sperm membrane to produce alkoxy and peroxy radicals that generate lipid peroxides that are highly reactive, alters hormonal levels, reduces enzyme activity, change sperm concentration and induce injury or necrosis (Sikka et al., 1995; Ogeturk et al., 2005). Protected the testes and other origins against antioxidant status induced by CCl_4 can be achieved by natural products containing antioxidant as shown through some studies (Khan et al., 2009; Cemek et al., 2010).

Apples may play a large role in reducing the risk of a wide variety of chronic disease, the phrase that says "An apple a day keeps the doctor away" is quite popular but many studies have provided the scientific backing for both of these very common phrases. Apples also, maintain a healthy lifestyle in general (Boyer and Liu, 2004).

Many studies reported that grapes have antioxidant properties (Park et al., 2003; Dani et al., 2007; Buchner et al., 2014) and this antioxidant properties could be at least attributed to the high phenolic content present in grape juice (Dani et al., 2008) where others refer to grape juice modulates as apoptosis but not oxidative stress (Oshima et al., 2015). Many cultures indicated that pomegranate fruit has been used as a natural medicine; the antioxidant defense mechanism was augmented by pomegranate juice against CCl_4 -induced reproductive toxicity, pomegranate juice provides evidence that it may have a therapeutic role in free radical mediated diseases

(Al-Olayan et al., 2014). In these days, pollution is widespread with chemical materials which cause oxidative stress in the human body and has an effect on reproduction. So the aim of this study is to investigate these effects, and reduce it with a natural material like fresh fruit juice.

MATERIALS AND METHODS

Fruits, rats and chemicals

Fruits [fresh yellow apples (*Malus domestica*, L.), red grapes containing seeds (*Vitis vinifera*, L.) and pomegranates (*Punica granatum*, L.)] were obtained from the local market, Tanta city, El-Gharbia governorate, Egypt. Normal male albino rats ($n = 75$) of Sprague Dawley strain weighing 200 ± 10 g used in this experiment were obtained from the laboratory animal colony, Helwan Farm, Vaccine and Immunity Organization, Cairo, Egypt. Casein, DL-methionine, choline chloride, vitamins, minerals, cellulose, CCl_4 and other required chemicals were obtained from Elgomhorya Company for Chemicals and Drugs, Cairo, Egypt. Corn starch and soybean oil were purchased from the local market, Tanta city, El-Gharbia governorate, Egypt.

Preparation of fruit juices

Samples of fruits were cleaned and free from evidence of insect infestation and objectionable materials. Afterwards, grape and pomegranate fruits were homogenized in the blender individually without water or sugar. In contrast, apple juice was prepared using water (30g/100 ml) and with zero sugar. Pomegranate peels, in particular, were removed before homogenization and homogenized with their seeds, whereas apple homogenized with their peels and without seeds. After that, each juice was cleared and administered to rats immediately as a fresh juice.

Biological experiment

Male albino rats ($n = 75$) of Sprague Dawley strain weighing (200 ± 10 g) were housed in well aerated cages under hygienic conditions and fed on basal diet for one week for adaptation. Basal diet was prepared from fine ingredients per 100 g. The diet had the following composition: Casein (>85% protein) 14%, soybean oil 10%, cellulose 5%, salt mixture (Hegsted et al., 1941) 3.5%, vitamin mixture (Campbell, 1961) 1%, choline chloride 0.25%, DL-methionine 0.3% and corn starch up to 100g (AIN, 1993). Low-fat diet was prepared from fine ingredients per 100 g. The diet had the following composition: Casein (>85% protein) 14%, soybean oil 4%, cellulose 5%, salt mixture, Hegsted et al., 1941, 3.5%, vitamin mixture (Campbell, 1961) 1%, choline chloride 0.25%, DL-methionine 0.3% and corn starch up to 100 g (Reeves et al., 1993). Dextrose was added to complete the weight of vitamin mixture to one kilogram. Vitamins A and D were supplied by adding 0.5 g of cod liver oil to the diet. Vitamin E was supplied from sunflower oil to give the final concentration 50 mg/kg diet. After this period, rats were divided into two main groups. The first main group (5 rats) was kept as a negative control group (con-) and fed on basal diet only. The second main group (70 rats) was injected subcutaneously with CCl_4 in paraffin oil (50% v/v, 2 ml/kg body weight) twice a week for two weeks to induce chronic damage in the liver according to Jayasekhar et al. (1997). To examine the effectiveness of induction, blood samples were withdrawn from eye plexus of veins and the activities of transaminases (AST "SGOT" and ALT "SGPT") were

Table 1. The treatments and its abbreviations.

No.	Treatment	Abbreviation
1	Basal diet only "Bd"	con-
2	Bd with CCl ₄ "BdC"	con+
3	BdC with Apple juice 2 ml	appB2
4	BdC with Apple juice 4 ml	appB4
5	BdC with grape juice 2 ml	grpB2
6	BdC with grape juice 4 ml	grpB4
7	BdC with pomegranate juice 2 ml	pomB2
8	BdC with pomegranate juice 4 ml	pomB4
9	low-fat diet with CCl ₄ "LdC"	con++
10	LdC with Apple juice 2 ml	appL2
11	LdC with Apple juice 4 ml	appL4
12	LdC with grape juice 2 ml	grpL2
13	LdC with grape juice 4 ml	grpL4
14	LdC with pomegranate juice 2 ml	pomL2
15	LdC with pomegranate juice 4 ml	pomL4

determined in serum (Reitman and Frankel, 1957). AST activity in CCl₄-injected group was 160.57±5.68 U/L versus 61.71±9.99 U/L in normal control group, while ALT activity in CCl₄-injected group was 85.88±5.98 U/L versus 21.65±3.45 U/L in normal control group. After that, injected rats were divided into 14 equal subgroups. Of which, 7 groups were fed on basal diet only (positive control group "I" or basal diet) plus either apple, grape or pomegranate juices in two doses (2 and 4 ml), while the others were fed on low-fat diet (4% soybean oil) only (positive control group "II" or low-fat diet) plus either apple, grape or pomegranate juices in two doses (2 and 4 ml) Table 1. Juices were administered using a stomach tube and given once daily. After 28 days, animals were sacrificed under other anesthetized and testes were removed by careful dissection, and one of two testes was immersed in formalin solution (10%) to be examined histopathologically, while the others were taken for DNA analysis.

Histopathological examination

Testis of rats were taken and immersed in 10% buffered neutral formalin solution. After immersing in formalin, the fixed specimens were trimmed, washed and dehydrated by passing in serial concentrations in ascending grades of alcohol. Furthermore, testis were then cleared in xylol and embedded in paraffin. They have now been cut in sections with thickness of 4-6 microns and stained with haematoxylin and eosin as described by Drury and Wallington (1980).

Total Genomic DNA Extraction

To extract the total genomic DNA, aTIAamp genomic DNA Kit (Cat. no. DP304, TianGen) was used according to the manufacturer's protocol. Briefly, rat tissue (10 mg) was washed, resuspended and centrifuged at 10,000 rpm for 1 min. Proteinase K were added in 20 µl to samples and incubated at 56°C until completely lyses. Finally, DNA was dissolved in 80 µl TE buffer.

Semi-Random Amplified Polymorphic DNA-Polymerase Chain Reaction (Semi-random RAPD)

Seven Semi-Random RAPD (intron-exon splice junctions (ISJ))

Table 2. Primers, their codes and sequences used in this study.

Number	Primer Code	Sequence (5'→3')
1	ISJ-3	TGC AGG TCA G
2	ISJ-4	GTC GGC GGA CAG GTA AGT
3	ISJ-5	CAG GGT CCC ACC TGC A
4	ISJ-6	ACT TAC CTG AGC CAG CGA
5	ISJ-7	TGC AGG TCA GGA CCC T
6	ISJ-11	TGC AGG TCA AAC GTC G
7	ISJ-12	GGA CTG GAG CAG GTA AGT

primers were screened against the pooled rats DNA. The list of primers and their sequences are presented in Table 2.

The optimization of PCR conditions for each primer was performed in a 20 µl reaction volume including 1 µl of isolated template DNA. Final concentration of each reaction was 1x master mix (MyTaq™ Red Mix, Bioline, England), 0.8 µM primers. Amplifications were carried out in a thermal cycler PCR machine according to the instructions of the manufacturer as follows: the initial amplification program started with 95°C to denaturation for 2 min, followed by 35 cycles consisting of denaturation at 95°C for 15 s, annealing at 30 - 50°C according to the primer for 20 s and elongation at 72°C for 1 min.

The program ended with a final elongation step for 5 min at 72°C. The amplified products were separated on 1.2 % agarose gel, stained with ethidium bromide and photographed with UV-Gel documentation. A known DNA Ladder (O'GeneRuler DNA Ladder Mix ready-to-use, Cat-no: #SM1173, Thermo Scientific) was run against the PCR products, Weining and Langridge (1991), Sawicki and Szczecinska (2007).

Data analysis

Genomic template stability (GTS) has been calculated as follows: $GTS (\%) = (1 - a/n) \times 100$; where n is the number of total bands detected in the control and a is the number of polymorphic bands detected in each treated sample. Polymorphism observed in the semi-RAPD profile included appearance of a new band and disappearance of a normal band in comparison to control semi-RAPD profile (Luceri et al., 2000; Atienzar et al., 2002; Qari, 2010).

RESULTS AND DISCUSSION

Histological studies

A number of reports clearly demonstrated that CCl₄ does not only induce free radical attack against liver cells, but also against many tissues such as kidney, heart, lung, testis and brain, and may induce oxidative injury in these tissues (Dashti et al., 1989; Adewole et al., 2007). In this present study, histological and genetic investigations were carried out on rat testis to evaluate high toxicity on reproductive tissue and the role of natural juice in protecting against the side effects of CCl₄.

Microscopically, testis of rat from negative control "con-" revealed normal seminiferous tubules (Figure1a). Rat testis from positive control "con+" appeared to be spermatogoneal cells lining seminiferous tubules

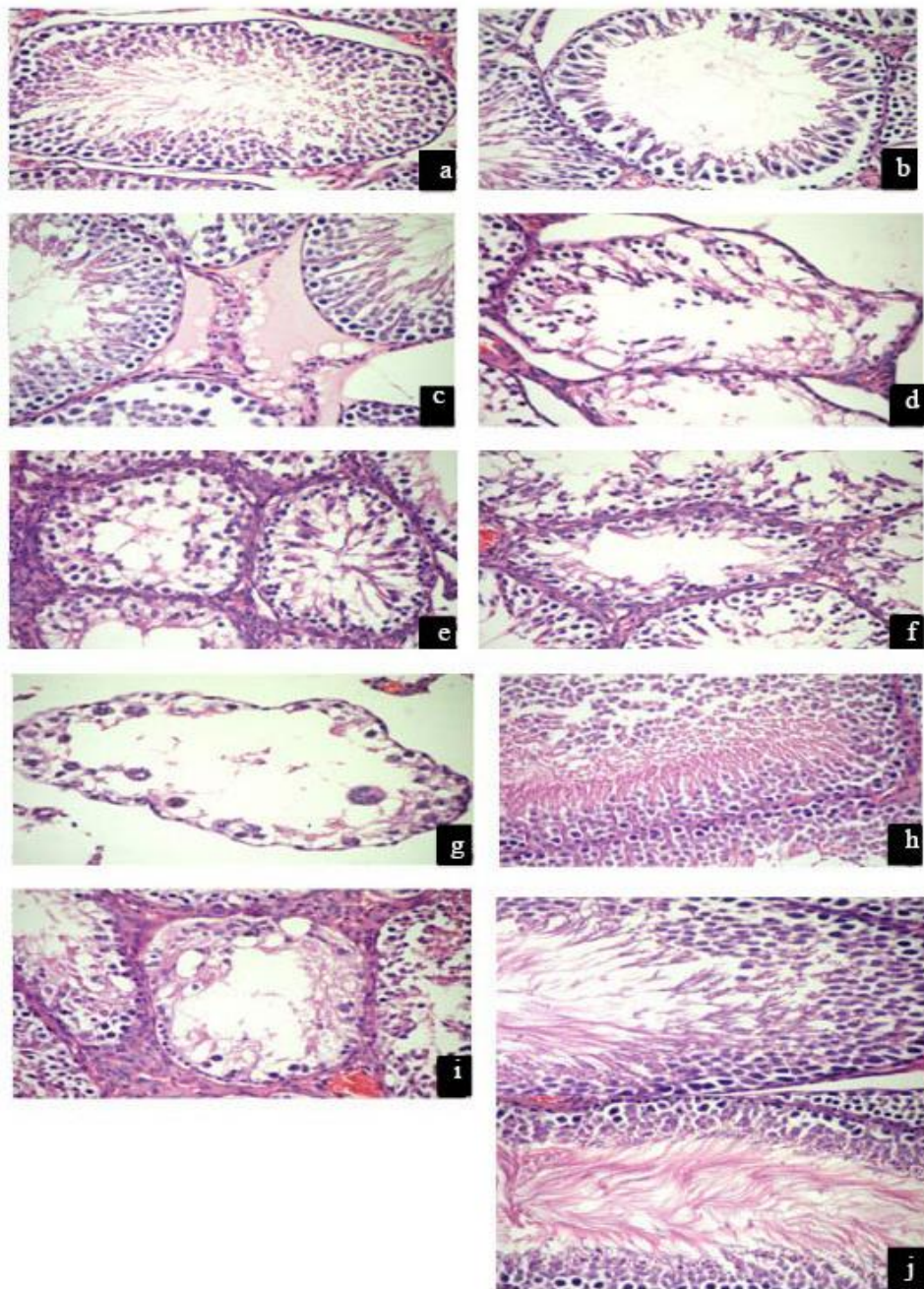


Figure 1 (a-j). Histological studies on rat testis damaged by carbon tetrachloride and treated with fruit juice. "a" con- showing normal seminiferous tubules; "b" con+ showing degeneration of spermatogonial cells lining seminiferous tubules; "c" con+ showing interstitial oedema; "d" grpB2 showing necrosis of spermatogonial cells lining seminiferous tubules; "e" grpB4 showing necrosis of spermatogonial cells lining seminiferous tubules; "f" pomB2 showing degeneration and necrosis of spermatogonial cells lining seminiferous tubules, "g" pomB2 showing spermatide giant cells in the lumen of seminiferous tubules; "h" con++ showing hyperactivation of spermatogonial cells lining seminiferous tubules; "i" grpL4 showing necrosis of spermatogonial cells lining seminiferous tubules; "j" pomL4 showing hyperactivation and hyperplasia of spermatogonial cells lining seminiferous tubules.

degeneration (Figure1b) in addition to interstitial oedema (Figure1c), whereas no histological changes were noticed

in testis of rats from appB2 and appB4 groups. Examined sections from grpB2 and some sections from grpB4

revealed spermatogoneal cells lining seminiferous tubules necrosis (Figure 1d and 1e) respectively, whereas no histological changes has been detected from other sections from grpB4. Tests of rat from pomB2 revealed necrosis and degeneration in spermatogoneal cells lining seminiferous tubules (Figure 1f) and spermatid giant cells in the lumen of seminiferous tubules (Figure 1g). However, testis of rat from pomB4 and some sections from low-fat diet with CCl₄ treatment "con++" revealed no histological changes whereas, other sections from con++ showed hyper-activation of spermatogoneal cell lining seminiferous tubules (Figure 1h). More also, testis of rat from appL2, appL4, grpL2 and some sections from grpL4 revealed no histological changes whereas, other sections from grpL4 showed necrosis of spermatogoneal cells lining seminiferous tubules (Figure 1i). No histological changes were noticed in testes of rat from pomL2. Meanwhile, tests of rat from pomL4 revealed hyper-activation of spermatogoneal cells lining seminiferous tubules (Figure 1j).

From previous results, we noticed that CCl₄ destroy testis in positive control, this result agree with Khan (2012) who reported that CCl₄ caused loss of germ cells, interruption in meiosis, sperm with abnormal shape, abnormality of germinative epithelium, fibroblast and inflammatory cells, as well as those caused by atrophy of somniferous tubules. Whereas CCl₄ with low-fat diet showed recovery in some section, the others did not. Orally, treatment with apple juice revealed a recovery of testicular abnormalities induced by CCl₄ in both concentrations 2 and 4 ml in both of basal diet and low-fat diet. Treatment with grape juice did not show any positive effect with concentration 2 ml in basal diet but it was more effective in low-fat diet. In concentration, 4 ml in some sections were recovered while others did not in both of basal and low-fat diet.

Pomegranate did not show any effect at a level of 2 ml but 4 ml had positive effect in basal diet. In low-fat diet, pomegranate at 2 ml was more effective and 4 ml was hyper effective as shown in Figure 1j.

From previous results, we can conclude that low-fat diet has obvious effects than basal diet in both of con++, grpL2, pomL2 and hyper effect in pomL4 while low-fat diet was in equal effect with basal diet in the other treatments, so it could be indicated that low-fat diet gave significant effects (about 75% in con++, appL2, appL4, grpL4, pomL2 recovery) while 25% began to recover as shown in pomB4 and grpL2.

Yang et al. (2010) reported that mice which had acute hepatotoxicity induction by CCl₄, gave a significant protective effect when treated with apple polyphenols and this effect may be due to inhibition of lipid peroxidation, its free radical scavenging effect, and ability to increase antioxidant activity. On the other hand, Khan (2012) examined the protective effects of *Launaea procumbens* on testis against oxidative stress of CCl₄ in male rat which also improved the levels of antioxidant enzymes in

CCl₄ administered rats as a result of the presence of phenolic and polyphenolic constituents.

RAPD polymorphism and GTS % among treatments in testes

DNA alteration can be detected by many laboratory technique, one of them is random amplified polymorphism DNA " RAPD", which was developed by Williams et al. (1990) and Welsh and McClelland (1990). It can be used with any organism without prior information on the nucleotide sequences, and this is one of its advantages.

In this experiment, we study the protective role of fruit juice on testis after exposure to CCl₄ as a toxin through oxidative stress. Seven (intron-exon splice junctions (ISJ)) primers have been used with random sequences as shown in Table 2. These primers gave total of fragments 714, and total of 58 bands (Table 3). Twenty five bands out of them were polymorphic with the percentage of 43.1%, whereas thirty three bands were monomorphic (common) for all treatments. The highest level of polymorphism (75 %) was observed with primer ISJ-4. Moreover, the lowest level of polymorphism was 0% with primer 3 as shown in Table 3.

Results from semi-RAPD profile which appeared in Table (3) refer to changes between control and other treatments, while negative control showed in total 45 bands resulted from seven primers, number of these bands ranged from three bands with primer ISJ-3 and 4 to twelve bands with primer 5. The positive control gave 6 variable bands (polymorphic bands include appearance of new bands and disappearance of normal bands) and GTS% was 86.7 in addition to treatments 2 and 12 where showed results similar to positive control. Treatments appB4, appL4, grpL2, pomL2 and pomL4 showed lowest polymorphic bands 5 and GTS% was 88.9 compared with negative control. The highest polymorphic bands were in grpB2 which gave thirteen polymorphic bands, GTS % was 71.1% compared with the negative control.

The results showed high increase in band intensity in treatment pomL2 when compared with negative control, positive control and other treatments, which recorded 13 bands, followed by appL2 and appB4 treatments that recorded 16 bands increase in its intensity for both treatments (Figure 2). On the other hand, the decrease in band intensity was detected in 8 bands in the positive control whereas the recorded high number of other treatments was compared with negative control followed by treatment grpL4 which decrease in 6 bands than negative control.

In this study, the results showed that the decrease in polymorphic bands (5) and increase in GTS % (Table 4) which was 88.9% and increase in intensity was in treatments appB4, appL4, grpL2, pomL2 and pomL4 in rats with toxicity in testis which was induced by CCl₄, the treatment appB4 with basal diet and the others were in

Table 3. Level of polymorphism among treatments in testes compared with control- on RAPD analysis.

Primer	TAF	PB	MP	P%	con-	con+	appB2	appB4	grpB2	grpB4	pomB2	pomB4	con++	appL2	appL4	grpL2	grpL4	pomL2	pomL4	
3	3	0	3	0	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
4	4	3	1	75	3	4	4	3	2	4	3	4	3	3	3	3	3	3	3	3
5	17	8	9	47.1	12	12	12	12	12	12	12	15	11	13	12	12	12	11	11	11
6	12	3	9	25	10	9	12	12	12	12	12	12	12	12	12	12	12	12	12	12
7	7	1	6	14.3	7	6	7	7	7	7	7	7	7	7	7	7	7	7	7	7
11	8	5	3	62.5	4	4	5	3	5	4	5	7	4	4	5	3	4	3	3	3
12	7	5	2	71.4	6	7	6	6	7	7	6	8	6	9	6	4	6	7	7	7
Total	58	25	33	43.1	45	45	49	46	48	49	48	56	46	51	48	44	47	46	46	46
Total												714								

TAF, Total amplified fragment; PB, polymorphic bands; MP, monomorphic bands; P%, polymorphism %.

Table 4. Changes in DNA semi-RAPD profile in rat's testes with CCl4 induced toxicity and treated with fruit juices.

Primer	No. of bands in cont-	con+				appB2				appB4				grpB2				grpB4				pomB2				pomB4			
		a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
3	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	2	0	0	0	1	0	0	0	0	1	0
4	3	1	0	1	0	1	0	1	0	0	0	1	0	1	2	0	0	1	0	0	0	0	0	0	1	1	0	0	0
5	12	0	0	1	2	1	1	0	1	1	1	0	0	2	2	0	0	3	3	0	0	1	1	0	1	3	0	0	0
6	10	0	1	5	1	2	0	9	0	2	0	10	0	2	0	10	0	2	0	10	0	2	0	10	0	2	0	10	0
7	7	0	1	0	4	0	0	1	0	0	0	3	0	0	0	3	0	0	0	1	0	0	0	0	0	0	0	0	0
11	4	1	1	0	0	1	0	0	0	0	1	0	0	2	1	0	0	1	1	0	0	2	1	0	0	4	1	0	0
12	6	1	0	0	0	0	0	0	0	0	0	2	0	1	0	0	1	1	0	0	0	0	0	1	0	2	0	0	0
Total	45	3	3	7	8	5	1	11	1	3	2	16	0	8	5	13	2	8	4	13	0	5	2	12	2	12	1	11	0
	a+b	6				6				5				13				12				7				11			
	GTS %	86.7				86.7				88.9				71.1				73.3				84.4				75.6			

Primer	No. of bands in cont-	con++				appL2				appL4				grpL2				grpL4				pomL2				pomL4			
		a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
3	3	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	1	0	0	1	1	0	0	1	1
4	3	1	1	0	0	0	0	1	0	0	0	1	0	0	0	1	0	1	1	0	2	0	0	1	0	0	0	0	0
5	12	1	2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	1	0	0
6	10	2	0	10	0	2	0	10	0	2	0	10	0	2	0	10	0	2	0	10	0	2	0	10	0	2	0	10	0
7	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	4	1	1	0	0	1	1	0	0	2	1	0	0	0	1	1	0	1	1	1	0	0	1	1	0	0	1	1	0
12	6	1	1	0	3	3	0	4	0	0	0	0	0	0	2	2	0	0	0	0	3	1	0	2	0	1	0	2	0

Table 4. Contd.

Total	45	6	5	11	3	7	1	16	0	4	1	12	0	2	3	15	0	4	2	12	6	3	2	17	1	3	2	14	1
a+b				11				8				5				5				6			5					5	
GTS %				75.6				82.2				88.9				88.9				86.7			88.9					88.9	

a: appearance of new band, b: disappearance of normal band, c: increase in band intensity, d: decrease in band intensity, a+b: polymorphic bands, GTS: genomic template stability.

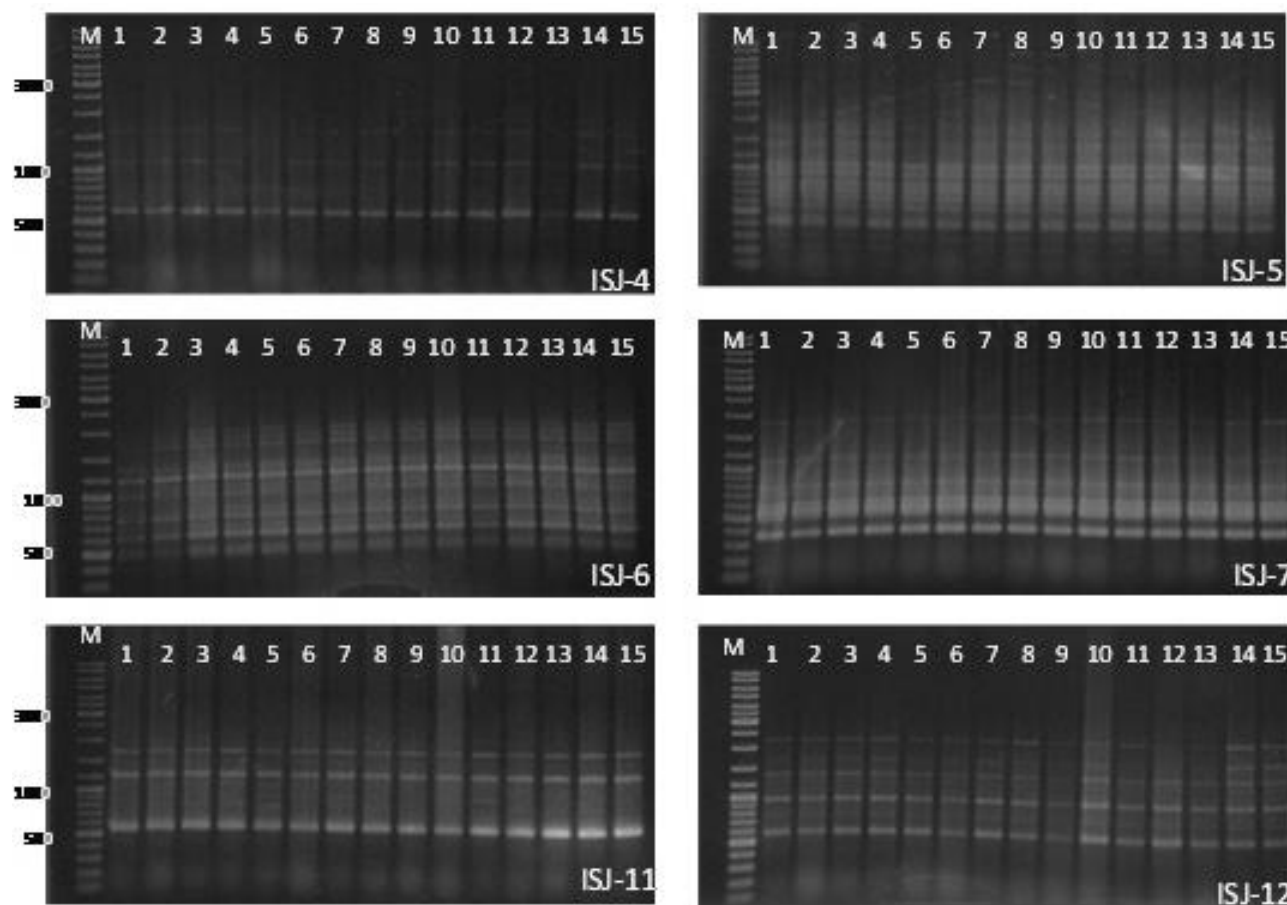


Figure 2. DNA-semi-random RAPD patterns generated by seven arbitrary primers (ISJ) with fifteen sample (1= con-; 2= con+; 3= appB2; 4= appB4; 5= grpB2; 6= grpB4; 7= pomB2; 8= pomB4; 9= con++; 10= appl2; 11= appl4; 12= grpL2; 13= grpL4; 14= pomL2; 15= pomL4; (M) refers to the DNA ladder.

low-fat diet and this indicated that low-fat diet had a positive effect against CCl₄ induced toxicity with fruit juice. Low-fat diet only with CCl₄ gave negative effects, and this agreed with Gomes et al. (2014), who refer to low-fat diet as one that can change the metabolic parameter and cause changes in hormonal milieu which in turn affects reproduction.

The increase of apple juice dose in both basal and low-fat diet increased GTS to 88.9 % in comparison with grape juice in basal diet and increment of dose in low-fat diet (Table 4). The results refer to negative effect by increasing the dose of grape juice, whereas, pomegranate juice gave positive effect than grape juice which may be attributed to its antioxidant potency in pomegranate juice than in grape juice. Pomegranate juice sugar-containing polyphenolic anthocyanins and polyphenols were considered to confer pomegranate juice the antioxidant capacity (Rozenberg et al., 2006).

With regard to pomegranate juice, this result agrees with Ebtesam et al. (2014). in the low-fat diet, it gave changes less than positive control and this didn't happen in basal diet, it was reported that the effects of *P. granatum* juice on lipid peroxidation, and nitric oxide contents in testes of rats treated with CCl₄ were low in treatment about positive control and high about negative control, but this results disagree with basal diet. The protective effects of pomegranate on carbon tetrachloride mediated reproductive toxicity come from tannins (Amakura et al., 2000; Yehia et al., 2011), phenols (Lansky et al., 2005) and flavonoids (Van Elswijk et al., 2004). These components in pomegranate can directly or indirectly reduce oxidative damage by preventing the excessive generation of free radicals (Ebtesam et al., 2014).

These results agree with histological studies, where more effects have been observed in low-fat diet. This indicated that fruit juice has positive effect in low-fat diet against toxicity induced by CCl₄ in testes of rats.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Confirmation of antibodies against L-tryptophan-like epitope in human African trypanosomosis serological diagnostic

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Antibodies directed against L-tryptophan epitope (WE - W for tryptophan, E for epitope), a constant epitope borne by variant surface glycoproteins (VSG), have been detected in sera of all 152 Human African Trypanosomosis (HAT) patients from Angola. The WE is present in VSG hydrophobic regions of the C terminal domains. In the assay, L-tryptophan was linked to bovine serum albumin (BSA) with glutaraldehyde to synthesize W-G-BSA conjugate which was used in an enzyme-linked immunosorbent assay (ELISA) to detect the antibodies. A significant difference was found between HAT patients and controls confirming previous results obtained with a lower number of patients in Congo. A diagnostic test based on this synthetic epitope, especially in combination with other tests, might improve the HAT diagnostic test in field conditions.

Key words: Tryptophan, enzyme-linked immunosorbent assay (ELISA), human African trypanosomosis, serological diagnostic.

INTRODUCTION

Trypanosoma brucei gambiense (*T. b. gambiense*) and *T. b. rhodesiense*, the causative agents of Human African

Trypanosomosis (HAT) or sleeping sickness, are tsetse fly-transmitted protozoan parasites. Diagnosis of HAT

remains a challenge for disease control as HAT is characterized by a non-specific clinical presentation and its definitive diagnosis requires trypanosome detection by microscopy (WHO, 2013). Additionally, the detection of this parasite is difficult because of the low parasite concentration in circulating blood, despite the use of concentration methods such as the mini-anion-exchange centrifugation technique (mAECT) (Lumsden et al., 1979). Moreover, molecular diagnostics based on parasite DNA or RNA detection are more sensitive but are not adapted to field conditions.

Serological diagnostic test of HAT, caused by *T. b. gambiense*, had been performed in the field during medical surveys since 1978 using the Card Agglutination Test with stained Trypanosomes (CATT) (Magnus et al., 1978). CATT/*T. b. gambiense* is still the reference test in the field conditions although, it requires refrigeration and an electric rotator, with drawbacks of false positive and negative results (Magnus et al., 1978). Trypanosomes produce a set of variant surface glycoproteins (VSG). Thus, new diagnostic approaches are based using native surface glycoproteins although the variable nature of the VSG is not satisfactory for a serological diagnostic test (WHO, 2013).

Following an initiative of Foundation for Innovative New Diagnostics (FIND), a rapid diagnostic test is now available (Standart Diagnostic SD Bioline HAT, Sternberg et al., 2014). However, this latter is very useful for passive mode detection but not for mass screening survey (Büscher et al., 2014). The search of new biomarker candidates will permit an improvement to HAT diagnostic test based on CATT (Bonnet et al., 2015). Proteomic investigations are identifying potential immunodiagnostic parasite protein antigens and several biomarkers that are in preliminary evaluation or design for new diagnostic tests (Holzmüller et al., 2013). It has also been discussed how molecular diagnostics may contribute to the elimination of HAT (Büscher and Deborggraeve, 2015).

A tryptophan-like epitope (WE) was found in VSGs from the *T. brucei* group (Semballa et al., 2007). The tryptophan residue is one of the conserved flanking residues of cysteine residues in their hydrophobic regions in the C terminal domain. Antibodies directed to WE were previously found in the sera of HAT patients detected during medical survey (active mode detection), but they were not detected in HIV infection, malaria, Chagas and Parkinson's diseases (Okomo-Assoumou et al., 1995). This latter investigation was performed in the Bouenza focus in the Republic of Congo. No other study has been done in a different HAT focus.

In the present study, anti-WE detection in sera of HAT patients from Angola detected by passive mode at

the Viana hospital compared to negative controls, in order to confirm the potential value of this immune marker in another endemic country, use in setting up a new test for serological HAT mass screening.

METHODS

Patients

Informed consent was obtained from all individual participants which were included in the study. Patients were diagnosed by passive mode detection in Viana hospital in Angola (Truc et al., 2012). Only CATT positive patients with trypanosomes that were detected in one body fluid (blood, lymph juice, cerebrospinal fluid) were selected. Stage determination of the disease was done by searching trypanosomes and white blood cells count in CSF: 13 in stage 1 (early period) and 139 in stage 2 (neurological period). A clinical examination allows detection of neurological signs. Sera from CATT negative subjects living in the same endemic area were used as controls. These controls were uninfected volunteer persons. Serum aliquots were kept at -80°C until use.

ELISA based on L-tryptophan conjugates

Enzyme-linked immunosorbent assay (ELISA) was performed as previously described (Okomo-Assoumou et al., 1995). Briefly, W and BSA (bovine serum albumin) were dissolved in 1.5 M acetate buffer, pH 8 before the addition of glutaraldehyde. After dialysis, the conjugate (W-G-BSA) or control (G-BSA) was added into polystyrene well plate. The well plate was filled with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-Tween), 10% glycerol and BSA (5 g/L). The well plate was incubated for 1 hr at 37°C to saturate it. The plate was rinsed twice with PBS-Tween. The well plate was then filled with 200 µL of diluted (2,000-fold) serum plus PBS-Tween containing BSA (5 g/L) and 10% glycerol. After washings, horseradish peroxidase-conjugated goat antibodies were added to human immunoglobulins. Thereafter, substrate solution and stop solution were successively added. The absorbance in the well plate was measured at 492 nm. The specific immunologic binding of sera was obtained by subtracting blank values read on well plates coated with BSA-G from experimental absorbance values.

Statistical method

Results are expressed in OD value for each individual. Comparison between the patients and the control groups was made using the nonparametric Wilcoxon–Mann–Whitney test with median and quintiles. The difference was considered significant when $p < 0.05$. This comparison was illustrated by a graph performed with Graph Pad V6.0.

RESULTS AND DISCUSSION

Anti-WE antibodies were detected in serum of 152 patients (Table 1) and in 10 controls (Table 2). The limited

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Table 1. Values of anti-WE antibodies in optic density and clinical stage for each patient.

Patient Code	Stage	OD Units (anti-WE)	Patient Code	Stage	OD Units (anti-WE)
V31	1	0.546	V117	2	0.750
V34	1	0.475	V119	2	0.725
V36	1	0.536	V122	2	3.808
V38	1	0.442	V124	2	0.546
V40	1	0.291	V125	2	1.346
V42	1	0.447	V128	2	0.722
V91	1	0.345	V129	2	0.635
V198	1	0.404	V130	2	0.451
V201	1	0.225	V133	2	0.716
V203	1	0.320	V134	2	0.676
V215	1	0.259	V135	2	0.602
V217	1	0.382	V137	2	0.593
V131	1	0.267	V138	2	0.604
V32	2	0.500	V139	2	0.505
V63	2	0.514	V141	2	0.592
V120	2	0.371	V142	2	0.779
V231	2	0.540	V143	2	0.780
V02	2	0.311	V144	2	0.736
V03	2	1.317	V145	2	0.634
V05	2	1.848	V146	2	0.592
V07	2	0.345	V147	2	1.288
V10	2	1.251	V150	2	0.707
V13	2	0.628	V151	2	0.852
V15	2	0.772	V152	2	0.509
V16	2	0.914	V153	2	0.724
V17	2	0.730	V156	2	0.777
V18	2	0.911	V157	2	0.557
V19	2	1.107	V159	2	0.691
V23	2	0.792	V160	2	0.504
V24	2	0.693	V162	2	0.622
V25	2	0.426	V164	2	0.871
V26	2	0.598	V165	2	0.602
V27	2	0.733	V166	2	0.512
V35	2	0.554	V167	2	1.234
V37	2	0.683	V168	2	0.730
V43	2	0.783	V170	2	0.760
V44	2	0.784	V171	2	0.715
V46	2	1.036	V172	2	0.850
V47	2	0.775	V173	2	0.530
V48	2	0.687	V174	2	0.517
V49	2	0.588	V176	2	0.642
V52	2	0.655	V177	2	0.736
V53	2	0.603	V178	2	0.546
V54	2	0.736	V179	2	0.794
V57	2	0.856	V180	2	0.577
V61	2	0.915	V182	2	0.607
V64	2	0.728	V183	2	1.055
V65	2	0.664	V187	2	0.573
V66	2	0.582	V188	2	0.719
V67	2	0.652	V189	2	0.746
V69	2	0.659	V191	2	0.700

Table 1. Contd.

V70	2	0.846	V192	2	0.744
V75	2	0.561	V194	2	0.677
V80	2	0.684	V195	2	0.624
V82	2	1.389	V196	2	0.514
V83	2	0.685	V199	2	1.034
V85	2	0.643	V200	2	0.490
V86	2	0.594	V202	2	0.325
V87	2	1.015	V206	2	0.835
V88	2	0.558	V207	2	0.559
V89	2	0.604	V208	2	0.741
V90	2	0.898	V209	2	0.571
V92	2	0.841	V210	2	0.589
V93	2	0.843	V212	2	0.518
V94	2	0.781	V214	2	0.489
V96	2	0.364	V216	2	0.649
V98	2	1.637	V221	2	0.498
V100	2	0.996	V223	2	0.733
V104	2	0.802	V226	2	0.385
V105	2	1.099	V228	2	0.655
V106	2	0.944	V232	2	0.880
V107	2	0.700	V235	2	0.555
V109	2	0.758	V238	2	0.439
V110	2	3.090	V239	2	0.571
V113	2	0.796	V240	2	0.438
V114	2	0.719	V241	2	0.518

Table 2. Values of anti-WE antibodies in optic density afor each negative control individual.

Control Code	OD Units (anti-WE)
AG	0.109
AR	0.108
EB	0.096
MR	0.114
NF	0.089
BA	0.111
BH	0.192
MF	0.121
OE	0.117
OC	0.110

number of the control group was due to the difficulty in obtaining the people's consent at the Viana Hospital. In a survey carried out in Congo, including 22 controls and 76 patients (52 and 24 in stages 1 and 2 respectively), a very low immunological signal was detected in controls.

The comparison between patients (median= 0.6622, n=152) and controls values is highly significant $P < 0.0001$ (Figure 1). This confirms the use of anti-WE as a potential

biomarker in HAT serological diagnostic test. Further investigations are required to compare the present results with the post-cure HAT persons and other parasitic diseases. Furthermore, among 139 stage 2 patients, 86 presented neurological disorders. There is no correlation between OD values and presence of neurological disorders $P=0.58$. Thus, the anti-WE value is not correlated to patient clinical state.

Rapid tests are now available and are very promising but have been employed mainly for passive mode detection (Büscher et al., 2014). However, their observed specificity was relatively low. A larger study on their use in field condition in combination and in comparison with immune trypanolysis, the reference test, is under progress.

L-Tryptophan epitope conjugate production by peptide synthesis is now cheaper and easily compared to CATT antigen production, requiring *T. b. gambiense* infection in laboratory rodents, or production of recombinant proteins to develop diagnostic kits. Moreover, WE conjugate is thermo-stable and can be developed in a rapid test format. For instance, as most anti-WE antibodies are of IgM isotype, an agglutination assay is feasible. Reliably and easy to use, diagnostic tools are necessary to eliminate HAT as a public health problem in the year 2020 and zero transmission in the year 2030.

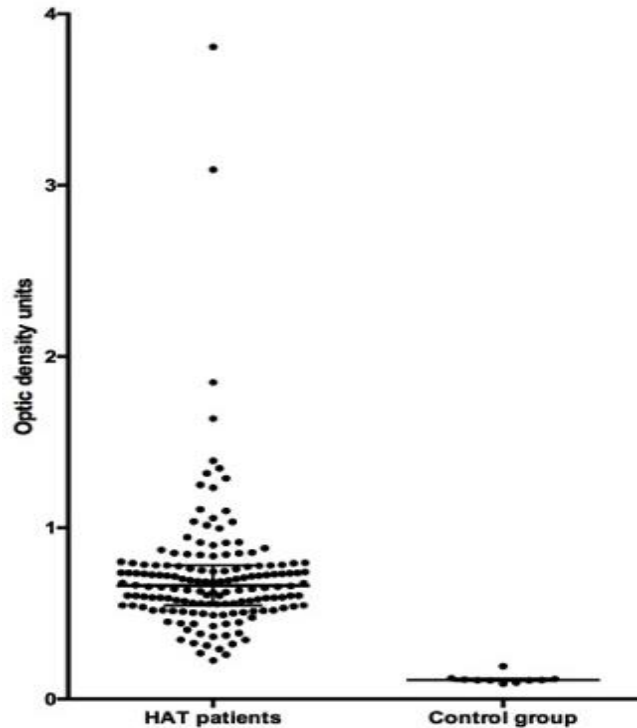


Figure 1. Level of anti-WE antibodies, as assayed by ELISA, in the HAT patients and control groups. Black lines indicates median and interquartiles (Q25 and Q75).

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of phenolic compounds of two *Lygeum spartum* L. cytotypes

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Lygeum spartum represents a natural barrier in the Algerian high plateaus against the advance of sand and the desertification. The plant is of interest due to its tolerance to environmental stress. There are two levels of ploidy in this species; the diploid cytotype $2n = 16$ which is confined in the locality of Ain Benkhilil and the polyploid cytotype $2n = 40$ which thrives in semi-arid climates and has a large ecologic plasticity. The aim of this study was the evaluation of the phenolic compounds and flavonoids in both cytotypes. The antioxidant activity was also measured with results showing differences in the level of phenolic compounds; thus, these compounds could serve as biomarkers to distinguish the two cytotypes.

Key words: *Lygeum spartum* L, total phenols, flavonoids, 2,2-diphenylpicrylhydrazyl (DPPH), lipid peroxidation.

INTRODUCTION

Lygeum spartum L. (Poaceae) is a native species in Algerian steppe, widely distributed in semi-arid Mediterranean areas. The plant has considerable ecological importance due to its tolerance to environmental stress and represents a natural barrier against the advances of sand and desertification (Nedjimi, 2009). Harche et al. (1990) showed that as the Alfa (*Stipa tenacissima* L.), leaves of *L. spartum* have fibrous walls rich in polysaccharide compounds which give the paper pulp. Studies have shown the existence of two levels of ploidy in this species in Algeria; a diploid with $2n = 16$ located in arid regions and a polyploid with $2n = 40$ present in the semi-arid zones of the littoral

(Benmansour and Kaid-Harche, 2001; Djabeur et al., 2008; Boughanmi-Abdeddaim, 2010). Cytotype polyploid ($2n = 40$) is widespread in Algeria and is also present in Egypt (Ramanujam, 1938) and Spain (Lorenzo-Andreu and Garcia-Sanz, 1950). According to recent findings, the second cytotype diploid ($2n = 16$) seems to be located on the high Algeria trays, only in Ain Benkhellil and Kheiter (Benmansour and Kaid-Harche, 2001; Djabeur et al., 2008; Boughanmi-Abdeddaim, 2010). Djabeur et al. (2008) showed that the two cytotypes differ at the anatomical level in the spikelet morphology and reproductive capacity. The diploid cytotype has more vigorous fructifications with lemmas characterized by

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shorter distal parts, a character which can be used for an easy recognition of the cytotype in the field. Conesa et al. (2007) found that the *L. spartum* could be found in soils containing heavy metals, or soils rich in NaCl (Nedjimi, 2009) and this adaptation may be related to phenolic compounds and their antioxidant activity (Boscaiu et al., 2010). Phenolic compounds such as phenolic acids and flavonoids have been found to be the most widespread substantial groups of plant secondary metabolites produced from the shikimate-phenylpropanoid biosynthetic pathway (Torrás-Claveria et al., 2012; Ma et al., 2014). These molecules have been described as markers of biotic and abiotic stress tolerance in plants (Lattanzio et al., 2006). Abiotic stresses cause changes at the morphological and molecular levels that adversely affect plant growth. Drought, high temperature, flooding, chilling, salinity, high light, and heavy metals are the major abiotic stresses that affect plants (Bita and Gerats, 2013). The present study is intended to identify the possible secondary metabolites present in the aerial part of both cytotypes by performing phytochemical screening of saponins, flavonoids, tannins, alkaloids and free quinines. An evaluation of total phenols and flavonoids by spectrophotometric assay was conducted. Antioxidant activity was measured by 2,2-diphenylpicrylhydrazyl (DPPH) radical inhibition test and lipid peroxidation was estimated by a 2-thiobarbituric acid reactive substances (TBARS) test.

MATERIALS AND METHODS

Plant material

The aerial parts (leaves, stems) of the two cytotypes (diploid and polyploid) were harvested at the Department of Biotechnology at the University of USTO in March 2014; the complete aerial part of the plant were then dried at 45°C for 48 h and ground with electric mill to give fine powder.

Preparation of plant extracts

The extraction of the grounded plant's aerial parts (leaves, stem) was carried using a Soxhlet apparatus. Six successive extractions were performed with the following organic solvents: Hexane, dichloromethane, chloroform, butanol methanol and ethyl acetate. In detail, 100 g powder of the plant was introduced into a filter cartridge adjusted to the size of the apparatus. The flask contained 1000 ml of the solvent; each solvent was brought to extraction for 48 h. The six different solvent extracts were obtained using a rotary evaporator and stored in a dry and dark place.

Determination of totals phenol contents

Assay for determination of total phenol contents of both populations in the butanolic, ethyl acetate and methanolic extracts was carried out using the colorimetric method of Folin-Ciocalteu (Singleton and Rossi, 1965). The amount of total phenolic compounds in each of the extracts was later extrapolated from a standard curve made with different concentrations of gallic acid.

Determination of flavonoids contents

The level of flavonoids was measured according to the method of Kim et al. (2003). The amount of flavonoids was determined by a standard curve made with different concentrations of catechin.

DPPH radical inhibition test

Evaluation of antioxidant activity was performed by the 2,2-diphenyl-1-picrylhydrazil free radical (DPPH) inhibition test (Brand-Williams et al., 1995). 50 µL of various concentrations of the extracts were added to 1950 µL of DPPH solution (0.025 g/L in methanol). After a 30-min of incubation at room temperature, the absorbance was read at 515 nm against a blank containing all reagents apart from the test compound. The ascorbic acid was used as positive control. Each sample was measured in triplicate. The results were expressed as percentage of scavenging activity (% I). $I\% = [(Abs\ blank - Abs\ sample) / Abs\ blank] \times 100$. Extract concentration providing 50% inhibition (IC50) values were determined from the graph against extract concentrations.

Lipid peroxidation

Lipid peroxidation values (measured as levels of malondialdehyde, MDA) were estimated by a thiobarbituric acid reactive species (TBARS) test described by Hernandez et al. (2001). 50 mg of the sample (in dry weight) was ground and then homogenized in 2 mL of 1%w/v trichloroacetic acid (TCA). The homogenate was centrifuged at 15000 g for 10 min at 4°C and 0.5 mL of the supernatant was mixed with 1.5 mL of thiobarbituric acid (TBA) prepared in 20% TCA and incubated at 90°C for 20 min. The absorbance was read at 532 nm using a spectrophotometer. The content of MDA was determined using the extinction coefficient at 155/mm/cm. All the experiments were conducted in triplicate and data presented as mean values ± standard deviation (SD).

RESULTS AND DISCUSSION

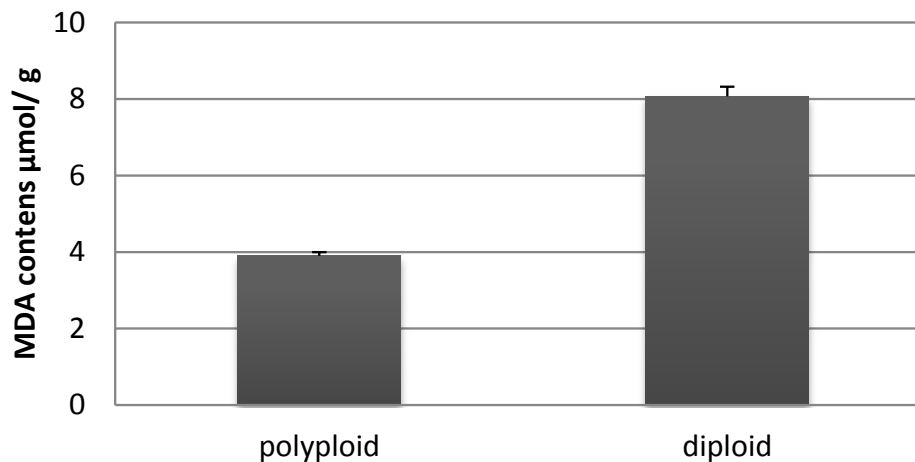
The study focused on finding the major phenolic compounds and the antioxidant activity of both *L. spartum* L. cytotypes. The phytochemical screening revealed the presence of flavonoids, tannins, saponides and free quinones in both cytotypes (Table 1).

Estimation of total phenols showed that extracts from diploid cytotype were richer in phenolic compounds in the methanol and butanol extract, while the ethyl acetate fraction showed that polyploid cytotype contained more polyphenols. The quantitative estimation of total flavonoids by the trichloride aluminum method showed that the methanolic extract of both cytotypes was higher, but the diploid cytotype had a greater concentration of flavonoids. Previous studies have shown that extrinsic factors (such as geographic and climatic factors), genetic factors, and also the degree of maturation of the plant and the storage duration have a strong influence on the content of polyphenols (Aganga and Mosase, 2001). Quan et al. (2016) showed that phenolic acid plays a role in drought-tolerance of rice. In white clover, the high levels of flavonoids (flavonols, quercetin and kaempferol) contents were associated with enhanced stress tolerance

Table 1. Concentration of total phenolics, flavonoids, and antioxidant activity (IC₅₀ values) of both *Lygeum spatum* L. cytotypes.

Extract	Butanolic extract	Ethyl acetate extract	Methanolic extract
Total phenols (mg GAE/ g of extract)*			
Diploid cytotype	82.85±0.04	25.66±0.002	79.63±0.08
Polyploid cytotype	27.55±0.01	48.68±0.005	57.61±0.05
Flavonoids (mg CE/ g of extract)**			
Diploid cytotype	14.16±0.01	7.24±0.02	38.49±0.08
Polyploid cytotype	11.43±0.02	20.63±0.13	21.58±0.02
IC 50 (µg DPPH /ml)			
Diploid cytotype	0.05±0.01	0.11±0.02	0.03±0.01
Polyploid cytotype	0.05±0.007	0.06±0.003	0.09±0.002

IC50 represents half maximal inhibition concentration. GAE, Gallic acid equivalent ; CE, catechin equivalent.

**Figure 1.** MDA content in µmol / g dry mass of the two *Lygeum spatum* L. cytotypes.

capacity of under UV-B radiation and drought conditions (Nichols et al., 2015).

Phenolic compounds may contribute directly to antioxidative action (Awika et al., 2003). The antioxidant activity of phenolic extracts shows variation depending on the solvent. Ethyl acetate fractions gave a higher antioxidant activity in diploid cytotype, while in polyploid cytotype, the methanolic extract gave a higher antioxidant activity. This can be explained by the fact that phenolic compounds vary between two cytotypes. The high antioxidant activities can be explained by the high reactivity of phenolic units, which may act as effective antioxidants (Jung et al., 2003). Many authors show that phenolic compounds and flavonoids have a protective effect against abiotic stress including antioxidant activity (Rice-Evans et al., 1997; Winkel-Shirley, 2002; Michalak, 2006). Indeed flavonols are able to inhibit the generation of reactive oxygen species (ROS) and as such they have

a very important antioxidant function in cells (Brunetti et al., 2013).

The degree of lipid peroxidation was determined by the level of malondialdehyde (MDA), since its accumulation is an indicator of the damage caused by oxidative stress. Diploid cytotype showed a higher level in MDA contents compared to polyploid cytotype (Figure 1). Many authors have shown that high level of lipid peroxidation is caused by abiotic stress (Ben Youssef et al., 2005; Amor et al., 2006; Moradi and Ismail, 2007; Liang et al., 2008). The presence of trace solvent may influence the composition. Abiotic stresses are caused by complex environmental conditions such as high and low temperatures, freezing, drought, salinity, heavy metals, strong light, ultra-violet (UV) light, or hypoxia (Hirayama and Shinozaki, 2010). In this case, adaptive capacity of plants is mainly related to genetic evolution where the duplicated genome allowed for the new polyploid species to populate new ecological

niches or have a broader response in abiotic and biotic stress (Wood et al., 2009). This explains the restriction of the diploid population in small area and the high plasticity of polyploid cytotype. The results obtained indicate that there is a high interspecific variation of phenolic content and antioxidant activity between the two cytotypes of *Lygeum spartum* and these molecules can be further used as stress biomarkers for their distinction. A further investigation is warranted to understand the role of total phenols and flavonoids in plant protection against abiotic stress (drought, salinity, UV).

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Sodium hypochlorite sterilization of culture medium in micropropagation of *Gerbera hybrida* cv. Essandre

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Micropropagation requires controlling contamination that might compromise the success of the process. Thermal sterilization is traditionally used; however, costs deriving from equipment acquisition and maintenance render this technique costly. With the purpose of finding an alternative to thermal sterilization, this research aimed at assessing the efficiency and ideal concentration of sodium hypochlorite for sterilization of culture media and glassware used during rooting of micropropagated *Gerbera hybrida* cv. Essandre. Two experiments were carried out. In the first one, treatments consisted of control I (no sterilization), control II (thermal sterilization), and total active chlorine concentrations of 0.0005, 0.001, 0.002 and 0.003%. In the second experiment, based on the results observed in the first experiment, treatments consisted of control I (thermal sterilization) and II (chemical sterilization), and total active chlorine concentrations of 0.002, 0.0025 and 0.003%. Plant behavior was assessed based on the length of aerial part and roots, number of roots, and dry biomass of plants. Results showed that the addition of an active chlorine concentration of 0.003% to culture media provided total control of contaminants, and there were no significant differences regarding the variables analyzed between plants obtained with thermal sterilization and with sodium hypochlorite sterilization. Thus, chemical sterilization can be used as a replacement for thermal sterilization of nutrition media for rooting of gerbera *in vitro*.

Key words: Sodium hypochlorite (NaOCl), tissue culture, contamination, chemical sterilization, autoclaving.

INTRODUCTION

In the commercial production of gerberas, micropropagation enables the large-scale production of plants in a limited space, as well as the obtaining of disease- and pest-free uniform plants (Bhargava et al., 2013). This technique also ensures precision in

production schedules and product quality, regarding plant homogeneity and vigor. Nevertheless, the occurrence of contamination in an *in vitro* culture is frequent, and might result in high damage. The method traditionally used in contamination control consists of autoclave-sterilization of

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culture media and glassware. Nonetheless, it is costly due to equipment (autoclave) acquisition and too high electric power consumption, which might lead to nutrient decomposition in culture media (Ribeiro, 2006; Weber et al., 2015).

Due to the advantages of micropropagation and to the need for reducing the costs involved, some alternatives to thermal sterilization have been examined. Latimer and Matsen (1977) suggested microwave sterilization, although they observed this method was inefficient in the sterilization of liquids due to medium overflow with elevation of temperature (Tisserat et al., 1992; Teixeira et al., 2005b). Another alternative is the sterilization of culture media by filtering, but it has proven to be inadequate when used for large volumes because it increases consumables and labor costs, and is time-consuming (Tisserat et al., 1992).

Chemical sterilization with sodium hypochlorite (NaOCl), proposed initially by Teixeira et al. (2005a, b), provided satisfactory results for pineapple (*Ananas comosus* L. cv Smooth Cayenne) (Teixeira et al., 2006), sequoia (*Sequoia sempervirens* L.) (Ribeiro et al., 2011), and eucalyptus (*Eucalyptus pellita* L. and *Eucalyptus benthamii* Maiden et Cambage) (Teixeira et al., 2008; Brondani et al., 2013). Recently another chemical compound, chlorine dioxide (ClO₂), a stabilized gas, used by Cardoso (2009) in the sterilization of culture media for anthurium (*Anthurium andraeanum* Lind.) and gerbera, eliminated contamination without causing phytotoxicity to plants (Cardoso, 2009; Cardoso and Silva, 2012).

Among all chemical products tested, sodium hypochlorite stands out because it is of low cost and easy to acquire. Thus, this research aimed to determine the ideal NaOCl concentration and its effectiveness in culture media sterilization when growing *in vitro* *Gerbera hybrida* cv. Essandre.

MATERIALS AND METHODS

The influence of active chlorine in the sterilization of culture media and glassware in *G. hybrida* cv. Essandre cultivated *in vitro* was assessed during the rooting phase. Sodium hypochlorite (NaOCl) was used as a source of active chlorine, available in Qboa[®], a marketed household bleach with 2% total active chlorine in its composition.

Two experiments were carried out with the purpose of assessing the most efficient total active chlorine concentration to obtain culture media sterilization. In the first one, treatments consisted of control I (no sterilization), control II (thermal sterilization), and total active chlorine concentrations of 0.0005, 0.001, 0.002 and 0.003% (m/v). In the second experiment, which was conducted based on the results observed in the first experiment, treatments consisted of control I (thermal sterilization) and II (chemical sterilization), and total active chlorine concentrations of 0.002, 0.0025, and 0.003%. The preparation of culture media and sterilization of glassware and water in NaOCl sterilized treatments followed the protocol developed by Teixeira et al. (2006), with addition of 0.003% active chlorine to the medium. In control II, autoclaving was used for a period of 40 min to sterilize glassware and water and 20 min to sterilize the culture medium at a temperature of approximately

121°C at 1 kgf/cm². In control I (sterilization), the glassware was washed with detergent and rinsed with distilled water. The water used in the preparation of the culture medium was distilled and deionized, and no active chlorine was added to the culture medium.

The culture medium consisted of inorganic salts MS (Murashige and Skoog, 1962), White's vitamins (White, 1943), 100 mg L⁻¹ i-inositol, 30 g L⁻¹ sucrose, solidified with 10 g L⁻¹ of agar. When autoclave was used to sterilize, the media used had pH 5.7 ± 1, and in NaOCl sterilized treatments pH was 6.0 ± 1.

After preparation, the nutrient medium was distributed in 20 ml aliquots per culture flask (250 ml), which were then left open inside the laminar flow cabinet for 10 min for chlorine to volatilize. Explants were inoculated in the flasks that did not show visible microorganism growth after 48 h. Three-leaf gerbera shoots deriving from stock culture, in the stage of multiplication, were placed in the culture flasks and left in the growth room for 30 days at a temperature of 26 ± 1°C, with photoperiod of 16 h and irradiance of 19 mol.m⁻².s⁻¹.

Plant behavior was assessed based on length of aerial parts and roots, number of roots, and plant dry biomass. The flasks with visible growth of microorganisms were considered only as presence of contamination.

Experiments were conducted in a completely randomized design, with six treatments in experiment I and five in experiment II, both with five replications and three experimental plots; each plot was represented by one flask with one explant. Data were submitted to a variance analysis and means were compared using Tukey's test with 5% of significance with the help of the SAS program for Windows, version 9.2, 2002-2008.

RESULTS AND DISCUSSION

Contamination rate decreased as active chlorine concentration increased and the 0.003% concentration provided total sterilization of the culture medium (Table 1). In the treatment with no sterilization, there was 100% of contamination after 24 h, thus preventing the inoculation of explants.

Regarding mean length of aerial part, mean root length, mean number of roots, mean number of leaves, and plant dry biomass, there was a significant difference only in plant dry biomass, which was higher in the treatment containing 0.0005% of total active chlorine. However, this concentration did not totally prevent contaminants. In the other NaOCl concentrations, plant behavior was similar to the autoclaved control.

The contamination data in Table 1 showed that the ideal concentration for the total sterilization of the culture medium would be between 0.002 and 0.003% of active chlorine in the medium, and should be no less than 0.002%, as this concentration resulted in 13.3% of loss from contamination. Hence, experiment II (Table 2) was conducted with the purpose of determining a more precise sterilizing concentration, using shorter intervals than the ones used in experiment I.

In the second experiment data (Table 2), it was observed that all chlorine concentrations achieved efficient sterilization, but the 0.002% concentration showed 13.3% of losses in the first experiment. Therefore, contaminant control was obtained starting at 0.0025% of active chlorine in the culture medium.

Table 1. Contamination (number), mean length of aerial part (MLAP), mean root length (MRL), mean number of roots (MNR), mean number of leaves (MNL), and plant dry mass (PDM) in different active chlorine concentrations added to culture medium in experiment I for *Gerbera hybrida* cv. Essandre.

Active chlorine concentration (m/v)	Contamination		Variables				
	Flasks before inoculation	Flasks after inoculation	MLAP (cm)	MRL (cm)	MNR	MNL	PDM (g)
No sterilization	15	0	-	-	-	-	-
Autoclaved	0	1	4.5 ^a	2.1 ^a	1.2 ^a	6.46 ^a	0.027 ^b
0.0005%	5	2	5.27 ^a	2.18 ^a	2 ^a	8.53 ^a	0.044 ^a
0.001%	5	-	4.9 ^a	2.34 ^a	2 ^a	7.47 ^a	0.024 ^b
0.002%	2	-	4.53 ^a	2.67 ^a	2.26 ^a	8.2 ^a	0.025 ^b
0.003%	-	-	4.99 ^a	2.18 ^a	1.8 ^a	5.67 ^a	0.028 ^b
CV (%)			24.3	15.3	46.2	31.0	35.3

Mean values followed by the same letter in the column do not differ statistically in Tukey's test with 5% significance.

Table 2. Contamination (number), mean length of aerial part (MLAP), mean root length (MRL), mean number of roots (MNR), mean number of leaves (MNL), and plant dry mass (PDM) in different active chlorine concentrations added to culture medium in experiment II for *G. hybrida* cv. Essandre.

Active chlorine concentration (m/v)	Contamination		Variables				
	Flasks before inoculation	Flasks after inoculation	MLAP (cm)	MRL (cm)	MNR	MNL	PDM (g)
No sterilization	15	0	-	-	-	-	-
Autoclaved	0	1	3.63 ^a	3.45 ^a	1.47 ^a	6.27 ^a	0.025 ^a
0.002%	0	0	3.23 ^a	2.86 ^a	1.73 ^a	6.33 ^a	0.026 ^a
0.0025%	0	0	3.82 ^a	3.18 ^a	1.8 ^a	5.93 ^a	0.027 ^a
0.003%	0	0	3.28 ^a	2.84 ^a	1.33 ^a	6.07 ^a	0.022 ^a
CV (%)			6.7	18.7	30.4	15.7	28.8

Mean values followed by the same letter in the column do not differ statistically in Tukey's test with 5% significance.

Regarding the variables analyzed, there were no significant differences between the results obtained with thermal sterilization and the three active chlorine concentrations of chemical sterilization.

However, since the 0.003% concentration provided to plants the same development in relation to autoclaved treatment in three experiment replications, this concentration can be recommended for gerbera micropropagation. The concentration 0.003% also was effective in sterilizing culture media for sequoia propagation (Ribeiro et al., 2011). However, Teixeira et al. (2006) working with *in vitro* pineapple tree cultures (*Ananas comosus* cv Smooth cayenne), obtained successful sterilization with a lower active chlorine concentration (0.0003%) with no damage to the plant's development; on the contrary, it promoted an increase in biomass and in the number of shoots. On the other hand, when sterilizing media to obtain Brazilian ginseng (*Pfaffia glomerata*) calluses, Ribeiro et al. (2009) obtained satisfactory results with 0.05% NaOCl concentration and did not observe any changes in callus biomass.

Differences observed among several authors regarding

the ideal sterilizing concentration of sodium hypochlorite occurred according to laboratory asepsis conditions and the plant species used. This becomes evident in studies in which two species of the genus *Eucalyptus* were reported; whereas 0.005% concentration was effective in sterilizing *E. pellita* (Teixeira et al., 2008); it caused the number of shoots to decrease in *E. benthamii* (Brodani et al., 2013).

The effects of NaOCl sterilization are related to chlorine ions, which trigger oxidative reactions responsible for enzymatic inactivation and lipid and fatty acid degradation; hence, its biocide properties (Saran et al., 1998; Estrela et al., 2002; Emmanuel et al., 2004).

Regarding plant behavior in chemical sterilization, Cardoso and Silva (2012) observed a higher vigor in gerbera sprouts when the medium was sterilized with chlorine dioxide (ClO₂). In sodium hypochlorite (NaOCl) sterilization, the same behavior was observed in rooted plants either in hypochlorite- or autoclave-sterilized media.

The present results corroborate data observed by other authors, who verified that chemically-sterilized tissue

culture proved to be effective in the micropropagation of different species (Brondani et al., 2013; Weber et al., 2015). Weber et al. (2015) stated that laboratories in Kenya, Africa, routinely use this technique in potato micropropagation, which proves that there is a practical and promising use for chemical sterilization.

Conclusions

1. Chemical sterilization can replace thermally-sterilized nutrition media in rooting of gerbera *in vitro*.
2. Addition of total active chlorine concentration of 0.003% to the nutrition medium completely eliminates contaminations and allows for the satisfactory development of rooting of gerberas *in vitro* without causing phytotoxicity.
3. Gerberas grown *in vitro* in chemically sterilized nutrition media using 0.003% of total active chlorine resulted in plants with mean length of aerial parts, mean root length, mean number of roots, mean number of leaves, and plant dry biomass similar to plants grown in autoclave-sterilized media.

Conflict of interests

The authors have not declared any conflict of interests.

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