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Full Length Research Paper

The urothelium enhancive polarization in *CfTX-1* peptide intervened toad urinary bladder

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Toad urothelium barrier is the model to mimic and investigate urothelium permeability. Thiazide blocked ionic transportation in polarized membrane state. Jellyfish venom causes pores to be adjusted to urothelium permeability which improved polarization. This study aimed at *CfTX-1* peptide in urothelium permeability evoked polarizations. Thiazide pretreated toad urothelium permeability to ions were investigated in modified Ussing chamber. Thiazide urothelium were further intervened by *CfTX-1* peptide and treated with G-protein receptor agonists. 0.1 mol $CaCl_2$ activated transurothelium potential differences were recorded by unipolar lead and computerized by fast Fourier transform technique. Apical chamber was settled as anode. The amplitude of potential differences were evaluated to determine the urothelium polarizations. The results indicated that $CaCl_2$ activation induced a positive monophasic wave in thiazide urothelium, which suggested the urothelium was slightly polarized and significantly enhancive in adrenergic receptor treated urothelium. Furthermore, *CfTX-1* peptide enhanced transurothelium potential difference in thiazide urothelium, therefore, urothelium were supra polarized. NPPB treatment significantly attenuated this supra polarization, which suggested that the Cl^- influx was the main stream ionic compound of this polarization. It is concluded that *CfTX-1* peptide was considered to generate supra polarization in thiazide urothelium. This mechanism is useful to study the improvement of drug delivery crossing urothelium barrier.

Key words: *CfTX-1* partial sequence, toad urothelium, supra polarization, Cl^- permeability.

INTRODUCTION

Aurelia aurita nematocysts were dominated by large quantities of secretory proteolytic enzymes, displayed a wide spectrum of toxic activities that determined clinical relevance of the neurotoxicity, myotoxicity and hemolysis. *Aurelia aurita* crude venom was found to affect irreversible depolarization of the muscle membrane, increase in membrane permeability to sodium ions (Kihara et al., 1988), cell membrane lipid metabolism

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#Authors contributed equally to this study

(Helmholz et al., 2007), have a preferentially postsynaptic action at the neuromuscular junction by inhibiting acetylcholine binding to postsynaptic acetylcholine receptors (Ponce et al., 2013). Jellyfish venom, such as *Carybdea rastoni*, *Chiropsalmus quadrigatus*, contained diversified toxic isoforms were classified into at least two distinct subfamilies, type I and II. The box jellyfish *Chironex fleckeri* (*C. fleckeri*) toxin 1 and 2 were the short name of *CfTX-1* and *2*, two highly abundant toxins in the *C. fleckeri* venom, which was confirmed causing rapid cardiovascular collapse accompanied by hemolysis in anaesthetized rats (Yanagihara and Shohet, 2012). *CfTX* toxins shared high structural similarity with pore-forming three-domain Cry toxins produced by the bacterium *Bacillus thuringiensis* that possess the potent insecticidal activities (Podobnik and Anderluh, 2017)). The structural homology of *Chironex fleckeri* toxins and Cry toxins suggest that the toxins may have a similar pore-forming mechanism of action involving helices from the N-terminal domain (Brinkman, 2014), while structural variability within the toxin family may modulate receptor specificity. Therefore, *CfTX*-like toxins may be involved in oligomerization and pore formation not only on erythrocytes, but also other susceptible cells (Brinkman, 2007).

In isolated amphibian urinary bladder wall, mucosal and serosal surfaces have different permeability to solutes and water. Mucosal surface is normally impermeable to water (Peachey and Rasmussen, 1961; Parisi et al., 1981); however urothelium plasma membranes on the serosal surface are normally relatively permeable to circulation released stimulations (Michailova and Usunoff, 2006). Water permeability barrier is at mucous urothelium surface with striking properties of its polarity and the tight junctions (Parsons, 2007), while ionic traverse the barrier are involved in the ionic transporters located in apical membrane (Witten et al., 2018). The active transport in basal membrane induced variation of the permeability is evoked by Ca^{2+} and other solutes across the blood capillaries in serous. It has not been established whether one or the other of these layers is responsible for the unusual permeability characteristics of this cell surface. Cytological evidence indicated that the urothelium were structured by filamentous layer in the outside of the plasma membrane. That was the place to maintain the tight lateral attachment near the mucosal surface to assure an almost leak-proof bladder. Loosely interlocking folds of the lateral membranes probably help to have osmotic equilibrium with the serosal fluid crossing the urothelium barrier. Furthermore, serous as the urothelial sheet were more effect than mucous side in solutes transporting via the circulatory system into the urinary bladder sac. Ions either enter the cell through basal membrane surfaces or act at these surfaces lead to the permeability changes. The amplitude of transurothelium electrical potential difference is proportional to the ionic movement.

Based on the above previously knowledge, ionic permeability induced urothelium potentials in $CaCl_2$ activation in basal membrane on serosal side was investigated. Moreover, the $CaCl_2$ evoked transurothelium electrical potential difference in partial sequence of CfTX-1 pretreated urothelium preparations was analyzed.

MATERIALS AND METHODS

Aurelia aurita nematocysts crude venom production

The crude venom were extracted from nematocyst of *Aurelia aurita* tentacle that obtained from Qiongzhou strait, Hainan, China. After homogenization nematocyst solution was centrifuged at 20,000×g for 1 h at 4°C. The proteins concentration in resultant supernatant was primarily determined by Bradford method, then immediately frozen at -80°C in condenser chamber and vacuum to extremely dry (VirTis BenchTop freeze dryer, SP industries, Inc., Pennsylvania, U. S. A.). Lyophilized concentrated crude venom was stored in -20°C for further analysis.

CfTX-1 peptide identification and synthesize

10% acrylamide gel was used to separate lyophilized concentrated crude venom by SDS-PAGE protocol. The target 43 kDa band cut off. The proteins were digested by trypsinase and the peptide fragments were entered into mass spectrometry analysis. The LC-MS/MS and peptide mapping method were used to identify *CfTX-1* N-terminal fragment. Once the sequence of *CfTX-1* was identified, this partial sequence of *CfTX-1* were synthesized by using commercial resin solid-phase protocol and purified and final quality control by HPLC and electrospray Ionization Tandem Mass Spectrometry (ESI-MS).

Toad urothelium preparations

The toads were from the Hainan provincial drug safety evaluation center, Hainan province, China. The study protocol was approved in advance by ethics committee, Hainan Medical College. Toads were fed up in a humidity cage in the dark cycle prior to pithing. All toads were maintained under the surveillance of a veterinarian and adherence to policies and procedures from regulations for the administration of affairs concerning experimental animals.

The hemibladder wall were from double pithing toad, then prepared into approximately 9 mm x 9 mm in size, and balanced in isotonic amphibian Ringer's solution for 5 min to maintain the natural existence of urothelium osmotic gradient. The control preparation to the Thiazide pretreated was the urothelium only activated by $CaCl_2$ but had no pretreatment and intervention; the control preparation in Thiazide pretreated was the urothelium only activated by $CaCl_2$ but had no intervention by *CfTX-1* peptide, acetylcholine, norepinephrine or 5-nitro-2-(3-phenylpropylamino) benzoic acid.

Thiazide (HCTZ) pretreated and *CfTX-1* peptide intervened urothelium preparations were detected by using the hemibladder from the same toad urinary bladder. Tissue from at least four hemibladders with appropriate control preparations for comparison was subjected to each experimental condition that was studied. All experimental manipulations were performed within 2 h for avoiding the influences of membrane bioactivities.

Toad urothelium transurothelium potential difference analysis in mini Ussing chamber

The toad urothelium transurothelium potential differences

measurement referenced Dunning-Davies method (Dunning-Davies et al., 2013). After rinsing with 0.9% saline solution, the intact urothelium layer were apical side down mounted on 80 mm^2 circle surface on the modified mini Ussing chamber, where the dual chambers were vertically separated by horizontal mounted urothelium barrier to become apical chamber and basal chamber. The chambers were fulfilled with 0.9% saline solution and stabled in room temperature before investigations. For investigating the ionic crossing urothelium layer induced transurothelium potential difference (TPD), the preparations were initially activated urothelium basal membrane by 0.1 mol $CaCl_2$ in basal chamber to obtain a primary TPD, then inspected the TPD in hydrochlorothiazide (HCTZ) pretreated urothelium preparations (HCTZ urothelium). The ionic crossing urothelium flowing induced TPD were measured by unipolar lead, and recorded with BL-420S biological signal acquisition system (Chengdu Taimeng Technology Ltd., Chengdu, China). The signals were computerized by fast Fourier transform technique with TM_WAVE software (Chengdu Techman Software Co. Ltd., Chengdu, China); apical side was settled as anode. Tissue from at least four hemibladders with appropriate control preparations for comparison was subjected to each experimental condition that was studied. All experimental manipulations were performed within 2 h to avoid the influences of membrane bioactivities (Kerec et al., 2005; Cohen et al., 2007).

CfTX-1 peptide evoked transurothelium potential difference in thiazide pretreated urothelium preparations

The HCTZ urothelium were further intervened by *CfTX-1* peptide in basal chamber with dosage of 10 μg/ml. The muscarinic receptor agonist Acetylcholine (*Ach*, 2 mmol) or adrenergic receptor agonist norepinephrine (*NE*, 3 mmol) were treated to observe activating guanine nucleotide-binding proteins signaling induced permeability effect in *CfTX-1* peptide intervened preparations. For understanding the ionic flow components that caused TPD in urothelium, *5-Nitro-2-(3-phenylpropylamino) benzoic acid* (*NPPB*, 2mmol) were used to block the Cl^- channel.

RESULTS

CfTX-1 identified in *Aurelia aurita* concentrated crude venom

In Figure 1a, lane 1 was Molecular PageRuler (Thermo Scientific, Inc Lithuania), lane 2, 3 and 4 were the reference bands of bovine serum albumin with the concentration of 10, 5 and 2.5 μg respectively. Lane 5, 6 and 7 were concentrated crude venom of 10, 5 and 2.5 mg, respectively. The compound in 43 kDa band of Figure 1a, lane 5 (the band with the arrow) was the target band for identifying sequence by LC-MS/MS. An 11 amino acid peptide sequence which had a high overlap with the positive strain of amino acid sequences 304 to 314 (*IFNFFDLmKVK*) of *CfTX-1* N-terminal were confirmed (Figure 1b). This specific partial sequence was additionally synthesized by resin solid-phase synthesis. It was final purified to 99.75% (abbreviated as *CfTX-1* peptide in the later). The quality control of peptide was monitored by HPLC and ESI-MS. Figure 1c was the 701.9Da peak value of purified *CfTX-1* 11 amino acid peptide product (Figure 1c).

CfTX-1 peptide in *HCTZ* urothelium preparations

In *HCTZ* urothelium, $CaCl_2$ activation induced an increasing ionic current crossing the preparation, further generated a positive amplitude increasing of transurothelium potential difference (Figure 2a, 20.05±1.75 μV, n=5). Because *HCTZ* blocked the Na^+/Cl^- symporter in the apical membrane of urothelium, the remaining main ionic current were the ionic influx from the serosal basal side to the apical side (ionic influx). Furthermore, in *CfTX-1* peptide intervened *HCTZ* urothelium, $CaCl_2$ evoked significant increase in ionic influx current. This further induced secondary enhanced positive amplitude of transurothelium potential difference (Figure 2b, 120.74±23.66μV). This result suggested that *CfTX-1* peptide significantly enhanced the ionic permeability in urothelium.

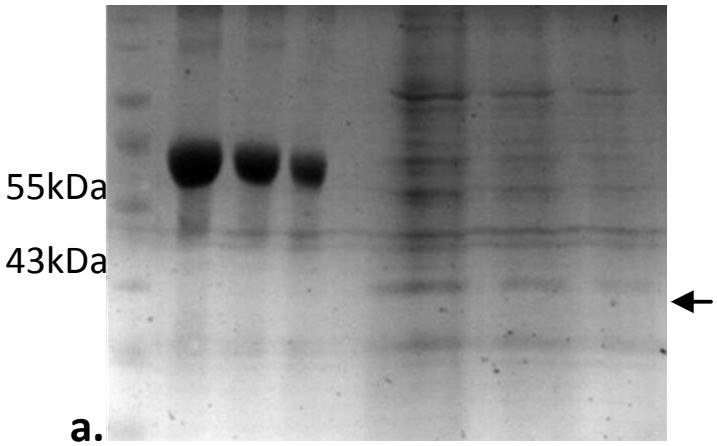
As the anode were settled in the apical of the urothelium in the Ussing chamber, the main stream of the ionic influx have been generated by the anion influx from the basal membrane.

NPPB attenuated transurothelium potential difference in thiazide pretreated and *CfTX-1* peptide intervened preparation

NPPB was an inhibitor of many different Cl^- channels and inhibit ATP release mediated by Cl^- channels. It is an inhibitor of channel-mediated ATP release. In *HCTZ* pretreated and *CfTX-1* peptide intervened preparations, NPPB significantly reduced *CfTX-1* peptide induced ionic current influx and the amplitude of TPD (Figure 2c). The amplitude of TPD were attenuated to 26.67±8.62 μV, reduced 450% when compared with Figure 2b. NPPB attenuated TPD in Figure 2b suggested that *CfTX-1* peptide triggered anion influx and TPD was Cl^- influx.

CfTX-1 peptide intervened TPD attenuated in adrenergic receptor activated *HCTZ* urothelium

Figure 2d was the $CaCl_2$ activated ionic permeability increasing induced amplitude of TPD in *HCTZ* urothelium that cholinergic receptor activated by *Ach*. In *HCTZ* urothelium, $CaCl_2$ induced slightly but observable increased positive amplitude of potential difference. However, as shown in Figure 2f, *HCTZ* urothelium activated by adrenergic receptor agonist *NE* induced enhancement amplitude of TPD which suggested adrenergic receptors were the excitatory factor to increasing polarization in *HCTZ* urothelium. In *HCTZ* urothelium, *Ach* treated obtained 18.72±3.11□V of TPD, moreover 82.40±9.55μV of TPD in *NE* treated *HCTZ* urothelium. A significantly results of *CfTX-1* peptide intervene *HCTZ* urothelium was $CaCl_2$ evoked TPD enhancement neither in *Ach* activated *HCTZ* urothelium



a.

1 2 3 4 5 6 7

```
        10         20         30         40         50
MVKMLFFAFL PLLFMTGIAA ESTISSGLNS LKTKIDAKMP SGKQLFDKVV
        60         70         80         90        100
EMQKQIDAKF SNDDERAKVM GAIGSLSTAV GKFQSGDPAK IASGCLDILV
       110        120        130        140        150
GISSVLKDFA KFSPIFSILS LVVGLFSGTK AEESVGSVVK KAVQEQSDQE
       160        170        180        190        200
LQEALYGVKR EYAVSKAFLD GVRNETSDLS PTEVSALAAN VPIYQGVRFI
       210        220        230        240        250
AMVVQRIKYI KPKTESEIKR MLTMLELFTD LCSLRDLILL DLYQLVATPG
       260        270        280        290        300
HSPNIASGIK EVSNLGREEY KKVFEDLLKN DDKETYLFLS YLYPREKNEQ
       310        320        330        340        350
SRKIFNFFDL MKVKYDDRLK QDLTGVKIFS NVHWPNYFMC SSNDYLALIC
       360        370        380        390        400
TKPYGSLKLD KLNDGYYSIK TTQHDPKICH RYGNYILFTH KRNDDLEKFN
       410        420        430        440        450
FVPVKLEKRE IYLLSSKESP NKFAYVPQNA DGALFFVDGI PSKVGYGNQG
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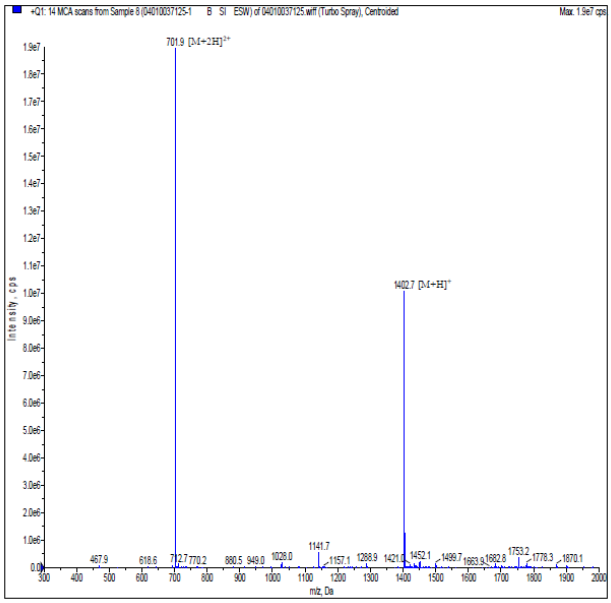
b. YFTLVE



c.

Figure 1. *Aurelia aurita* concentrated crude venom and *CfTX-1* partial sequence identification. (a) The candidate proteins in 43kDa band were confirmed from *Aurelia aurita* lyophilized concentrated crude in 10% acrylamide. The arrow marked band location in lane 5, 6, 7 was the 43 kDa band of crude venom. The sampling gradient concentration was 10, 5 and 2.5 µg, respectively. Lane 2, 3, 4 are bovine serum albumin with the sampling gradient concentration 10, 5 and 2.5 µg, respectively. Bovine serum albumin band was the reference band for optical density calculation to confirm the grossly candidate proteins in 43 kDa band (the optical density calculation is not shown). (b) The partial sequence of *CfTX-1* N-terminal fragment were identified from this band. ifnffdlmkvk of Toxin *CfTX-1* was the specific sequence confirmed from Hainan local *Aurelia aurita* crude venom. (c) This partial sequence of *CfTX-1* was laboratory synthesized and final quality control by HPLC and electrospray Ionization Tandem Mass Spectrometry (ESI-MS). The peak value of 1402.7 was the synthesized and purified *CfTX-1* peptide.

nor in *NE* activated *HCTZ* urothelium (Figure 2e and 2g, marked with vertical arrows), which suggested the *CfTX-1* peptide intervene evoked enhancement of urothelium polarization were inhibited by G-protein coupled receptors.

Summary of above results indicated that (1) *CfTX-1* peptide intervene can evoke significantly urothelium polarization in *HCTZ* urothelium preparation. Furthermore, the main compound of ionic current induced supra polarization was Cl^-, because *NPPB* can block this specific supra polarization. (2) *CfTX-1* peptide evoked Cl^- current was inhibited by G-protein coupled receptors.

DISCUSSION

In this study abundant of *Aurelia aurita* tentacles nematocyst proteins were identified from polyacrylamide

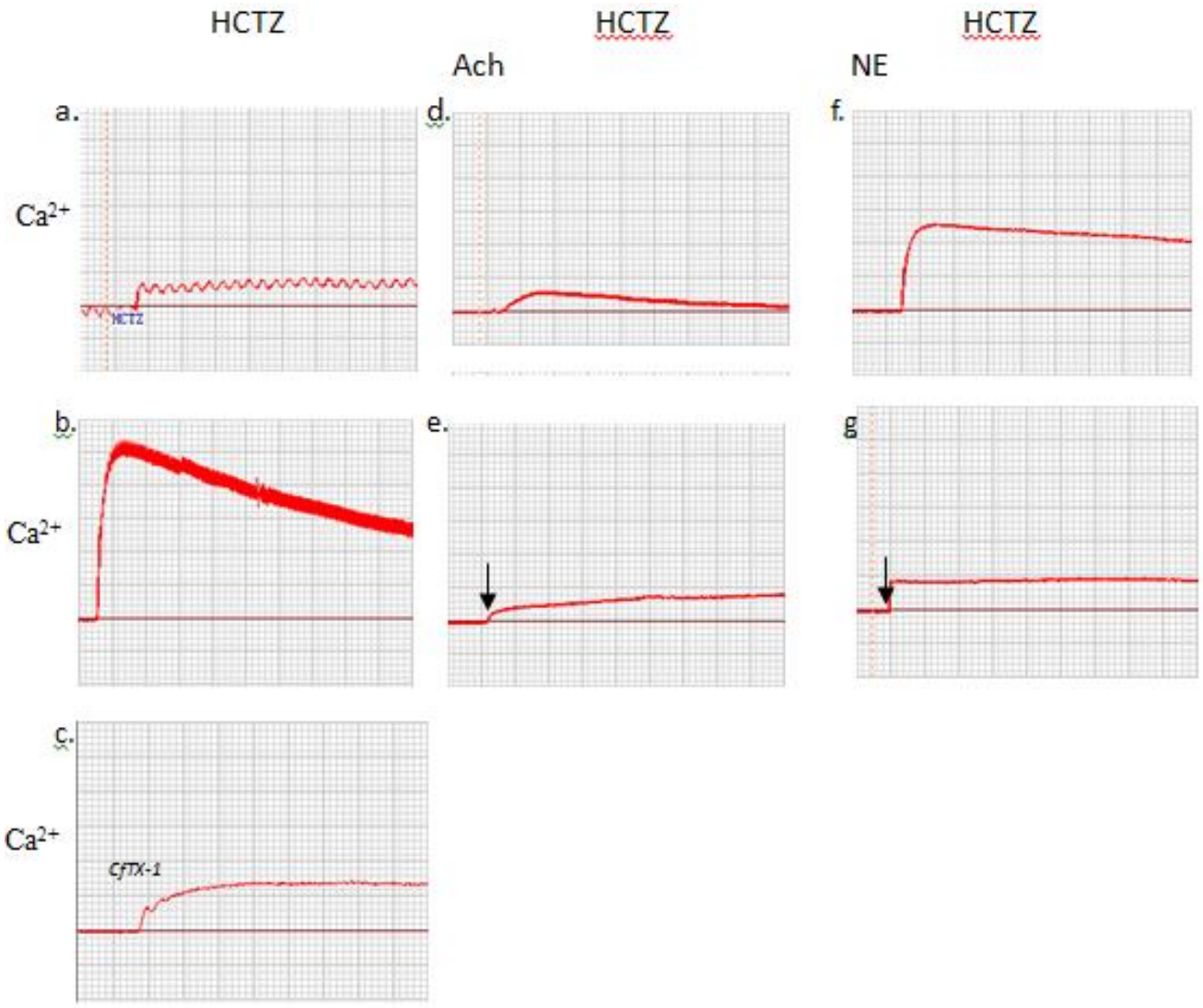


Figure 2. *CfTX-1* peptide evoked transurothelium potential difference in *HCTZ* urothelium. (a) $CaCl_2$ activated ionic current and amplitude of TPD in *HCTZ* urothelium, slightly polarized *HCTZ* urothelium. (b) *CfTX-1* peptide intervened evoked an enhancement of TPD in *HCTZ* urothelium. This generated supra polarization on *HCTZ* urothelium. (c) Cl^- channel blockade *NPPB* treatment induced recognized attenuated amplitude of TPD in *CfTX-1* peptide intervened *HCTZ* urothelium. (d and f) $CaCl_2$ activation induced TPD in G-protein receptor agonists treated *HCTZ* urothelium. TPD in *Ach* treated had no significant changes, however *NE* treatment induced a dramatically enhancement of TPD in *HCTZ* urothelium and the urothelium was supra polarized. (**e** and **g)** The *CfTX-1* peptide intervened TPD in *HCTZ* urothelium were significantly attenuated after *Ach* and *NE* treatment.

gel. a specific sequence that belonged to a fragment of *CfTX-1* N-terminal was identified. As the *CfTX-1* N-terminal was function as the cellular outer membrane porin protein (OmpD), this study forward investigated this partial sequence of *CfTX-1* evoking ionic permeability induced TPD in *HCTZ* urothelium. The results revealed that the *CfTX-1* peptide represented enhanced positive peak potentials in anode on apical membrane, generated supra polarization on *HCTZ* urothelium. Moreover, the supra polarization was inhibited in adrenergic receptor activated *HCTZ* urothelium preparation. There is a precious reference report which suggested this result. Thurman and Higgins, 1988) reported that α2-adrenoceptor agonist was most potent for stimulating the amplitude of the short-circuit current in frog urothelium, and related to a simultaneous increase in the transepithelial flux of both chloride and sodium. Jellyfish proteins induced cell membranes pore formation was first reported in box jellyfish proteins (Nagai et al., 2000). Both *CfTX-1* and *CfTX-2* generated ring-shaped pores on cellular membranes which fold in two domains, larger N-terminal domain representing almost 75% of the structure, and a

smaller C-terminal domain. Using tandem mass spectrometry, the proteome of *Aurelia aurita* crude venom profiled in 43 kDa band extracted peptide has significant sequence similarity to *CfTX-1* N-terminal (Figure 1b). *CfTX-1* which belongs to a family of pore-forming cnidarian toxins with N-terminal domain composed exclusively of α-helices and connecting loops have significant structural similarity to N-terminal domains of pore-forming 3d-Cry toxins (Brinkman, 2007, 2012). As the domain I of Cry toxin shares a significant structural similarity with the pore-formation domain of β-PFTs colicin A, *CfTX-1* N-terminal estimated that might play an essential role in membrane penetration and pore formation after binding to the specific receptors.

Amphibian urinary bladder serosal surface was covered by an incomplete mesothelium, urothelium were water infiltrate in hypotonic in serous layer (Peachey, 1961). As is well known, Ca^{2+} applied directly to the mucosal surface has no effect, however, in the living toad the Ca^{2+} supply normally via the capillary network, which is on the serosal side of the epithelial sheet. Furthermore, since the mucosal surface of the bladder is normally impermeable to water, it does not seem that molecule as large as the ion could penetrate this barrier to express their activity. $CaCl_2$ was used to activate serous chamber (basement membrane); this protocol reported increases $[Ca^{2+}]_i$ through acting Na^+/H^+ transporter via basolateral membrane of the urothelium (Harvey and Ehrenfeld, 1988). Another cascade is the activation of phospholipase C and inositol 1,4,5-trisphosphate (IP_3) pathway (Hanna-Mitchell and Gebruers, 2006). It is final release of Ca^{2+} from cytoplasmic storage. This induced secondary influx of Ca^{2+} via calcium release-activated chloride channels, as well as serous Ca^{2+} induced ATP increased $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} (Brodin et al., 1996; Brodin and Nielsen 2000). The thiazide urothelium generated dramatic enhanced positive potential by *CfTX-1* peptide (Figure 2b), however, this enhancement were blocked by Cl^- channel blockade *NPPB* (Figure 2c). It could be summarized that the *CfTX-1* peptide significantly increased urothelium barrier permeability, increased Cl^- influx on urothelium basal membrane. The role of chloride uptake is probably carrier mediated and driven by symporter or counter transporter. In thiazide urothelium chloride may pass through the pore formation of *CfTX-1* peptide. Classic frog serous-urothelium membrane studies revealed that serous ATP activated in tight urothelium increased in $[Ca^{2+}]i$, influenced both apical Na^+ channels and basolateral K^+ channels, activates chloride channels, plasma membrane proteins Ca^{2+} activated Cl^- channels mediates the secretion of Cl^-, bicarbonate and thiocyanate. In smooth muscle and excitable cells of the nervous system, calcium-dependent chloride channel (CaCCs) have an excitatory role coupling intracellular Ca^{2+} elevation to membrane depolarization, causes the appearance of Ca^{2+}-activated Cl^- currents in native tissues (Ferrera et al., 2011). However, this classic mechanical is not enough to explain the extremely evoked urothelium hyperpolarization triggered by *CfTX-1* peptide. Cellular membrane pore-forming, resulting in increasing membrane chloride conductance and, thus, depolarization of the urothelium was further dependent on membrane electrochemical gradient.

Conclusion

CfTX-1 peptide is a partial sequence of *Chironex fleckeri* toxins, which was identified from *Aurelia aurita* nematocysts from Qiongzhou Strait, Hainan province, China. The primary activation with $CaCl_2$ indicated the increased permeability of anion accross thiazide urothelium, inducing secondary transurothelium potential enhancement. This supra polarization was probably induced by *CfTX-1* peptide improved high permeability to Cl^-. The Cl^--induced supra polarization mechanism is meaningful in improving drug delivery crossing the urothelium barrier, and in promoting the drug clearance crossing the barrier.

ABBREVIATIONS

CfTX-1, Chironex fleckeri Toxin 1; **NPPB,** 5-Nitro-2-(3-phenylpropylamino) benzoic acid; **Ach,** acetylcholine; **NE,** norepinephrine; **HCTZ,** hydrochlorothiazide.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effects of salt stress on antioxidant system activity and peroxidation damage in root tip cells of strawberry

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In this study, strawberry seedlings were treated with 250 mmol·L^{-1} NaCl solution; then the O_2^- production rate, the content of H_2O_2 and thiobarbituric acid reactive substance (TBARS), electrolyte leakage (EL), the enzyme activity of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) in the root tip cells were determined to be used for analysis after salt stress. The results revealed that the O_2^- production rate, EL, and the content of TBARS showed an increasing trend with the time of salt stress, while the content of H_2O_2, the enzyme activity of SOD, POD and CAT exhibited a pattern of increasing first and decreasing later under the salt stress. In conclusion, the results demonstrated that the oxidative damage in root tip cells of strawberry was increased with the time of salt stress, and the enhanced antioxidant activity was not enough to eliminate the peroxidation damage which was caused by 250 mmol·L^{-1} NaCl solution.

Key words: Strawberry root tip, salt stress, the activities of the antioxidant enzymes.

INTRODUCTION

Roots are not only the most important nutrient uptake organs of plants, but also the most sensitive parts of salt stress, and promote the plant to make a further response to stress. Roots response to salt stress mainly includes growth inhibition, osmotic regulation, protects the enzyme system to operate effectively, the metabolism of active oxygen, the changes of membrane structure and function in the root (Bohra and Doerffling, 1993). Salt stress leads to accumulation of a variety of reactive oxygen species (ROS) in plant cells, such as H_2O_2 and O_2^-; the excessive ROS is toxic to the plant cells and causes the peroxidation of membrane lipid, which leads to an increase in the level of EL level and the content of TRABS, and degradation of biological molecules such as proteins, DNA molecules and carbohydrates (Rusty and Regina, 2005; KOC, 2015; Yang and Guo, 2018), thus affecting the normal functions of these molecules, which interfere with the normal physiological activities of the cells. Further research indicates that the imbalance of ROS metabolism is the primary reason that salt stress leads to the over-oxidation injury in plant cells and cell death (Katsuhara and Kawasaki, 1996). There are non-enzymatic antioxidant systems, and antioxidant enzyme systems in the plants to eliminate the peroxidation damage caused by salt stress and to maintain the normal growth of plants.

The strawberry (Fragaria × ananassa Duch) is the collective name of Rosaceae family plants, belonging to perennial herbaceous plants, the fruits are red berries, soft and succulent, with unique flavor, so it is popular with people. With facilities in the development of agricultural technology, the cultivation facilities of strawberry have

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also been developed rapidly. However, secondary salinization of the soil gradually becomes an important factor limiting the greenhouse cropping system, which affects the improvement of the yield and quality of strawberry. Therefore, the study on the damage to strawberry growth and root system caused by high concentration salt stress becoming an important subject for the future development of strawberry industry. In this work, the strawberry root tip was used to analyze antioxidant activity and peroxidation damage under the salt stress, which provides a theoretical basis for the study of the effects of salt stress on the physiological characteristics of strawberry.

MATERIALS AND METHODS

Plant and treatment

Forty-five-days old strawberry (*Fragaria* × *ananassa* Duch. cv. Fengxiang) plants were used in this study; the uniform and healthy seedlings were pre-cultured with 1/2 strength Hoagland's nutrient solution for three weeks until the growth of roots at about 6 cm. Then, the strawberry seedlings were divided into two groups (control and stress treatment) and transferred into Hoagland's nutrient solution and high-salinity 1/2-strength Hoagland's nutrient solution (250 mmol·L^{-1} NaCl), and then the 1 cm roots tip samples were harvested at 0, 12, 24, 48 and 72 h, respectively. Then, the samples were frozen immediately in liquid nitrogen and stored at -80°C for the further experiments. The strawberry seedlings were kept in a growth chamber with a light/dark regime of 16 h/8 h at 24°C, 75% relative humidity, and a light intensity of 800 µmol m^{-2} s^{-1} of photosynthetically active radiation. The culture solution was as follows: 6 mmol·L^{-1} KNO_3, 4 mmol·L^{-1} $Ca(NO_3)_2$, 2 mmol·L^{-1} $NH_4H_2PO_4$, 1 mmol·L^{-1} $MgSO_4$, 50 µmol·L^{-1} KCl, 10 µmol·L^{-1} H_3BO_3, 2 µmol·L^{-1} $MnSO_4$, 2 µmol·L^{-1} $ZnSO_4$, 0.5 µmol·L^{-1} $CuSO_4$, 0.065 µmol·L^{-1} $(NH_4)_6Mo_7O_{24}$, and 40 µmol·L^{-1} Fe–EDTA (Hoagland and Arnon, 1950). The nutrient solution was ventilated for 30 min every 2 h by the combined action of the timer (Pinyi AL-06, China) and ventilation pump (SUNSUN ACO, China); the hydroponic system is shown in Supplementary Figure 1 and replaced once a day. The pH of all nutrient solutions was adjusted to 6.5 with 0.1 M KOH. Three biological replicates were used for each sample and 50 seedlings were used for each group.

Physiological measurement

The measurement of electrolyte leakage (EL) referred to the method used by Heath and Packer 1968), and the content of thiobarbituric acid reactive substance (TBARS) was measured by adding an equal aliquot of 0.5% TBA in 20% trichloroacetic acid to an aliquot of the incubation mixture containing a few grains of sand used as a boiling aid. The solution was heated 25 min at 95°C, and then centrifuged for 1 min in a clinical centrifuge to clarify the solution. Absorbancy was measured at 532 nm corrected for nonspecific turbidity by subtracting the absorbancy at 600 nm (Fu and Huang, 2001). H_2O_2 was extracted and measured by Mukherjee and Choudhuri (1983), and brief method is as follows: 0.5 mL potassium phosphate buffer with pH=7.8 was added to the test tube containing the sample, and then 0.1 mL with 10 mmol/L hydroxylamine hydrochloride were added to the experimental sample. After 20 min of water bath (25°C), 1 mL with 58 mmol/L of sulfonamide and 1 mL with 7 mmol/L of alpha-naphthalamine were added to the experimental sample. After 20 min of water bath (25°C), the light absorption value of the sample at 530 nm was measured. O_2^- production rate were measured by Elstner and Heupel (1976), and the brief method is as follows: isolation was made from 5 g of tissue in ice cold acetone. By addition of 5% (w/v) titanyl sulphate and conc. NH_4OH solution, the peroxide-titanium complex was precipitated. This was dissolved in 15 ml of 2 M H_2SO_2, marking the final volume 20 ml with cold water. The absorbance was read at 415 nm against a water blank. The H_2O_2 content was calculated from a standard curve prepared in a similar way. Activities of catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD) referred to the method used by Chen and Cheng (2003) and Giannopolitis and Ries (1977), respectively. For CAT, the decomposition of H_2O_2 was measured by the decline in absorbance at 240 nm for 1 min. The 3 ml reaction mixture contained 0.1 ml enzyme extract, 15 mM H_2O_2 and 50 mM phosphate buffer (pH 7.0), which initiated the reaction. For POD, the oxidation of guaiacol was measured by the increase in absorbance at 470 nm for 1 min. The reaction mixture contained 0.1 ml enzyme extract, 50 µl of 20 mM guaiacol and 2.8 ml of 10 mM phosphate buffer (pH 7.0). The reaction was started with 20 µl of 40 mM H_2O_2. For SOD, a 3 ml reaction mixture contained 20 µl of enzyme extract, 1.3 µM riboflavin, 63 µM NBT, 13 mM methionine, 0.1 mM EDTA, and 50 mM phosphate buffer (pH 7.8). The test tubes containing the mixture were placed under light at 78 µmol photons s^{-1} m^{-2} for 10 min and absorbance at 560 nm was recorded. Each treatment was repeated three times.

Statistical analysis

Three replicates (three plants per replicate) were used for all experimental treatment. Statistical analyses of the data were performed using the SPSS statistical package and the differences were statistically compared by employing the Duncan test with a significance level of $p < 0.05$.

RESULTS

To evaluate the degree of oxidation of strawberry root tip cells, the O_2^- production rate and the content of H_2O_2 were measured. As shown in Figure 1A, the production rate of O_2^- did not change notably in the control, however, the production rate of O_2^- went up steadily until reaching the peak value at 72 h in the strawberry root tip cells under the salt stress treatment, nearly 2.1 times higher than the control. There was no significant change in the content of H_2O_2 in the root tip of the control, while the content of H_2O_2 rose steadily at the root tip of strawberry until reaching the peak at 36 h under salt stress, which was 2.4-fold increase relative to the control, and the content of H_2O_2 decreased afterwards, but was still significantly higher than the control (Figure 1B).

TBARS was one of the main products of membrane lipid peroxidation, which shows the degree of oxidative damage in plant cells. As shown in Figure 2A, the content of TBARS had been higher than the control after 12 h salt stress treatment in the root tip cells of strawberry, and then continued to rise until reaching the maximum value at 72 h, almost 3 times as much as the control. In this period, there were few changes in the control (Figure 2A).

In addition, there was the same trend of the level of EL and the content of TBARS in both control and treatments,

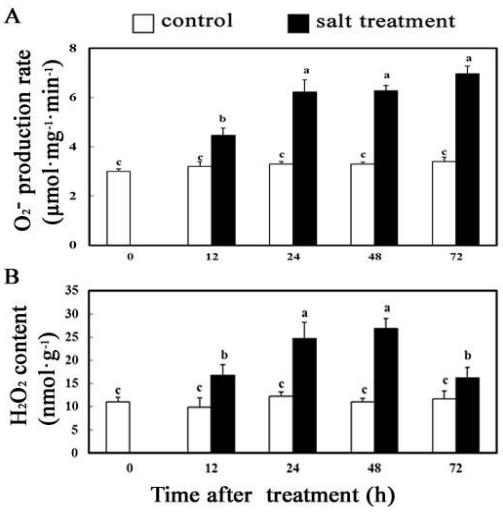

Figure 1. The changes of O_2^- production rate (A) and the content of H_2O_2 (B) in CK and salt stress treatment in the root tip cell of strawberry.

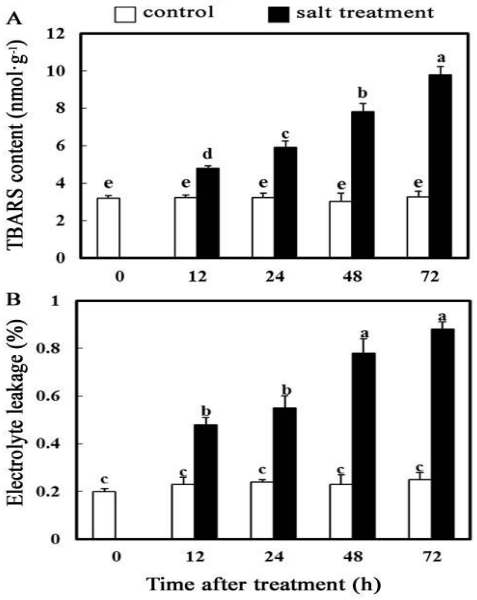

Figure 2. The content of TBARS (A) and the level of EL (B) in the strawberry root tips of strawberry under salt stress.

and the level of the EL reached the peak value at 72 h in the root tip cells of strawberry under salt stress, which was 3.5-fold increase relative to the control (Figure 2B). These results indicated that salt stress caused injury to the membrane in the root tip cells of strawberry, which increased the permeability of plasma-membrane leading to increase in the TBARS content and the EL.

Three significant antioxidant enzymes (SOD, POD and CAT) were important factors for scavenging ROS, which

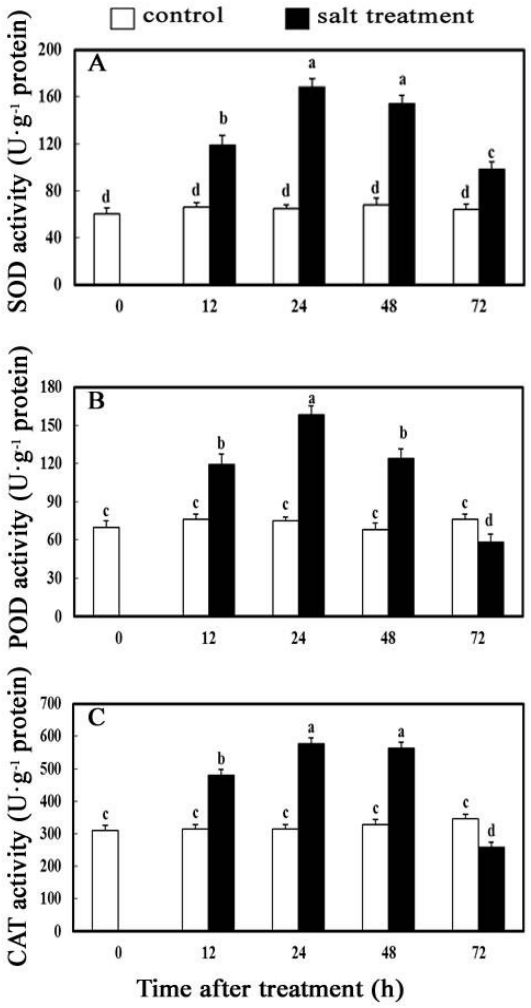

Figure 3. Activities of antioxidant enzymes in the root tip cells of strawberry under normal conditions and salt stress treatment. (A-C) Activities of SOD (A), POD (B) and CAT (C) in the root tip cells of strawberry under normal conditions and salt stress treatment.

proved to be the most effective in maintaining the balance of ROS in plant cells. In the present study, the activities of the three antioxidant enzymes were measured under normal condition and salt stress treatment with the extension of processing time. As can be seen in Figure 3A, the activities of SOD in the root tip cells of strawberry had no significant changes under normal condition, while the activities of SOD rise during early salt stress treatment, but tended to decline after 24 h and became significantly higher than the control at 72 h. The activities of POD and SOD in the root tip cells of strawberry showed similar tendency, except the activities of POD in control were higher than in the case of salt stress treatment at 72 h (Figure 3B). Besides that, the variation patterns of CAT enzyme activities were similar to that of POD enzyme activities (Figure 3C).

DISCUSSION

Salt stress causes a variety of injuries to plant cells, among which ion toxic, osmotic stress and the accumulation of ROS are the particular problems (Mittler, 2002). ROS is the secondary metabolite in plant photosynthesis and respiration, which primarily is generated in some oxidation of some mitochondrial enzymes, chloroplasts and peroxisome. Under normal growth conditions, the generation and depletion of ROS are kept in a state of dynamic balance, nevertheless, the state of balance is easy to destroy to generate excessive ROS in plant cells in the abiotic stress conditions which leads to oxidative stress occurrence and cell damage (Faghih et al., 2017). Under salt stress, the fixed rate of CO_2 is limited, leading to increase in the suppression of electron flow in the photosynthetic chains, thus excessive superoxide anion, other free radicals and active oxygen such as H_2O_2 and O_2^- are generated (Gill and Tuteja, 2010). In this research, we found that the O_2^- production rate and the content of H_2O_2 in the root tip cells of strawberry increased significantly as stress time increased, which is supported by earlier studies, such as soybean, wheat and chickpea. Under the condition of oxidative stress, ROS are so extremely active that lead to membrane lipid peroxidation. Fortunately, the existence of antioxidant enzymes such as POD, SOD and CAT in the plant cells can reduce the damage by the peroxidation. However, the present study shows that though the activities of antioxidant enzyme are elevated, but there are still high EL level and TBARS content in the root tip cells of strawberry under salt stress treatment, which are similar to the experimental results of Christou et al. (2014), showing that the antioxidant system does not provide enough protection to prevent the root tip cells of strawberry from the oxidative damage, and the results of this study are similar to the results reported by Rasool et al. (2013). In addition, although the activity of antioxidant enzymes increased in the early stage of salt stress, with the extension of stress time, the ability of antioxidant system to scavenge reactive species is gradually reduced, which further aggravates lipid peroxidation reaction in the root tip cells of strawberry, which are supported by Christou et al. (2014) and Gill and Tuteja (2010).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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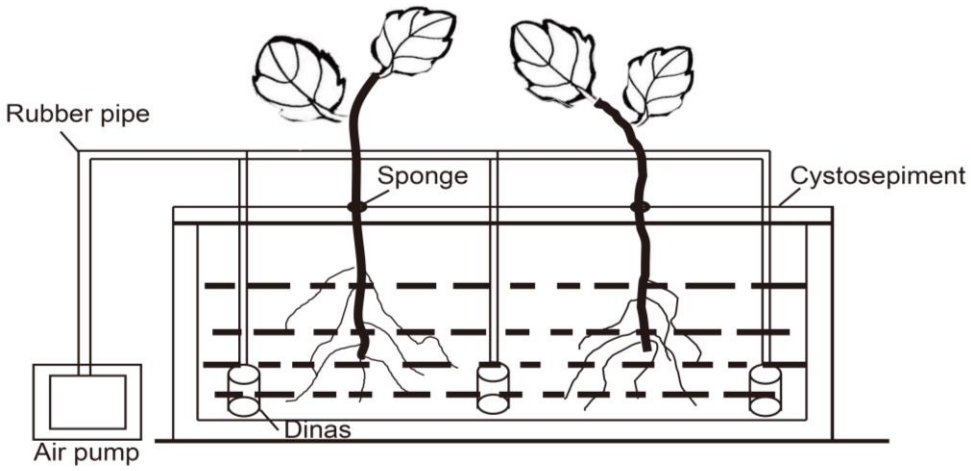

Supplementary Figure 1. A hydroponic plant schematic diagrams of the strawberry seedlings.

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Full Length Research Paper

Effect of explant source and different hormonal combinations on *in vitro* regeneration of *Heracleum candicans* Wall: An important medicinal herb

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***In vitro* regeneration of plants is influenced to a great extent by genotype, the type of explant used, media composition and plant growth regulators. This research was devised to study the effect of explant source and different hormonal combinations on the *in vitro* regeneration of *Heracleum candicans.* Two different explants viz; leaf and petiole were used to investigate their morphogenic response on MS medium supplemented with different concentrations of auxins (2, 4-dichlorophenoxyacetic acid and indole 3-acetic acid) and cytokinins (6-benzyl amino purine and kinetin) either individually or in combinations. Petiole explants were most responsive for callus production as well as shoot regeneration. 2,4-D at 3 mg/L proved to be the best concentration for callus induction. For shoot regeneration, MS medium supplemented with BAP at 3 mg/l and IAA at 2 mg/l proved to be effective in production of 21±0.7 mean number of shoots in 90% of cultures. Regenerated shoots were separated and rooted on full strength MS basal medium producing 6.9 average numbers of roots per shoot in 90% of cultures. The present research yielded a suitable explant and optimum concentrations of different plant growth regulators for *in vitro* regeneration of this important medicinal herb.**

Key words: *Heracleum candicans,* leaf, petiole, callus, shoot regeneration.

INTRODUCTION

Heracleum candicans is a perennial herb endemic to the northwest Himalayas and is found growing in alpine zones of West Pakistan, Nepal, Bhutan, Afghanistan and India (Sharma and Wakhlu, 2001). Propagation occurs by means of seeds, however, the seed germination is poor. This poor germination coupled with unsustainable harvesting of plants from natural habitats for commercial utilization threatens the existence of this plant species (Kaul, 1989). Being the main source of xanthotoxin, it has an immense demand in pharmaceutical industries (BCIL, 1996). Xanthotoxin is widely used to treat leucoderma and to prepare suntan lotions (Kaul, 1989). The fruit is used as an aphrodisiac and nerve tonic (Satyavati and Gupta, 1987). Activity-guided isolation has also shown

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heraclenin to be the anti-inflammatory principle present in *H. candicans.* Extracts of root and shoot showed antibacterial activity (Kaur et al., 2006). A lengthier vegetative growing phase coupled with low seed viability in this species (Butola and Badola, 2004, 2006) offer fewer chances to revive and shift into fresh habitats. Because of the high market price of its rootstock, it is one of the important medicinal plants exported from India (BCIL, 1996). Owing to its unsustainable harvesting in nature, this plant is categorized as endangered for northwest Himalayas (Anonymous, 1998; CAMP, 2003), and vulnerable for the Jammu & Kashmir State in India (Ved et al., 2003).

The instantaneous rising demand of herbal medicine is creating heavy pressure on some selected important medicinal plants in the wild due to overexploitation. Several of these medicinal plant species have slow growth rates, low population densities and narrow geographic ranges (Kala, 2009); therefore, they are more likely to undergo extinction (Anandanayaki, 2010). The vanishing of such dynamic and huge amounts of biodiversity presents one of the serious challenges for the world community to halt the devastation of plant diversity that is essential to meet the present and future needs of mankind. To regenerate organs from plant tissues, media components and hormone concentrations can be optimized. Tissue culture is not only a necessary enabling technology for transgenic plant production but is also used for *in vitro* propagation of valuable plants. Large-scale propagation is a prerequisite to meet the pharmaceutical needs, and also for effective conservation of endangered plant species. Tissue culture, from conservation point of view is important as it requires a small amount of propagules for mass multiplication providing huge quantity of raw material to herbal drug industry. Micropropagation of plant species has been found to be explant based. In case of Apiaceae family, different explants namely intermodal segments (Yoshimatsu and Shimomura, 1991), shoot tips (Wakhlu and Sharma, 1998; Rachetti and Biradar, 2016; Srilakshmi et al*.,* 2016), single-node explants (Chandrika et al., 2015; Tuncer, 2017), hypocotyl explant (Thapa et al*.*, 2015; Mandal and Sharma, 2016), leaf explant (Rao et al*.,* 2015; Soorni and Kahrizi, 2015) and petiole explant (Askari-Khorasgani et al*.*, 2013; Torabi et al*.,* 2014; Sharma, 2015) have been used for *in vitro* regeneration of different plants. The present study is aimed at identifying the best type of explant as well as the most efficient growth regulator concentration and combinations for shoot formation and regeneration of *H. candicans.*

MATERIALS AND METHODS

For standardization of *in vitro* regeneration protocols, two different explants viz; leaf and petiole were used. Leaf and petiole explants obtained from Kashmir University Botanical Garden (Voucher Specimen Number 2616-(Ref.No.F/Herbarium-Specimen COPT) KU/2017) were firstly washed with tap water in order to remove dust, dirt and other undesirable materials followed by washing with detergent solution (Labolene) containing 4 drops of Tween 20. This was followed by washing with tap water to eliminate the detergent. The explants were washed 2 to 3 times with double distilled water in a laminar air flow cabinet. Finally, the explants were sterilized with 2% sodium hypochlorite solution for different time durations. The surface disinfected explants were then washed 3 to 4 times with autoclaved double distilled water to remove the last traces of the sterilants. The disinfected plant material was then put into pre-autoclaved petri-dishes, cut into appropriate size and finally aseptically cultured on the medium. Each treatment involved about 10 to 30 explants and each experiment was repeated twice. Explants were inoculated onto MS basal medium (control) and MS medium fortified with different plant growth regulators both individually and in combinations. Throughout the experiments, culture room was kept entirely germ-free and explants were cultured under cool white light with 1500-3000 Lux light intensity. The temperature of the room was maintained between 22±4°C with 60-70% relative humidity and photoperiod of 16 h.

The cultures were regularly observed, changes in explant were recorded on weekly basis and the data was put into a suitable arrangement in tabulated form. The parameters recorded were induction of callus, texture of callus, shoot regeneration and root regeneration. Each experiment was repeated twice, data was analyzed by calculating Standard Error (SE) of various treatments and means were analyzed by analysis of variance (ANOVA).

RESULTS

During the present study, the comparative effect of two different explants viz. leaf and petiole and concentrations of auxins and cytokinins on the morphogenesis of *H. candicans* was analyzed. The results are summarized in Tables 1 to 4. The data revealed that there were significant differences in the type of explant used and the effect of different concentrations and combinations of 2,4-D, IAA, BAP and Kn.

Evaluation of the effect of explant type and hormonal concentration on callus induction

During the present investigation, callus induction from leaf and petiole explant was obtained on MS medium supplemented with auxins both individually and in combination with cytokinins. Among auxins, five different concentrations of 2,4-D were used for callus induction. Callus induction was observed in both the explants and callus induction percentage varied significantly among the two explants. The 2,4-D at 3 mg/L was the optimum or threshold concentration for callus induction in both the explants, however the number of days taken for callus induction were shortest in the case of petiole explants. In order to examine the effect of auxin-cytokinin combinations on callus induction, leaf and petiole explants were inoculated on MS medium supplemented with 2,4-D (3 mg/L) in combination with five different concentrations of Kn. The 2,4-D at 3 mg/L and Kn at 2 mg/L proved to be the optimal concentration for maximum callus induction, with 100% culture response occurring in the petiole explant. By increasing the

Table 1. Effect of explant type and hormonal concentration on callus induction.

Explant	2,4-D concentration (mg/L)	Initiation of callus (days)	% Culture response
Leaf	1	38	50
	2	33	70
	3	30	90
	4	40	30
	5	0	0
Petiole	1	21	40
	2	19	80
	3	12	100
	4	20	70
	5	25	60

Table 2. Effect of explant type and auxin-cytokinin combinations on callus induction.

Explant	2,4-D concentration mg/L	Kn concentration (mg/L)	Initiation of callus (days)	% Culture response
Leaf	**3**	1	24	40
	3	2	20	90
	3	3	25	80
	3	4	27	70
	3	5	29	60
Petiole	**3**	1	0	0
	3	2	14	100
	3	3	25	80
	3	4	28	50
	3	5	30	30

Table 3. Effect of explant type and hormonal concentration on shoot regeneration.

Explant	BAP concentration (mg/L)	Average number of shoots±SE	Average height of shoots (cm)±SE	Initiation of callus (days)	% Culture response
Leaf	1	0	0	0	0
	2	3 ± 0.2^{a}	2.6 ± 0.1^{a}	18	70
	3	5.9 ± 0.5^{c}	5.7 ± 0.2^{c}	15	80
	4	4.6 ± 0.3^{bc}	4.0 ± 0.2^{b}	21	50
	5	3.6 ± 0.3^{ab}	2.1 ± 0.2^{a}	23	30
Petiole	1	3.1 ± 0.3^{a}	1.3 ± 0.0^{a}	21	30
	2	9.2 ± 0.5^{ab}	5.8 ± 0.1^{ab}	18	60
	3	15.2 ± 0.6^{b}	7.6 ± 0.2^{c}	16	100
	4	6.7 ± 0.2^{a}	4.4 ± 0.1^{bc}	20	50
	5	2.2 ± 0.2^{a}	1.5 ± 0.1^{ab}	23	30

Different letters on the values indicate that the means are significantly ($P < 0.05$) different (Tukey's HSD test).

concentration of Kn further, there occurs a continuing decrease in callus induction, because of the fact that increasing the ratio of cytokinin to auxin favors shoot regeneration.

Table 4. Effect of explant type and auxin-cytokinin combinations on shoot regeneration.

Explant	BAP concentration (mg/L)	IAA concentration (mg/L)	Average number of shoots±SE	Average height of shoots (cm)±SE	Initiation of callus (days)	% Culture response
Leaf	3	1	9.3±0.7 ^{cd}	6.3±0.1^{bc}	15	70
	3	2	11.2±0.6 ^{d}	6.8±0.1^{c}	10	80
	3	3	7.5±0.4 ^{bc}	6.0±0.1^{bc}	16	60
	3	4	6.3±0.4 ^{b}	5.4±0.3^{b}	19	50
	3	5	3.2±0.2 ^{a}	1.4±0.1^{a}	21	30
Petiole	3	1	11.3±0.5^{c}	3.9±0.1^{c}	14	60
	3	2	21±0.7^{d}	4.9±0.1^{a}	11	100
	3	3	6.6±0.6^{b}	3.5±0.2 ^{bc}	19	70
	3	4	3.8±0.2^{a}	3.0±0.1^{b}	24	40
	3	5	3.1±0.3^{a}	2.2±0.0^{a}	28	30

Different letters on the values indicate that the means are significantly ($P < 0.05$) different (Tukey's HSD test).

Evaluation of the effect of explant type and hormonal concentration on shoot regeneration

During the present study, shoot regeneration was witnessed from both the explant types on MS medium supplemented with cytokinins individually and in combination with auxins. When MS medium was fortified, five different concentrations of BAP individually, BAP at 3 mg/L proved to be best medium for shoot regeneration with maximum number of shoots obtained from petiole explant in minimum number of days. Among five different auxin and cytokinin combinations used, BAP at 3 mg/L and IAA at 2 mg/L gave best results in both the explant types with maximum number of shoots regenerated from petiole derived calli. By increasing the auxin concentration from 2 to 5 mg/L in the medium, a significant decrease occurred in the frequency and number of shoots produced per explant with an increase in the amount of callus. For root formation, the *in vitro* raised shoots were subcultured on MS basal medium and MS medium containing IAA, NAA and IBA individually. In the present study, MS basal medium was effective for root regeneration. Among hormone supplemented medium, the most effective concentration at which maximum root induction was achieved was IAA at 2 mg/L.

DISCUSSION

The explant source is a vital factor for *in vitro* growth and development of plant species affecting callus production, shoot bud induction as well as regeneration and multiplication. During the present research, statistical analysis revealed that there were significant differences between the two explant types used. Petiole explant proved to be the most effective explant for both callus induction as well as shoot formation (Figure 1). Patra et al. (1998) developed a successful protocol for *in vitro* plant regeneration from callus derived from leaf explants in *Centella asiatica* (L.). Mohapatra et al*.* (2008) also performed *in vitro* studies in *C. asiatica* using leaf explant. The study revealed that frequency of multiple shoot regeneration was much higher on MS medium supplemented with combination of auxin and cytokinin than cytokinins used individually. Sharma and Wakhlu (2001) obtained similar results for adventitious shoot induction from petiole explants of *H. candicans* Wall. However, the researchers used 2,4-D rather than IAA used in the present study. Makunga et al*.* (2003) obtained direct shoot regeneration from petiole explant of *Thapsia garganica* on MS medium supplemented with 0.5 mg /L NAA and 1.5 mg/l BAP which is in contrast with our results. Sharma (2009) achieved callus initiation from petiole explants of *H. candicans* on MS medium supplemented with 0.5 mg/L 2,4-D and 0.5 mg/L BAP. In addition, the best combination of growth regulators for maximum callus growth was obtained upon subculture of callus to a medium supplemented with 1 mg/L 2,4-D and 0.25 mg/L Kn. Askari-Khorasgani et al*.* (2013) also used petiole explant for direct regeneration of *Kelussia odoratissima.* The maximum rate of shoot multiplication was achieved by inoculation of petiole explants on MS medium fortified with 2 mg/L BAP and 0.1 mg/L NAA. Sharma (2015) performed studies on callus induction from petiole explants in *Ferula jaeschakeana* in which explants were cultured on MS medium supplemented with 2,4-D for callus initiation. The best results of callus induction were obtained on medium fortified with 1 mg/L 2,4-D. The addition of Kn in combination with 2,4-D enhanced the callus formation from the explants.

Conclusion

The present study yielded a suitable explant and

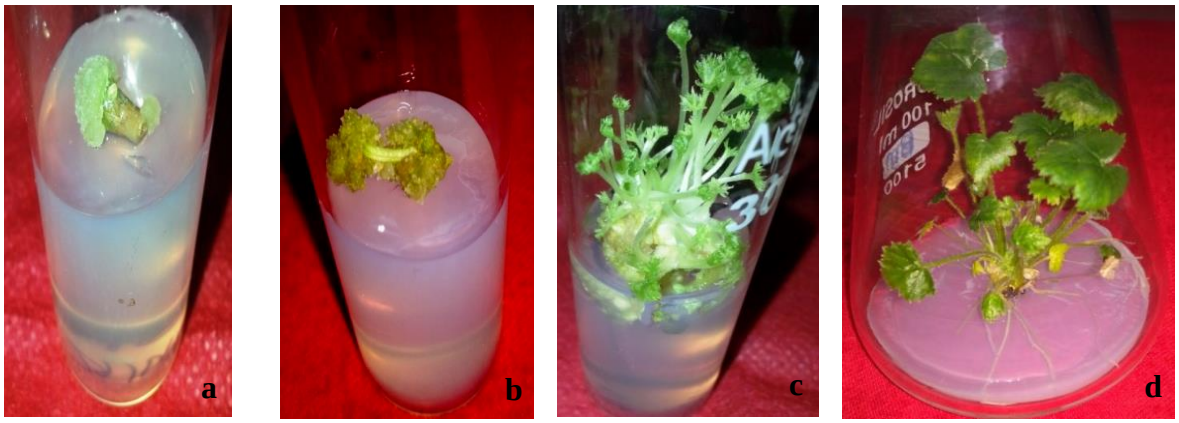

Figure 1. (a) and (b) callus induction from petiole explant (c) Shoot regeneration from petiole derived calli (d) Root regeneration.

optimum concentrations of different plant growth regulators for *in vitro* regeneration of this important medicinal herb.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Anti-*Xanthomonas* activity of Antarctic fungi crude extracts

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Agriculture suffers considerable losses in its production caused by plant diseases. In the citrus culture some problems were associated with citrus canker caused by *Xanthomonas citri* subsp. *citri.* That same way, sugarcane plantations are impaired to leaf scald disease caused by *Xanthomonas albilineans*. One of the measures used to contain citrus canker is the spray of cupric bactericides; however for scalding leaves there are no satisfactory control. Due to the phytosanitary problem caused by *Xanthomonas* (*X. citri* and *X. albilineans*) and by the difficulty of its control, the search for new forms of defense, less harmful to the environment, has become increasingly required. Thus, the aim of this work was to obtain crude organic extracts from filamentous fungi isolated from Antarctic soil samples and assess its bioactivity potential against *X. citri* and *X. albilineans*. One hundred and twenty-two extracts were tested, seven extracts inhibited the cell growth of *X. citri*, one was bioactive only against *X. albilineans* and one extract inhibited the cell growth of both bacteria. The bioactive extracts had a mean inhibition value of 96% against both bacteria. The values of MIC90 and MBC of bioactive extracts were also determined; for *X. citri* the isolate 3.1 Fe presented the lowest values of MIC90 (0.28 mg/mL) and MBC (1.0 mg/mL) and the two bioactive isolates for *X. albilineans* (1.1-Fe and B-Fe) presented the same values of MIC90 (1.4 mg/mL) and MBC (1.5 mg/mL). The filamentous fungi that produced positive extracts were identified as belonging to the genus *Pseudogymnoascus* (n=8) and *Cladosporium* (n=1). The filamentous fungi isolated from Antarctic soil produced compounds with bioactivity against phytopathogens from the *Xanthomonas* genus.

Key words: Antarctic fungi; antibacterial action; citrus canker; leaf scald; natural extracts.

INTRODUCTION

Xanthomonas are rod-shaped Gram-negative and obligate aerobe bacteria, which have a polar flagellum (Tessmann, 2002). This genus comprises 27 species that cause serious disease in about 400 plants hosts (Ryan et al., 2011) and affect at least 124 monocotyledonous and 268 dicotyledonous plant species (Leyns et al., 1984).

All citrus species and varieties of commercial importance are affected by citrus canker caused by

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Xanthomonas citri subsp. *citri* (Schaad et al., 2006). Every year orchards around the world suffer losses due to citrus canker, mainly countries with favorable climates for the development of *X. citri*. The control of this disease is accomplished by uses of less susceptible varieties, eradication of contaminated plants, decontamination of seeds and equipment, use of windbreakers and mostly use of antibiotics and copper sprays. This causes damage to the environment due to its accumulative potential in soil and trophic chain (Rocha and Azevedo, 2017).

The leaf scald in sugarcane is a disease caused by *X. albilineans*. This infection can be characterized in three ways: Asymptomatic or latent form (plants can tolerate the pathogen for a few weeks without exhibiting symptoms); chronic form (characterized by white or yellow streaks parallel to the main vein on leaves); acute form (presents attacks on xylem reducing sucrose production, and when in advanced stages can kill the plant) (Tokeshi and Rago, 1980). The leaf scald control is still scarce; it is only advisable to clean equipment and seedlings to reduce the propagation of the pathogen.

Considering the phytosanitary problems caused by *Xanthomonas* (*X. citri* and *X. albilineans*), it becomes necessary to research new forms of control, more efficient and less damaging to the environment.

Substances obtained from natural sources are important given the variability of such products. In general, natural molecules show greater structural diversity and several bioactive functions (Feher and Schmidt, 2003; Dayan et al., 2009; Newman and Cragg, 2016).

New microorganisms and natural products can be isolated from extreme environments, such as Antarctica (Pikuta et al., 2007). Many studies highlight the potential of the Antarctic environment in the production of bioactive metabolites (Furbino et al., 2014), antifungal substances (Svahn et al., 2015) and active compounds against *Xanthomonas* species, including *X. citri* (Moncheva et al., 2002; Encheva-Malinova et al., 2015; Puric et al., 2018; Vieira et al., 2018).

Thus, the present work is aimed to discover new fungi isolated from Antartic soils with potential for the production of bioactive compounds against *X. citri* and *X. albilineans.*

MATERIALS AND METHODS

Strain of filamentous fungi

Sixty-one filamentous fungi were used in this study. Among them, twenty-three were isolated from soil under an iron bar in Deception Island (South Shetland Islands, Antarctica) (60º41'459"W), twenty-three from soil at 4.0ºC (62º05'092"S) and fifteen from soil at 1.6ºC (57º56'763"W), in King George Island (South Shetland Islands, Maritime Antarctica). All of filamentous fungi are being maintained by cryopreservation at -80ºC and Castellani at 4ºC at Central of Microbial Resources (CRM-UNESP).

Crude extracts production

The fungi were reactivated on agar malt broth petri dishes (2% malt and 1.5% agar) at 15ºC for 10 days. After their growth, disks of 5 mm of agar plus mycelium were transfered to 150 mL of 2% liquid malt broth in erlenmeyer flasks. This culture was shaken at 15ºC for 20 days at 150 rpm. After the period of incubation, the biomass and metabolic medium (supernatant) were separated using vacuum filtration.

Intracellular extracts

The biomass was extracted 3 times with 40 mL methanol by maceration.

Extracellular extracts

The supernatant was extracted 3 times with 60 mL ethyl acetate by liquid-liquid extraction. The extracts were concentrated in a low pressure rotary evaporator and dissolved in dimethylsulfoxide (DMSO) 10% (v/v) until the concentration of 30 mg/mL.

Cultivation of *Xanthomonas*

X. citri strain 306 (IBSBF 1594) was activated on agar NYG (3 g yeast extract, 5 g peptone and 20 mL glycerol) broth at 29ºC for 2 days. Then, bacterial cells were cultured in 30 mL of liquid NYG broth medium, shaken at 200 rpm and 29ºC until OD_{600} = 0.8.

X. albilineans (IBSBF 1387) was activated on agar YSG medium (5 g yeast extract, 5 g glucose, 0.5 g monobasic ammonium phosphate and 0.2 g anyhidrous dibasic potassium phosphate) broth at 29ºC for 5 days. Then, bacterial cells were cultured in 30 mL of liquid YSG broth medium, shaken at 200 rpm and 29ºC. The bacterial inoculum was standardized at 10^7 CFU/mL (OD_{600} = 0.9).

Anti-*Xanthomonas* activity assessment

The bioassay was accomplished in 96-well plates with triplicate of events and experiments, based in the Resazurin Microtiter Assay (REMA) method (Silva et al., 2013). For the bioassay against *X. citri* the maximum concentration tested of extracts was 2.1 mg/mL followed by microdilution until 0.016 mg/mL and for *X. albilineans* the maximum concentration tested of extracts was 3 mg/mL. Kanamycin (0.02 mg/mL) was used as positive control (PC), DMSO 1% (v/v) was used as vehicle control (VC) and negative control (NC) was made with medium (NYG) and inoculum. The inoculum was diluted (1:10) in NYG and aliquote of 10 µL was added to all wells used and standardized at 10^5 CFU/well. After a 16 h incubation at 29ºC 15 µL of resazurin 0.01% (w/v) was added and submitted to another incubation for about 1 h at the same temperature. The REMA was made in triplicate.

A Biotek Synergy H1MFD microplate reader was used to measure cell viability. It scans the fluorescence of the resorufin (resazurin reduced by microbial metabolism) at 530 nm (excitation) and 590 nm (emission). The fluorescence units (FU) detected by the reader were used to calculate growth inhibition in percentage, according to the formula:

$$\frac{[(average\ FU\ negative\ control) - (average\ FU\ test)]}{(average\ FU\ negative\ control)} \times 100$$

Inhibition rate above 90% was considered as positive.

The minimal extract concentration needed for inhibition of 90% of cells (MIC90) was calculated based on the equation obtained from the regression curve constructed with the Rema bioassay data.

The bactericidal assay was performed as follows: A small aliquot from each well of the REMA plates was inoculated in petri dishes (150 x 15 mm, NYG medium) with 96-pin microplate replicator. After incubation for 48 h at 29ºC, it was possible to visually analyze the bacterial growth. The minimal concentration in which there was no bacterial growth was considered the minimal bactericidal concentration (MBC) of the extract.

Identification of the Antarctic fungi

DNA extraction was performed directly from the mycelium after the fungal growth in PDA medium for 7 to 14 days at 15°C, following a modified version of the CTAB method (Möller et al., 1992; Gerardo et al., 2004). ITS amplification was performed using primers ITS4 (5'TCCTCCGCTTATTGATATGC3') and ITS5 (5'GGAAGTAAAAGTCGTAACAAGG3') (Schoch et al., 2012).

PCRs were performed in a final volume of 25 µL (4 µL of dNTPs [1.25 mm each]; 5 µL of 5x buffer; 1 µL of BSA [1 mg/mL]; 2 µL of MgCl2 [25 mm]; 1 µL of each primer [10 µm]; 0.2 µL of Taq polymerase [5 U/µL], 2 µL of diluted genomic DNA [1:100] and 8.8 µL of sterile ultrapure water). PCR conditions for ITS were 94°C/3 min followed by 35 cycles at 94°C/1 min, 55°C/1 min and 72°C/2 min. Amplicons were purified using the Exonuclease I and Alkaline phosphatase (Thermo Scientific, Massachusetts, USA), according to the manufacturer's protocol. Samples were then quantified in NanoDrop® (Thermo Scientific) and sequenced using BigDye Terminator® v.3.1 kit (Applied Biosystems, California, USA), following the manufacturer's instructions. Sequences obtained were compared with sequences deposited in the NCBI – GenBank database. Alignments were performed separately for each molecular marker using MAFFT v.7 (Katoh and Standley, 2013). The phylogenetic analyses were done using the program MEGA v. 7 (Tamura et al., 2013). The Kimura 2-parameter model (Kimura, 1980) was used to estimate evolutionary distance for the Neighbor-joining analysis. Branch support was calculated by bootstrap with 1,000 replicate runs.

RESULTS

A total of 122 extracts was produced. All of them were tested against *X. citri*. Eight extracellular extracts presented, in the maximum concentration, averages of fluorescence measurements (Table 1) which when applied in the formula to calculate growth inhibition in percentage showed inhibition rates above 90% (Table 1) and were considered positive. The intracellular extracts showed no antibacterial activity against the tested *Xanthomonas* species.

The MIC90 values (Table 1) for the extracts of 2-Fe, 6-Fe, B-Fe and G-Fe were around 2.0 to 1.7 mg/mL, three extracts (11-Fe, C-Fe and D-Fe) presented values between 0.80 and 0.60 mg/mL, and the extract of 3.1-Fe attained the lowest MIC90 (0.28 mg/mL).

In the bactericidal assay, four extracts (2-Fe, 6-Fe, B-Fe and G-Fe) presented bacterial growth in the highest concentration tested (2.1 mg/mL), therefore they were considered bacteriostatic. The extracts of C-Fe, D-Fe, 3.1-Fe and 11-Fe were bactericidal, highlighting 3.1-Fe and 11-Fe, which had the lowest MBC value (1.0 mg/mL).

In the experiments with *X. albilineans,* two of the 122 extracts were considered positive, the extracts of 1.1-Fe and B-Fe presented a percentage of 97% inhibition of bacterial growth in the concentration 1.5 mg/mL. The MIC90 values for both extracts (1.1-Fe and B-Fe) were about 1.4 mg/mL. In the bactericidal bioassay the both extracts were bactericidal up to the concentration of 1.5 mg/mL (MBC).

In this study, eight bioactive fungi (1.1- Fe, 2-Fe, 3.1-Fe, 6-Fe, 11-Fe, B-Fe, C-Fe and G-Fe) were identified as belonging to the genus *Pseudogymnoascus* based on molecular taxonomy and phylogentic analysis (Figure 1).

The bioactive fungus D-Fe was identified as belonging to the genus *Cladosporium* (Figure 2). The taxonomic marker used (ITS) showed to be very conservative, since different species of the genus *Cladosporium* grouped in the same cluster of the Antarctic-derived fungus D-Fe.

DISCUSSION

Positive results of extracellular extracts may be associated with defense mechanism, since some microorganisms can produce substances that inhibit potential competitors (Smid and Lacroix, 2013; García et al., 2017). The samples tested are crude extracts, a reduction in MIC90 value is expected after purification and isolation of the bioactive molecules.

Based on this work, *X. albilineans* may be considered more resistant than *X. citri*, since only two extracts were inhibitory against *X. albilineans*. This also can be noted by the lower inhibition capacity of the antibiotic used as positive control (kanamicin) for this bacterium. In plantations, this resistance can be observed, since leaf scalding has no effective chemical combat and it is more difficult to contain than citrus canker.

The extract produced by the fungus B-Fe (*Pseudogymnoascus*) showed satisfactory bioactivity against both bacteria, and it can be considered as a promising microbial genetic resource in the fight against *Xanthomonas*.

The bioactive extracts (Table 1) were also tested, in another study, against *X. euvesicatoria* (bacterial spot on pepper and tomato) and *X. axonopodis* pv. *manihotis* (cassava bacteriosis) and only the fungus D-Fe showed activity against *X. axonopodis* pv. *manihotis*.

About identification, the *Pseudogymnoascus* ITS-rDNA sequences were highly similar, suggesting that it could be representatives of the same species. Amplicons were compared to homologous sequences deposited in the NCBI-GenBank database. The isolates showed 100% similarity with different strains of *Pseudogymnoascus* sp. Data from phylogenetic analysis showed that the isolates were placed in a separate cluster in relation to the other *Pseudogymnoascus* species (bootstrap value of 71%). These results suggest that *Pseudogymnoascus* strains could represent a new species. However, as reported by Chaturvedi et al. (2010), others taxonomic markers have

Table 1. Extracellular bioactive extracts against *X. citri.*

Extract	Fluorescence unit*	Inhibition (%)*	MIC 90 (mg/mL)	R^2
2-FE	1621.2	96.02 ± 0.27	2.00	0.9997
3.1-FE	3671	91.00 ± 0.55	0.28	0.9806
6-FE	880	97.84 ± 0.17	1.99	0.9989
11-FE	1096.2	97.31 ± 0.26	0.80	0.9854
B-FE	1023.8	97.48 ± 0.25	1.71	0.9972
C-FE	898.8	97.80 ± 0.13	0.78	0.9956
D-FE	1067.6	97.38 ± 0.18	0.60	0.9850
G-FE	2172.8	96.68 ± 0.88	1.99	0.9992
Negative control	40805.2	-	-	-

*Mean values obtained at the extracts concentration of 2.1 mg/mL (maximum concentration)**.** MIC90: Extract concentration needed for inhibition of 90% of the cells, in mg/mL. R^2: shows how well regression curve fits the original curve obtained from the extracts, with a maximum value of 1.

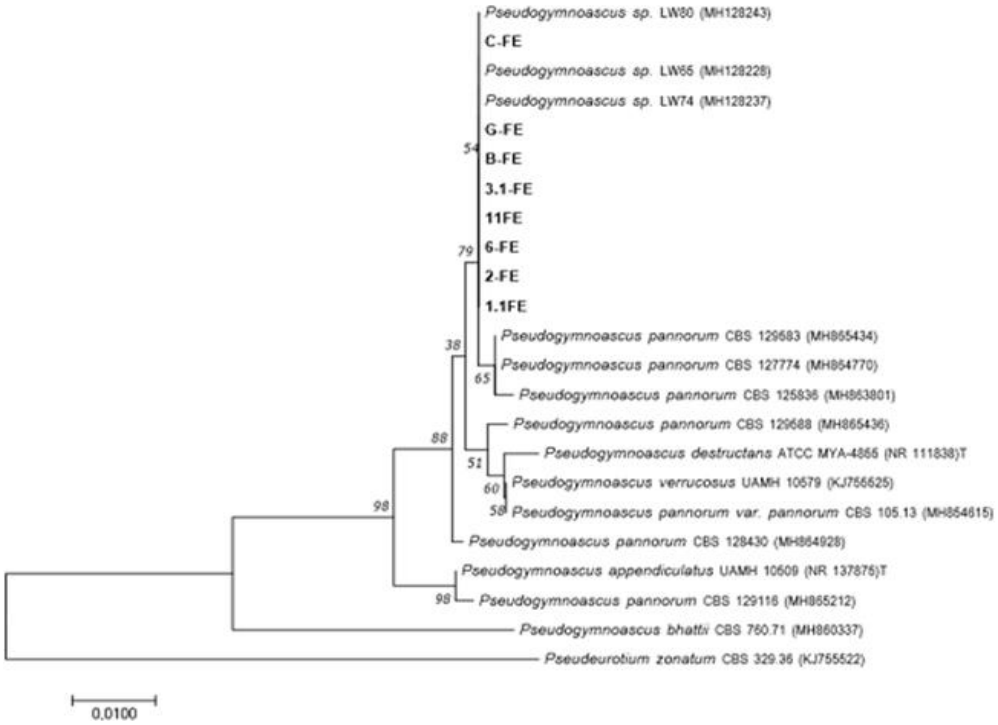

Figure 1. Phylogenetic analysis of *Pseudogymnoacus* species evaluated in this study with sequences of the closest species deposited in Genbank. The tree was built base on the ITS sequences using the Neighbor-joining method with Kimura 2-parameters nucleotide substitution model.

to be applied in order to get speciation for *Pseudogymnoascus/Geomyces*. Representatives of this genus are globally distributed and can develop in different conditions and extreme environment (Hayes, 2012), including Antarctic ecossystems (Arenz et al., 2006; Bridge and Spooner, 2012; Godinho et al., 2015). In the study reported by Wentzel et al. (2018) *Pseudogymnoascus* was the most abundant filamentous fungi found in antarctic samples of soil and marine sediments.

The isolates from *Pseudogymnoascus* have been reported as having activity against microorganisms (Furbino et al., 2014; Gonçalves et al., 2015). Figueroa et al. (2015) described four new nitroasterric acid derivatives with antibacterial and antifungal activity obtained from the cultures of a *Pseudogymnoascu*s sp.

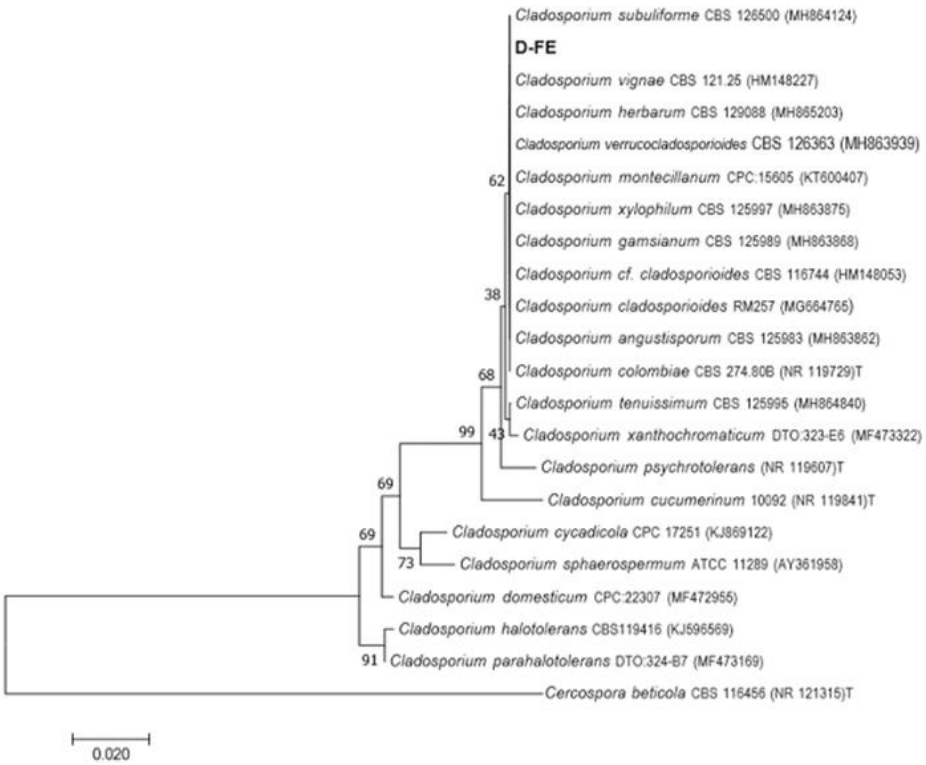

Figure 2. Phylogenetic analysis of Cladosporium sp. (D-FE) evaluated in this study with sequences of the closest species deposited in GenBank. The tree was built base on the ITS region sequences using the the Neighbor-joining method with Kimura 2-parameters nucleotide substituttion model.

fungus isolated from an Antarctic marine sponge. Other studies reported the bioactivity of this genus against *Xanthomonas* (Henríquez et al., 2014), including some from our research group, against *X. citri*, *X. euvesicatoria* and *X. axonopodis* pv*. passiflorae* (Vieira et al., 2018, Puric et al., 2018), but so far no molecules have been described.

Representatives of *Cladosporium* have also been reported in the Antarctic environments (Arenz et al., 2006; Bridge and Spooner, 2012; Wentzel et al., 2018). According to Tosi et al. (2002) *Cladosporium* is the most frequently observed genus in association with antarctic mosses. Antimicrobial molecules were reported as produced by representatives of this genus (Zhang et al., 2001; Xiong et al., 2009; Liang et al., 2018), but studies related to the bioactivity of *Cladosporium* against *Xanthomonas* are still scarce. In a previous study published by our research group, a representative of *Cladosporium* showed potencial to inhibit the cell growth of *X. citri, X. euvesicatoria* and *X. axonopodis* pv. *passiflorae* (Puric et al., 2018), corroborating the results achieved in the present study, where *Cladosporium* sp. (D-Fe) was able to produce bioactive molecules against *X. citri.*

The present study highlights the potential of bioprospecting of extrememophile microorganisms, in the search for new and more sustainable bioactive molecules, potentially able to substitute the use of copper in agriculture and in the combat of phytopathogens.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Studies on the sod culture and the management of soil moisture for the improvement of waxapple quality

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In Taiwan, Kaohsiung and Pingtung districts are the main cultivating areas of waxapple in Taiwan. The quality of waxapple is commonly involved in appropriate fertilization. Therefore, the demonstration orchards were established in Kaohsiung and Pingtung districts. This research was conducted to the effects of sod culture and management of soil moisture on the improvement of waxapple quality. The results showed that soil properties in the districts of two kinds of sod culture (*Alternanthera philoxeroidex* Mart. and *Wedelia chinensis* Merr.) were excellent than that of non-sod culture, and the harvesting fruit quality were higher for two areas of sod culture than non-sod cultivature. The treatment of moisture control by tensiometer (TM) on soil available elements, the concentration of leaf elements and fruit color were all highest, and fruits cracking percentage were lowest than other treatments in shallow soil. On the other hand, the effect of furrowed and immersed (FI) on soil available elements, the concentration of leaf elements and fruit color were all highest, and the fruit cracking percentage was lowest than other treatments in alluvial soil. The results showed that the quality of waxapple could be improved by treating with sod culture and the management of soil moisture.

Key words: Soil properties, tensiometer, furrow, immerse.

INTRODUCTION

Waxapple is one of the important fruits in Taiwan, they are cultivated mainly at the farmlands of Kaohsiung and Pingtung. High quality waxapples like" Black Pearl" and "Black Diamond" have been well known for more than a decade. However, the factors including climate (temperature, sunlight, and rainfall etc.), cultivation and management (pruning, flower and fruit thinning etc.), and appropriate fertilization significantly influence the growth and development, sugar content, color, and even abscission and cracking of the waxapple fruit (Kuo et al., 2004). In addition, sod culture influence the quality of waxapple fruit, it includes the soil properties and fruit weight, sugar degree, and so on (Lin, 2012, 2014). In the quality of waxapple, besides color and sugar degree, fruit cracking is another reference index for the quality of waxapple. Sugar content and color have been notably

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improved in the past years. However, fruit cracking is always a quality defects. High ratio of fruit cracking has a negative impact on the marketing of waxapple (Lai, 2005). Therefore, the problem needs to be solved urgently. There are a large number of achievements in fighting against fruit cracking for other fruits. For example, GA3 50 ppm + NAA 50 ppm is great for loquat in preventing fruit cracking (Yen, 1989). Calcium fertilizer improves the fruit cracking and malformed fruits of tomato as a result of calcium deficiency (Asen, 1976). For Fuyu sweet persimmon, an appropriate rates of N- and K-Fertilizer ($N:K_2O$=300:300 g/year/tree) is better than the high N- and K-Fertilizer rates ($N:K_2O$=450:300 g/year/tree), for increasing the quality of fruits and reducing the fruit cracking for 25% (Lai, 2001). The control of moisture content also has its impacts on fruit cracking. Markakis (1974) showed that it was possible to control the fruit cracking of pineapple by controlling the moisture content of the soil. During the period of fruit growing, instant rainfalls and bad management of moisture content in the soil may result in severe fruit cracking for Murcott Orange (Garcia-Luis et al., 1994). Soil should be kept from being excessively dry and with appropriate water content (Chang et al., 2005). For waxapple, most of the studies on the fruit quality are based on the utilization of nutritional agents and the cultivation management of the parts above ground in the past. For example, Lai (2005) showed that the fruit string at the outside of the crown demonstrated the highest fruit cracking ratio on a horizontal basis. Spraying 0.3% $CaCl_2$ or $CaSO_4$ on the surface of leaves had a remarkable effect against fruit cracking. Yen et al. (2004) believed that spraying Chung-Hsin 100 (plant extract) 1000X solution in the winter helped to reduce the fruit cracking ratio by 25%. Another reason that rainfalls in the mature stage of waxapple result in significant fruit cracking. This is because of the fast alternation between dryness and moisture at the roots (Lai, 2005). In this study, the effect of appropriate fertilization, sod culture and the moisture of soil are evaluated on the quality of waxapple (e.g. sugar content, size, weight, and fruit cracking ratio).

MATERIALS AND METHODS

Effect of sod culture on waxapple quality

The experimental design

The experiments of sod culture was proceeded at the waxapple orchards of Chianjhih, county, Pingtung. By evaluating, the two kinds of grass species (*Alternanthera philoxeroidex* Mart. and *Wedelia chinensis* Merr.) were excellent for the experiments of sod culture. The experiment was preceded in the vegetative growth period. Before experiment, other weeds were controlled by herbicide. After two weeks, two specific grass species were planted beside waxapple trees. By spreading of specific grasses, the rate of coverage grasses was high-density. The soil properties, the concentration of nutrients in leaves and fruit quality (e.g. fruit weight, width, length and sugar degree) were estimated and compared between the different areas of treatments.

Fourier-transform infrared spectroscopy (FTIR) analysis for the root surface of two kind of grass species

The root apice of two kind of grass species were sampled and oven-dried, and then powdered, and then 200 mg KBr after pre-dried at 110°C that was added and mixed for KBr pellet preparation by a die at 10,000~15,000 lb/in^2 pressure for 3 min. The KBr pellet was measured by Shinazu (Japan) FTIR spectrophotometer for its light transmission at wave member between 4000 and 400 cm^{-1}.

Efffects of the management of soil moisture on waxapple fruit quality

The soil properties of experimental orchards

The experimental orchards were located at Kaosu and Nantzu, Pingtung, Taiwan. The soil series in Kaosu is among Shashuipu series. It is shallow soil with very perfect drainage properties. This area can be characterized by rock layer or stone block layer beneath the surface soil. The soil in Nantzu belongs to Lantau series which is alluvial soil with limited drainage capability, based on duripan of iron and manganese accumulated by the ancient alluvial deposits of clay slates, which are alkalescent.

Experiments of soil moisture control for soil

During fruit setting stage, the experiment was treated accordingly, (1) FI: Digging a channel which is 20 cm in depth on the soil around the tree. Keep the water level in this channel as 5 cm in depth. (2) TM: The moisture of the root rhizophere at 30 cm under the ground is monitored with a tensionmeter as a reference for irrigation. If the value of tensionmeter is greater than 25 cbar, the irrigation was initiated. If the value is smaller than 15 cbar, the irrigation was stopped. (3) DR: There is no any irrigation after the fruit setting stage. (4) CK: The orchard was irrigated every 4 to 5 days to keep soil moist. Before the experiment and during the fruit setting stage, soil is sampled for different treatments. And the leaves from the branch of non-fruit branch were sampled for analysis. Sugar degree, average fruit length, average fruit width, average fruit weight, and cracking ratio at the harvested stage were investigated.

PRETREATMENT AND ANALYSIS OF SOIL AND PLANTS

Pretreatment and analysis of soil

After soils sampled, they were air dried and grinded. And then shaked the grinded soils through 2 mm sieve. The soil properties were analyzed as below, (1)The pH of soils were pretreated by water : soil = 1:1, and then measured by a pH meter (McLean, 1982). (2) Organic matter (OM) was measured by wet-oxidation method described by Nelson and Sommer (1982). (3) potassium (K), calcium (Ca), magnesium (Mg) were extracted from the soils by 0.1N HCl. And then the content of K, Ca and Mg were measured by an induced coupled plasma (ICP) spectrophotometer (Baker and Suhr, 1982). (4) phosphorus (P) was measured by molybdenum-blue method (Bray No.1) (Murphy and Riley, 1962). (5) iron (Fe), manganese (Mn), copper (Cu) and zinc (Zn) were extracted from the soils by 0.1 N HCl. And then were measured by an induced coupled plasma (ICP) spectrophotometer (Cope and Evans, 1985).

Pretreatment and analysis of leaves

At first, the dusts and chemical residues on the leaves were cleaned

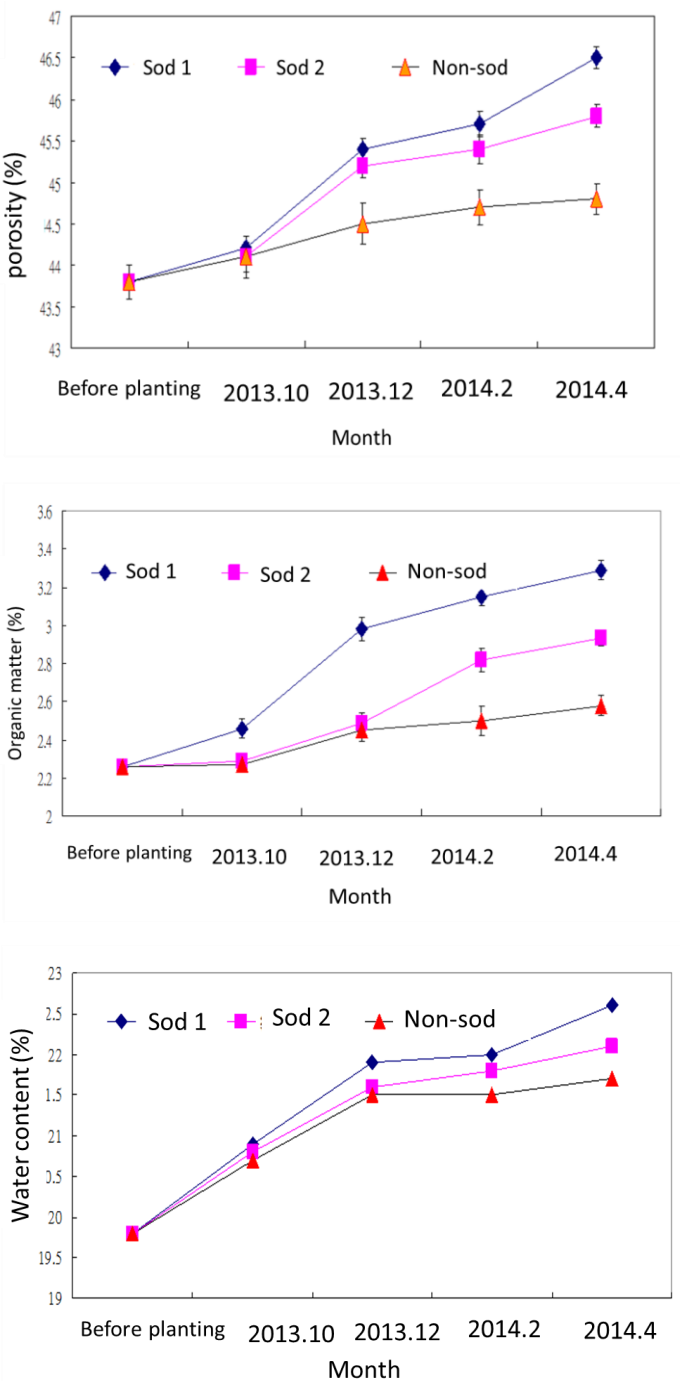

Figure 1. Effect of sod culture on soil properties in waxapple orchards (sod 1: *Wedelia chinensis* Merr. sod 2: *Alternanthera philoxeroidex* Mart.).

by distilled water. And then put the cleaned leaves were put into an oven (70-75°C). After 2 to 3 days, the dried leaves were grinded and put into decomposition tube. The leaves were then dissolved by concentrated sulphuric acid in high temperature and analyzed by the following methods. (1)N was measured by Kjeldahl method. (2) P was measured by molybdenum-yellow method (Bray No.1). (3) K, Ca, Mg, Fe and Mn were extracted from the soils by 0.1 N HCl. And then were measured by an induced coupled plasma (ICP) spectrophotometer (Cope and Evans, 1985; Chang, 1981).

Determination of fruits quality

In the mature stage of fruits, 20 fruits in similar size and are selected for determination of fruit length, width, weight and sugar content. Color of fruit peel was measured by the values of L (lightness), a (red representation), and b (yellow representation) via randomed selection for 20 fruits. 60 mature fruit samples were randomly selected from every treatment for the estimation of cracking ratio.

RESULTS AND DISCUSSION

Effect of sod culture on waxapple quality

The soil properties, nutrient concentration in leaves and fruit quality were shown in Figures 1 to 3. The results showed that soil porosity, organic matter and soil moisture were all higher at the area of two kinds of sod culture than non-sod culture. The average fruit weight was highest at the area of *A. philoxeroidex* Mart. Is highest between different treatments, and the sugar degree is highest in the area of *W. chinensis* Merr.. The fruit cracking is lowest in the area of *W. chinensis* Merr.. The concentration of potassium, calcium and maganisium is higher in waxapple leaves of sod culture is higher than that of non-sod culture. The results showed that the soil properties and fruit quality were improved by sod culturing of two specific grass species. Zou et al. (2016) showed that sod culture with white clover significantly increased the concentrations of easily extractable glomalin-related soil proteins (EE-GRSP), total GRSP (T-GRSP), and soil organic carbon (SOC), the distribution of water-stable aggregates in the size of 2-4, 1-2,and 0.5-1 mm, and the activity of soil peroxidase and phosphatase. They concluded that sod culture potentially improved soil properties in orchards (Zou et al., 2016). Wu et al. (2015) showed that sod culture can increase the activities of soil enzyme.

Based on FTIR spectroscopic analysis, the kind and intensity of functional groups from the roots of five specific grass species are shown in Figure 4. The main absorption zones of the spectrum include those located around 3300-3400 cm^{-1} (H bonds, OH group), 2850-2930 cm^{-1} (C-H asymmetric, C-H stretch of CH), 1690-1735 cm^{-1} (stretching mainly carboxyl-C or traces of ketones and esters C=O), 1595-1660 cm^{-1} (C=C in aromatic ring), 1510-1515 cm^{-1} (amide II bonds), 1420-1440 cm^{-1} (C-H deformation of CH_2 or CH_3 groups), 1372 cm^{-1} (COOH,CH_3), 1243 cm^{-1} (aromatic C, C-O stretch), 1157 cm^{-1} (aliphatic CH_2, OH or C-O stretch of various groups) and 1030-1145 cm^{-1} (C-O stretch of polysaccharite, Si-O stretch).

Inbar et al. (1989) compared some major spectroscopic intensities such as 1720 cm^{-1} (COOH with C=O bond), 1410 cm^{-1} (CH_2 and COO^-) and 1230 cm^{-1} (aromatic C

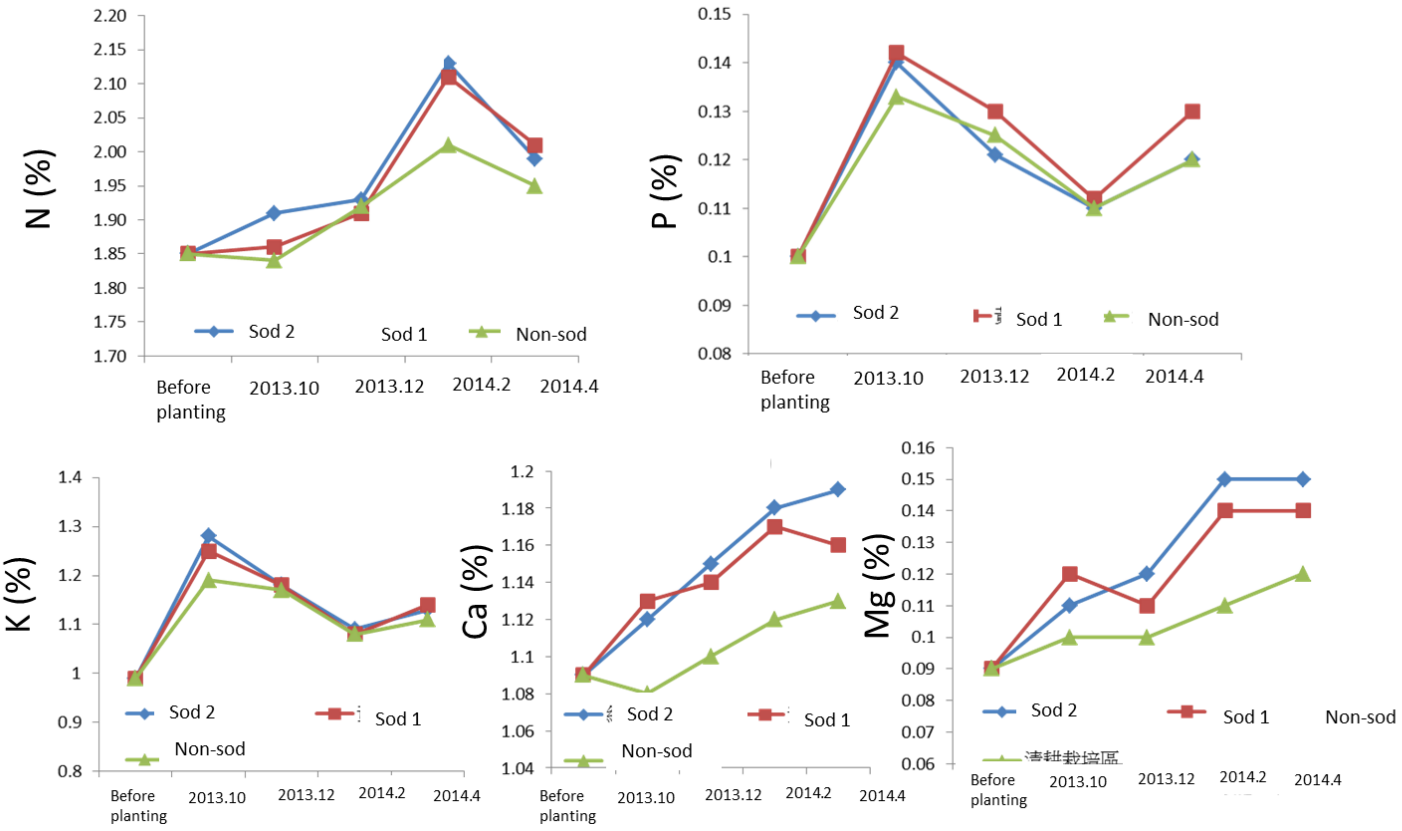

Figure 2. Effect of sod culture on nutrient elements (%) in leaves (sod 1: *Wedelia chinensis* Merr., sod 2:*Alternanthera philoxeroidex* Mart.).

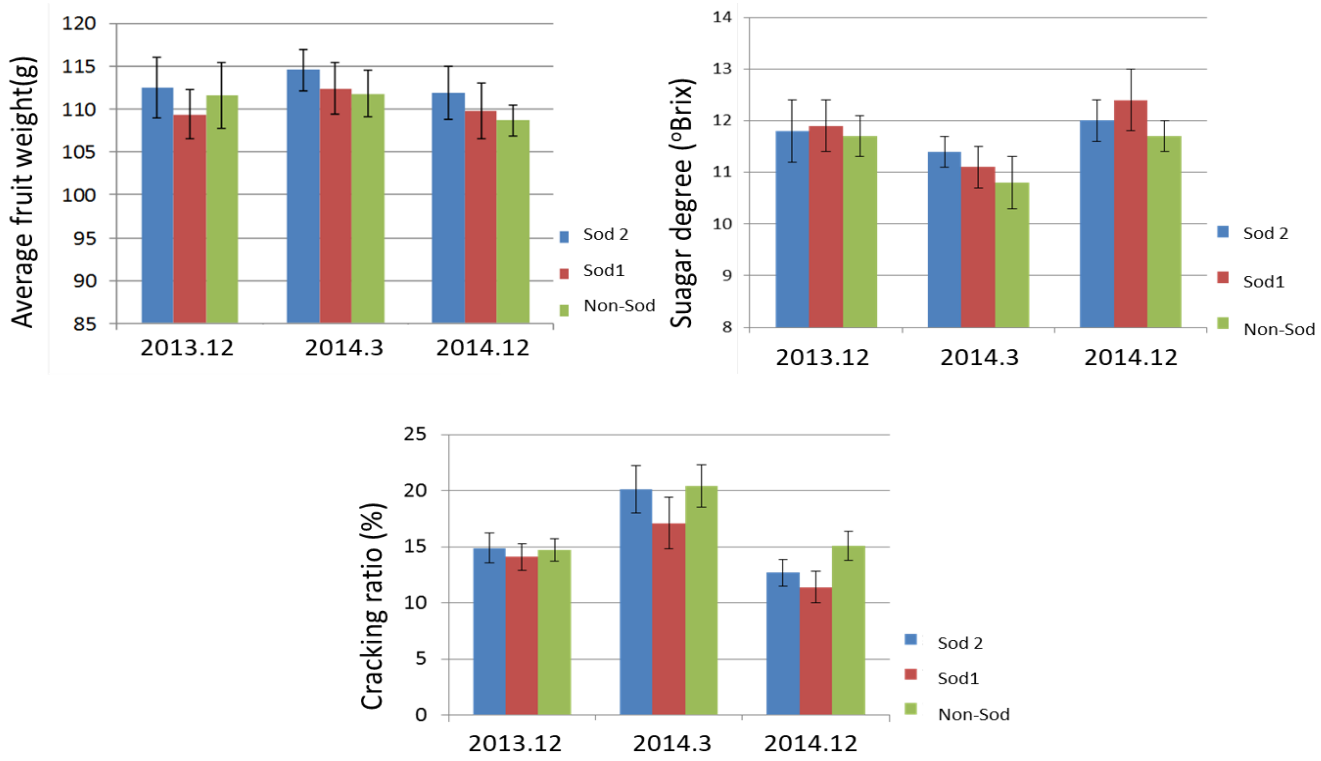

Figure 3. Effect of two different sod culture on fruit quality (sod 1:*Wedelia chinensis* Merr.,sod 2:*Alternanthera philoxeroidex* Mart.).

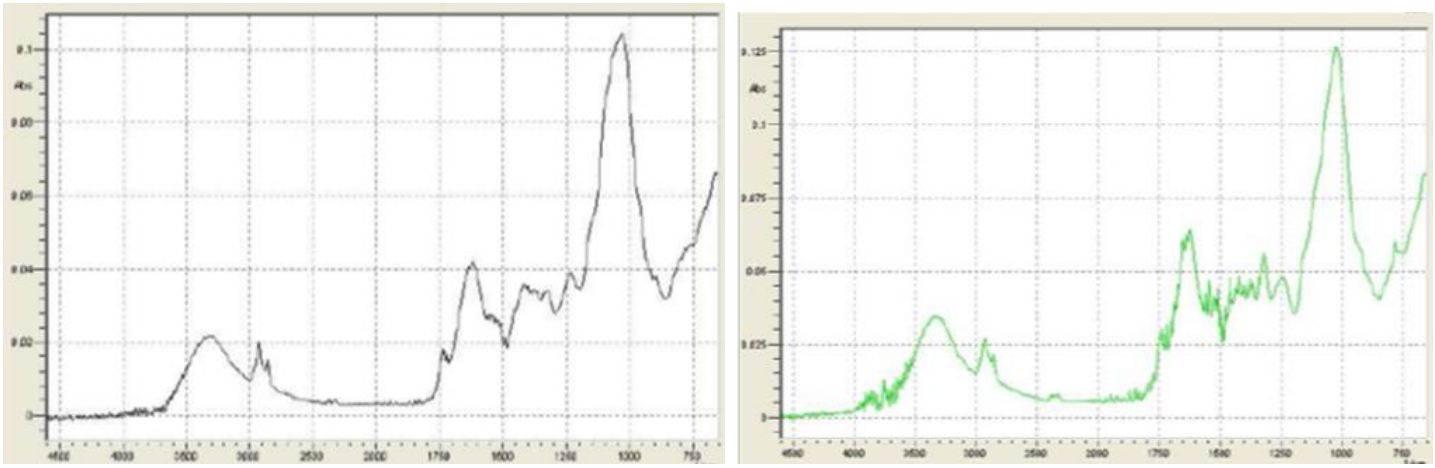

Figure 4. FTIR graphs of two kind of grasses on root surface (sod 1:*Wedelia chinensis* Merr., sod 2: *Alternanthera philoxeroidex* Mart.).

Table 1. The influence of different soil moisture managements on waxapple quality in Kaosu, Pingtung.

Treatments	Sugar degee (^{o}Brix)	Fruit length (cm)	Fruit width (cm)	Fruit weight (g/granule)	Cracking percentage (%)	Color		
						L	a	b
FI	8.8^{a}	62.3^{a}	72.1^{a}	134.1^{a}	38.3^{b}	29.9^{a}	12.6^{ab}	5.2^{a}
TM	9.0^{a}	63.2^{a}	67.5^{a}	115.4^{a}	35.0^{b}	29.9^{a}	15.5^{a}	6.3^{a}
DR	8.1^{b}	65.4^{a}	68.8^{a}	117.6^{a}	46.7^{a}	30.4^{a}	10.9^{b}	5.2^{a}
FU	9.2^{a}	62.0^{a}	68.2^{a}	123.9^{a}	43.3^{a}	31.9^{a}	13.0^{ab}	5.4^{a}

FI: Furrowing and Immersing, TM: Tensiometer monitoring, DR: Drought, FU: Farmer usage.

and C-O bond), and suggested that these spectroscopic intensities might represent the functional groups such as carboxylic group (-COOH) and hydroxyl group (-OH). In general, these carboxylic and hydroxlic groups may bond with metalic ions (Chang-Chien and Wang, 2003). In this study, the root of specific grass species were detected to have spectroscopic intensities at 1731 cm^{-1} (represent COOH with C=O bond), 1420 cm^{-1} (represent CH_2 and COO^{-}) and 1243 cm^{-1} (represent aromatic C and C-O bond), indicating existence of functional groups such as carboxyl and hydroxyl in the cell wall of their root apices. As dissociation of H^{+} from carboxyl and hydroxyl may increase negatively charged ions, the chance for bonding with nutrient ions is increased.

The influence of moisture management on fruit quality of waxapple in Kaosu

When *pomegranate* fruit was 14 day irrigative interval, it will resulted in significantly more cracking in pomegranate than 7 day interval (standard irrigation) (Ghanbarpour et al., 2019). These experiments were preceded during late-spring, the fruit cracking ratio of waxapple was higher season. In addition to fruit cracking, we investigated the other fruit quality, too. Table 1 showed that the treatment of TM and CK that had the highest sugar degree in the fruit at the harvested stage. That of DR was the lowest. For fruit length, DR showed the highest value than other treatments, and FI had the higher value in fruit width, however, the lowest value was found TM. But the narrowest value is from group TM. For average fruit weight, group FI has the heaviest value, but group TM is the lightest. For cracking ratio, the highest value was found in DR and the lowest value was found in TM. Based on the datas, the treatment drought result in insufficient water supply and therefore caused a negative impact on the photosynthesis in the leaves where carbohydrates were produced. As a result, the sugar degree in the treatment of drought was lowest than that of others. In the treatment of TM, the moisture in the soil was always sufficient. Therefore, the sugar higher was highest than other treatments. Although the fruits weight and length in DR were higher than other treatments, however, the fruit density may be lower. For cracking ratio, DR has a high value. Highest fruit cracking is probably dramatic variation of soil moisture by the suddenly rainfall before the fruits being harvested. Among all treatments, TM showed the

Table 2. The influence of different soil moisture managements on waxapple quality in Nantzu, Pingtung.

Treatments	Sugar degree (^{o}Brix)	Fruit length (cm)	Fruit width (cm)	Fruit weight (g/granule)	Cracking percentage (%)	Color		
						L	a	b
FI	10.5^{a}	66.8^{a}	74.5^{a}	136.2^{a}	10.3^{c}	30.2^{a}	14.3^{a}	4.8^{a}
TM	8.4^{b}	65.4^{a}	71.0^{a}	130.7^{a}	25.2^{b}	33.3^{a}	10.5^{b}	5.2^{a}
DR	8.0^{b}	64.5^{a}	68.3^{a}	127.3^{a}	33.3^{a}	31.4^{a}	11.3^{b}	4.2^{a}
FU	9.4^{a}	66.6^{a}	73.1^{a}	131.9^{a}	30.0^{a}	32.3^{a}	12.5^{ab}	5.1^{a}

FI: Furrowing and Immersing, TM: Tensiometer monitoring, DR: Drought, FU: Farmer usage.

lowest cracking ratio. It indicates that TM method may be the best method for controlling cracking ratio of waxapple in Kaosu. The control of soil moisture in the kind of soil texture may have increase the absorption of nutrients by waxapple. Advancely, the fruit quality was improved. Although the expression in the treatment of TM was not good performance in fruit length, width, and weight, however, the improving of sugar degree and the reduction of cracking ratio were significant. For the L value of fruit peel, CK had the highest value, however, that of FI and TM were low. For a value, TM is the highest and DR is the lowest. Similar condition can be found for b value. It indicates that although TM may not be the best method for fruit lightness, it was the perfect method for the improvement of red and yellow expression on fruit peel. Rain cracking of soft, fleshy fruits is thought to result from excessive water uptake through the wetted fruit surface and also through the vascular systems of the fruit pedicel (Winkler et al., 2016). In this experiment, the soil texture of Kaosu is sandy. When the irrigated interval was long period, the soil moisture will be dried easily. Hence, when the soil moisture was monitored by the tensiometer, and maintaining the soil moisture in the soil frequently, the fruit quality will be increased and the fruit cracking will be decreased.

The influence of moisture management on fruit quality of waxapple in Nantzu

Table 2 showed the comparison of fruit quality of waxapple fruit quality by different treatments of soil moisture. In the treatments of soil moisture in Nantzu, FI showed that the highest in sugar content, however DR was the lowest. Either for fruit size and weight. On the other hand, FI showed the lowest (10.3%) and group DR shows the highest (33.3%). Fruit cracking as shown in Figure 1, high fruit cracking may be the dramatic variation of soil moisture by the suddenly rainfall before fruits being harvested. The fruit cracking happened very serious in DR. The L value of fruit peel was highest in TM and lowest in FI. The “a” value was highest in FI and lowest in TM. The “b” value was highest in TM and lowest in DR. The results showed that although the perfect method for the lightness and “b” value on fruit peel, however, FI had highest performance for “a” value which was beneficial for red color on fruit peel. Yang et al., (2017) showed the fruit anthocyanin content was significantly positively correlated with irrigative frequency and pH of the irrigation water, so for maintaining higher anthocyanin content, fruit trees should be cultivated with soil moisture content between 60 and 90%, and the irrigation water should be neutral or alkaline. Rehman et al. (2019) showed that when cherry adapting certain management practices, of which irrigation management and mineral supplements, the fruit cracking will be decreased. In this experiment, we showed that when waxapple was planted on the clayey soil, the treatment of furrowing and immersing (FI) was excellent for increasing waxapple quality and decreasing fruit cracking.

Conclusion

In these researches, the management of sod culture on the orchards can increase the soil properties and the quality of waxapple that was found. On the other hand, the proper management of soil moisture in different soil texture during fruit-setting stage is important for waxapple, for example, the increase of sugar degree of fruit and color of fruit peel, the decrease of fruit cracking.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Morphological and molecular characterization of *Jatropha curcas* L. germplasm in Botswana

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***Jatropha curcas* L. is an undomesticated plant species that has recently received great attention for its utilization in biofuel production in many countries including Botswana. The experiment investigated 30 *J. curcas* accessions, and the results revealed high significant differences (P<0.01) among accessions for peduncle length, number of seed per fruit, length of fruit stalk, fruit length, fruit width, seed length, seed width, seed weight, leaf length, leaf width, petiole length, total flower per cluster, female and male flower per cluster, and number of fruit per cluster. The qualitative characters revealed substantial amount of variability among the accessions except in fruit shape, stem color and flower color. Multivariate cluster analysis based on morphological characters and molecular characterization grouped the accessions into four clusters at dissimilarity coefficient of 1.37 and 0.85, respectively. The clustering based on morphological characters slightly matched that of groupings derived through molecular analysis with group A and B having some similar accessions. One Ghana accession used as reference was clearly separated from other accessions by both morphological and molecular markers, and also by seed weight and inflorescence compactness. Eight morphological characters and six molecular markers correlated positively with correlation coefficient values ranging from 0.56 to 0.66.**

Key words: Characterization, genetic diversity, *Jatropha curcas*, molecular markers, morphological markers.

INTRODUCTION

Jatropha (*Jatropha curcas* L.) originated from Central America now found in both tropical and subtropical countries all over the world (Singh et al., 2010, Henning, 2009). More than 85% of *J. curcas* plantations are in Asia, chiefly Myanmar, India, China and Indonesia (Brittaine and Lutaladio, 2010). Africa accounts for only 12% or approximately 120,000 ha, mostly in Madagascar, Zambia, Tanzania and Mozambique (Renner et al., 2008). Latin America has approximately 20,000 ha of *J. curcas* plantation, mostly in Brazil (Brittaine and Lutaladio, 2010). Jatropha has been grown in the past mainly for producing oil for biofuels preferably in marginal environments in sub-Saharan Africa (Martin et al., 2019). The main problem of elevating *J. curcas* from a wild

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species to a profitable biodiesel crop is the low genetic and phenotypic variation of the accessions found in different regions of the world, hampering efficient plant breeding for productive traits (Montes et al., 2014).

Jatropha is an important and multipurpose plant with many attributes of considerable potential in bioenergy systems (Openshaw, 2000). The seed contain viscous, non-edible oil, which besides being a source of bio-diesel can also be used for manufacturing other useful products such as candle, high quality soap, cosmetics and can be used for healing several skin disorders (Openshaw, 2000). The seed cake can be used as organic fertilizer and feedstock for biogas production (Gavilanes et al., 2017; Staubmann et al*.,* 1997).

J. curcas is known for its ability to survive in very poor dry soils in conditions considered marginal for agriculture, and can even root into rock crevices, but survival ability does not mean that high productivity can be obtained from growing the plant under marginal agricultural environments (Brittaine and Lutaladio, 2010). The studies conducted previously had indicated that there was morphological variation in *J. curcas* germplasm growing in different parts of the world. Evaluation of genetic diversity through morphological traits is direct, in-expensive and easy. However, morphological estimations are more dependent on the environment and are more subjective than other measurements (Ye et al*.,* 2009). Morphological variability depends on a limited number of genes, and may not access much of the potential variability for the agronomic traits present in a crop (Mayes et al*.,* 2009). Gohil and Pandya (2008) studied fourteen characters in Indian *J. curcas* accessions finding moderate genetic diversity and none of the morphological variables had heritability of over 75%. In another study of Indian accessions, Saikia et al*.* (2015) compared 34 sources, finding moderate variation in plant height, stem girth, branches per plant and seed weight.

The molecular markers are now important tools in plant genetic diversity analysis due to their sensitivity and specificity. The use of DNA-based markers has precedence in genetic characterization of *J. curcas*. In India and China, a wide variety of molecular marker systems have been used to assess intra-specific genetic diversity within species of *J. curcas* involving accessions from different agro-climatic zones (Singh et al*.,* 2010). In this study, morphological and molecular markers were used to characterize *J. curcas* genotypes growing in Botswana that may serve as parental lines for breeding programs.

MATERIALS AND METHODS

Experimental site and plant materials

The molecular work was done at Botswana University of Agriculture and Natural Resources (BUAN) Tissue Culture Laboratory, and the morphological work was field based at Sebele Agricultural Research Station Nursery in 2015 and 2016. The nursery was established in 2011 with 86 *J. curcas* accessions that were collected by National Plant Genetic Resources Center (NPGRC) from Southern and Northern parts of Botswana and 1 accession from Ghana. Thirty *J. curcas* accessions from the nursery were selected for the study.

Morphological data collection and analysis

Three trees per accession were randomly tagged from the 30 selected accessions, and morphological data was collected only from the tagged trees. The accessions were selected based on their phenotype and location. A total of 24 of both qualitative and quantitative traits were recorded following the National Bureau of Plant Genetic Resources (NBPGR) minimal descriptor list for *J. curcas* as developed by Sunil et al. (2010). The data collected was subjected to analysis of variance (ANOVA) using the SAS version 9.3 General Linear Model (GLM) package to determine the statistical differences on the traits for the given accessions.

Cluster analysis and principal coordinate analysis

The data for each morphological character was first transformed using the STAND procedure in Numerical Taxonomy System-pc (NTSYSpc version 2.1; Rohlf, 2002) in order to eliminate the effects of different scales of measurement. Then the unweighted pair group method with arithmetic averages (UPGMA) cluster analysis and principal coordinates analysis were performed with NTSYSpc version 2.1 (Rohlf, 2002). For cluster analysis, average linkage based on Euclidean distance was used to produce a dendrogram to show the similarities and differences between *J. curcas* accessions. For the principal coordinate analysis, the data matrix was standardized by row, a matrix of distance was computed and distance matrix was double–centered, the double centered matrix was then factored and a plot was made showing the accessions in a 3-dimensional space.

Molecular data collection

The young leaves were collected from the tagged trees in the field and placed in liquid nitrogen, transported to the laboratory and stored in a freezer. The DNA was extracted following the CTAB protocol. A total of twenty-two (22) SSR primers pairs developed by Wang et al*.* (2011) were tested with 30 *J. curcas* DNA sample for reproducible amplification. The PCR conditions for the microsatellite analysis were the touchdown PCR program: Initial denaturation 94°C for 2 min, Denaturation at 92°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 30 s, and the procedure was repeated for 16 cycles; followed by 18 cycles of denaturalization at 92°C for 30 s, annealing at 45°C for 30 s and extension at 72°C for 5 min; then one final extension step at 72°C for 5 min. The PCR products were separated on 3% agarose gel and stained with 2.5 µL of ethidium bromide. The gel documentation and visualization was done using the Bio-Rad Gel Doc systems XR+ with image Lab Software.

Molecular data analysis

The amplified bands for simple sequence repeats (SSR) markers were scored as present (1) or absent (0) for each accession by manual inspection. The data were tabulated in a matrix using Microsoft Excel v2010. Data analysis was performed using Numerical Taxonomic and Multi-variant Analysis System (NTSYSpc version 2.1). The genetic similarity coefficient of pair-wise

Table 1. Standard deviation, range, coefficient of variation (CV), F-Value and P-Value of 30 *J. curcas* accessions assessed based on 16 quantitative traits.

Character	Mean	Std. dev	Range	CV%	F-Value	Pr>F
Peduncle length (PDL)	29.88	13.78	5-65	39.55	4.10	<0.0001
Number of seed/fruit (NSPF)	2.60	0.26	1-3.00	13.00	2.26	0.0036
Length of fruit stalk (LFS)	17.95	6.04	5-30	35.40	4.48	<0.0001
Fruit length (FL)	20.16	3.52	15-35	17.80	9.46	<0.0001
Fruit width (FW)	18.34	2.73	13-25	15.73	6.64	<0.0001
Seed length (SL)	14.49	1.73	10-20	11.91	13.92	<0.0001
Seed width (SW)	9.91	1.78	8-19	17.13	1.90	0.0003
Seed weight (SWHT)	1.41	0.36	0.61-2.05	25.53	1017.7	<0.0001
Leaf width (LW)	85.14	11.35	65-130	14.46	9.17	<0.0001
Leaf length (LL)	79.24	13.09	71-140	15.48	12.54	<0.001
Number of leaf lobes (NLL)	1.22	0.18	1-2	9.13	1.01	0.4705
Petiole length (PL)	90.17	14.74	65-140	16.55	7.15	<0.0001
Total flowers/cluster (TFC)	51.08	20.53	30-109	38.75	4.46	<0.0001
Number of female flowers / cluster (NFFpc)	2.95	1.30	1-7	42.90	2.35	0.0024
Number of male flowers /cluster (NMFpc)	48.18	20.21	26-103	40.52	4.19	<0.0001
Number of fruits per cluster (NFpc)	2.62	0.40	1-2	33.00	3.80	0.0001

Std. dev = Standard deviation.

comparisons among the *J. curcas* accessions analyzed were calculated based on Dice coefficient within the similarity for Qualitative data module of NTSYSpc version 2.1 (Rohlf, 2002). Clustering was performed using the generated coefficient according to the unweighted pair group mean algorithm (UPGMA) within the Sequential Agglomerative Hierarchical Nested analysis module. The dendrogram was drawn in the tree format using the tree plot module of NTSYSpc version 2.1 (Rohlf, 2002). The cophenetic correlation was estimated to measure goodness of fit among matrices generated using the matrix comparison module. The principal coordinated analysis was determined using the same procedure as for morphological markers above.

RESULTS

Characterization of *Jatropha curcas* accessions using morphological characters

There were significant differences (P<0.01) among the *J. curcas* genotypes in the characters that were analyzed, except for number of leaf lobes (Table 1). This indicated substantial amount of genetic variability among the traits analyzed for this set of accessions. The coefficient of variation (CV) revealed higher variations in the number of female flowers per cluster (42.90%), number of male flowers per cluster (40.52%), peduncle length (39.55), total flowers per cluster (38.75%), length of fruit stalk (35.40%) and number of fruit per cluster (33.00%). Lower coefficients of variation were shown in number of leaf lobes (9.13%), seed length (11.91%) and number of seed per fruit (13.00%) which indicates the small variation for these traits.

The *J. curcas* accessions differed in some qualitative characters (Table 2). All the accessions had a grey stem, cream yellow flower and an oval fruit shape. The pigmentation of emerging leaves was distributed as 71% dark greyed purple, 22.6% greyed purple and 6.4% red among the accessions. About 90.3% displayed brown pigmentation at the base of the petiole while only 9.7% displayed green petiole base. Inflorescence compactness was distributed as 77.4% compacted and 22.6% loose compact while the flowering occurrence was distributed as 51.6% flowered twice, 38.7% flowered once and only 9.7% had continuous flowering. The proportion of female flowers to male flower was distributed as 38.7% (2.1:11-20), 35.5% (3.1:>20) and 25.8% (1<1:10).

Morphological cluster analysis showed four clusters (A, B, C and D) at dissimilarity coefficient of approximately 1.37 (Figure 1). The first cluster (A) comprised of one accession which comes from Ghana (GJC1). The accession was separated from the rest of the accessions by seed weight and inflorescence compactness (Table 3).

Cluster B was the biggest with 23 accessions as follows: BJC5, BJC7, BJC13, BJC18, BJC14, BJC20, BJC51, BJC43, BJC65, BJC67, BJC57, BJC26, BJC33, BJC34, BJC17, BJC22, BJC10, BJC24, BJC25, BJC62, BJC19, BJC30 and BJC45. The major characters that led to the grouping were fruit stalk length, pigmentation on the emerging leaves, fruit length, fruit width, female to male flower ratio, pigmentation of peduncle base and number of seed per fruit. Within these cluster accession BJC14 and BJC20 were related with the distance coefficient of about 0.51.

Cluster C was the second largest with five accessions (BJC66, BJC68, BJC71, BJC75 and BJC79). The major

Table 2. Description of qualitative characters adopted and frequency of occurrence of the categories in the 30 accessions of *J. curcas.*

Description	Categories	Relative frequency (%)
Stem color	Grey	100
Flower color	Cream yellow	100
Fruit shape	Oval	100
Pigmentation of emerging leaves	Greyed purple (code 185A,B)	22.6
	Dark greyed purple (code 187A)	71.0
	Red (code 44)	6.4
Petiole base pigmentation	Green	9.7
	Brown	90.3
Inflorescence compactness	Semi-loose	22.6
	Compact	77.4
Female : Male flower ratio	1<1:10	25.8
	2.1:11-20	38.7
	3.1:>20	35.5
Flowering occurrence	One flush	38.7
	two flushes	51.6
	continuous flushes	9.7

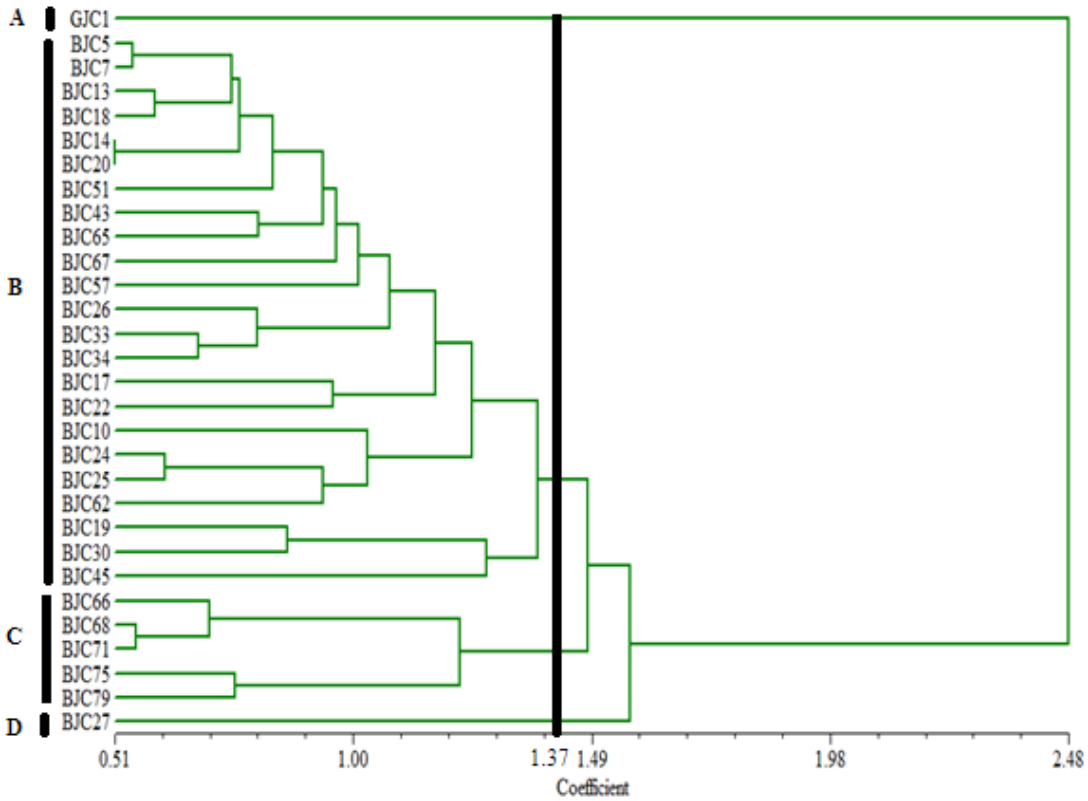

Figure 1. Hierarchical clustering from morphological data using the distance coefficient.

characters that led to the grouping were female to male flower ratio, number of seeds per fruit, number of male flower per cluster and total flower per cluster. Cluster D comprises of one accession (BJC27) from the southern region and it was separated from the rest by leaf length, leaf width and petiole length.

Table 3. Distribution of 30 *Jatropha curcas* accessions in four clusters based on their 20 morphological characters.

Cluster	Number of accession	Cluster identification	Cluster member
A	1	Seed weight and Inflorescence compactness	GJC1
B	23	Fruit stalk length, pigmentation on emerging leaves, fruit length, fruit width, female to male flower ratio, pigmentation of peduncle base and number of seed per fruit	BJC5, BJC7, BJC13, BJC18, BJC14, BJC20, BJC51, BJC43, BJC65, BJC67, BJC57, BJC26, BJC33, BJC34, BJC17, BJC22, BJC10, BJC24, BJC25, BJC62, BJC19, BJC30 and BJC45
C	5	Female : male flower ratio, number of seeds per fruit, number male flower per cluster and total flower per cluster	BJC66, BJC68, BJC71, BJC75, and BJC79
D	1	Leaf length, leaf width and petiole length	BJC27

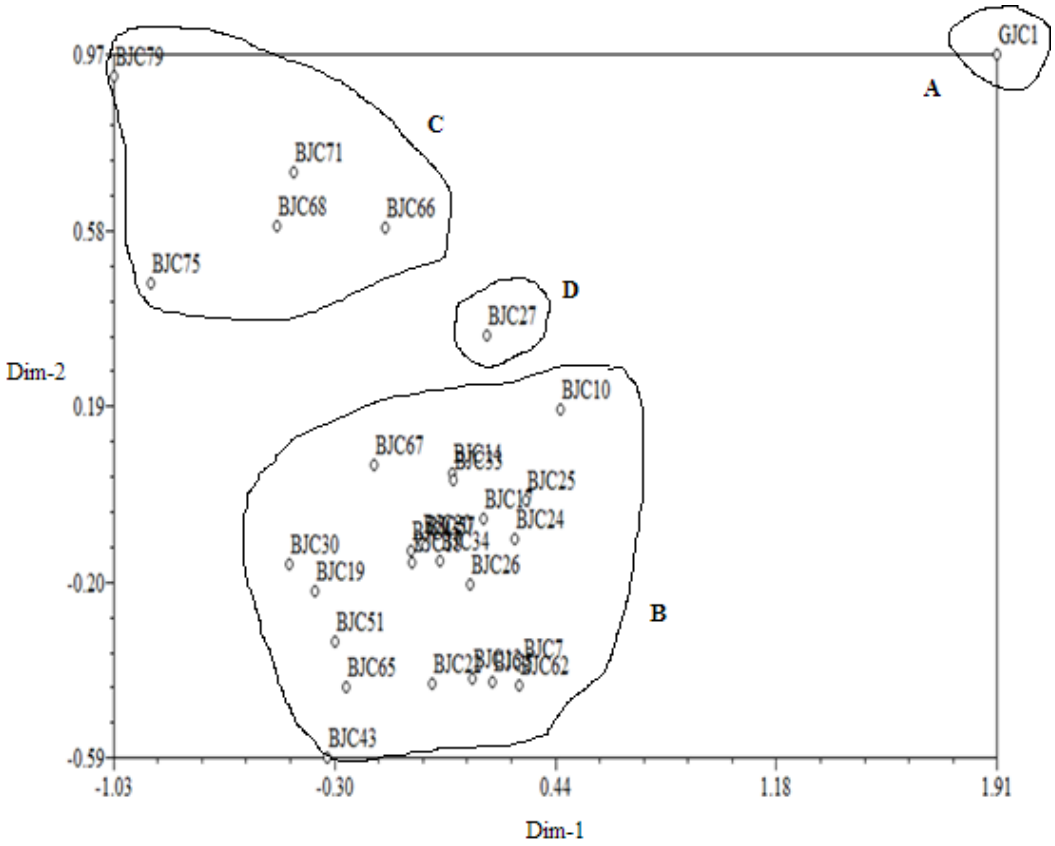

Figure 2. Principal Coordinates Analysis (PCoA) plot based on 20 morphological markers revealing four major clusters.

The Principal Coordinates Analysis (PCoA) revealed exactly the same four groupings obtained by cluster Analysis (Figure 2). The clusters were also designated by letter A, B, C and D. Cluster B being the biggest with 23 members followed by C with five accessions and lastly A and D comprising of only one accession each. The PCoA also clearly separated GJC1 (Ghana accession) from the rest of the accessions.

Characterization of *Jatropha curcas* accessions using Simple Sequence Repeat (SSR) markers

The genetic relationships among the 30 *J. curcas* accessions are shown in Figure 3. The amplitude in the similarity coefficient ranged from 0.71 to 1.00. The dendrogram obtained revealed four distinct clusters at 0.85 similarity coefficient, and grouping is from right

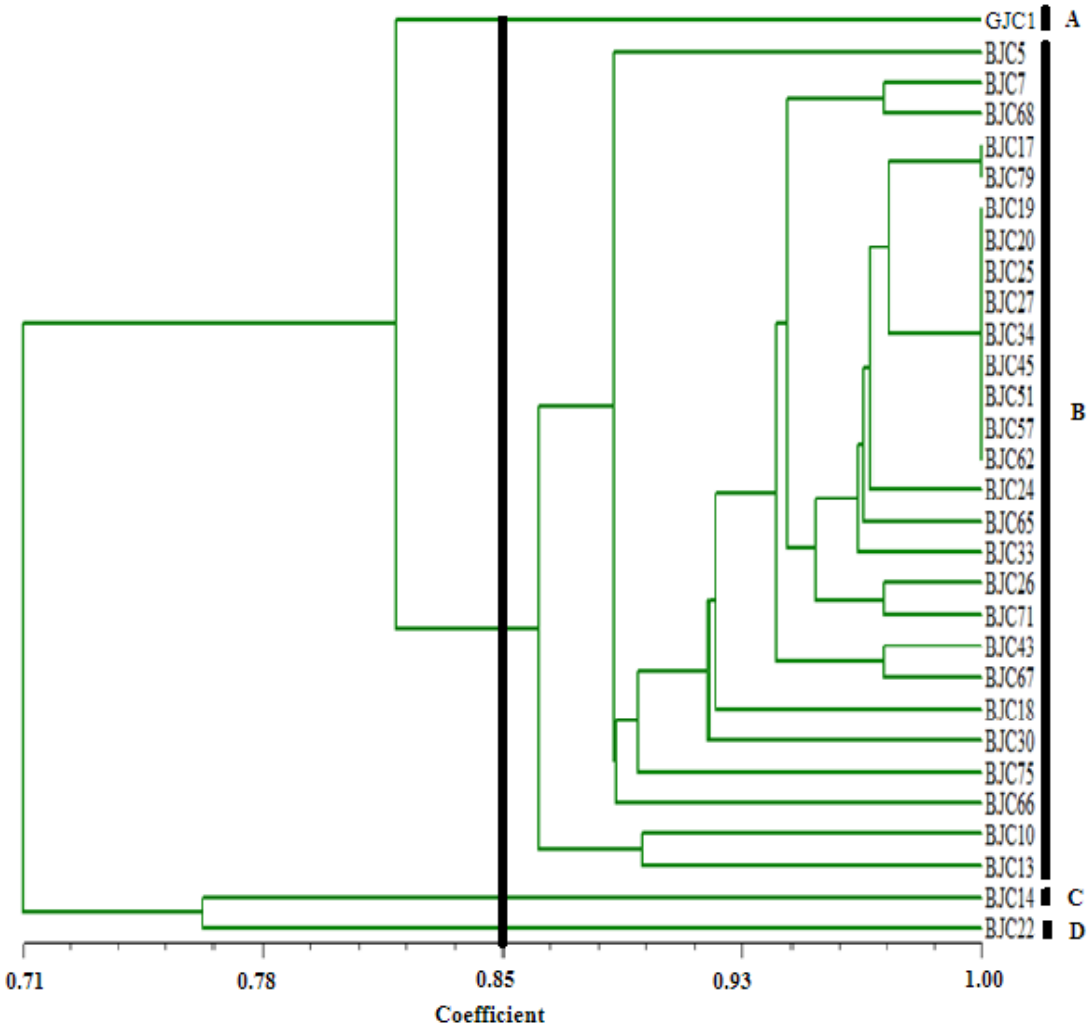

Figure 3. Hierarchical clustering from molecular markers using the genetic similarity coefficient.

to the left. The first cluster (A) comprised of one accession (GJC1 - Ghana). The second cluster (B) comprised of twenty-seven accessions (BJC5, BJC7,BJC68, BJC17, BJC79, BJC19, BJC20, BJC25, BJC27, BJC34, BJC45, BJC51, BJC57, BJC62, BJC24, BJC65, BJC33, BJC26, BJC71, BJC43, BJC67, BJC18, BJC30, BJC75, BJC66, BJC10 and BJC13) which are all Botswana accessions but collected from different villages in the north and south. Eleven accessions which are BJC17, BJC79, BJC19, BJC20, BJC25, BJC27, BJC34, BJC45, BJC51, BJC57 and BJC62 had the highest similarity coefficient of 1.00, which the used markers failed to distinguish them from one another and treated them as duplicates.

The third cluster (C) comprised of one accession (BJC14) which came from the northern region and the forth cluster (D) comprised also of one accession (BJC22) which came from the Southern region.

The Principal Coordinates Analysis (PCoA) revealed exactly the same four groupings obtained by cluster analysis (Figure 4). The clusters were also designated by letter A, B, C and D. Cluster B is the biggest with 27 members followed while cluster A, C and D are the smallest with one accession each.

Correlations between morphological and molecular markers

The correlation analysis showed correlation and no correlations between morphological and molecular markers. Eight morphological characters and six molecular markers showed moderate correlation (Table 4). The markers that showed positive correlation were peduncle base color with molecular marker Jcuint018 (0.63), Jatr691 (0.56) and Jatr209 (0.56). The seed size (length and width) correlated positively with molecular marker Jatr684 (0.66) and (0.58). The number of leaf

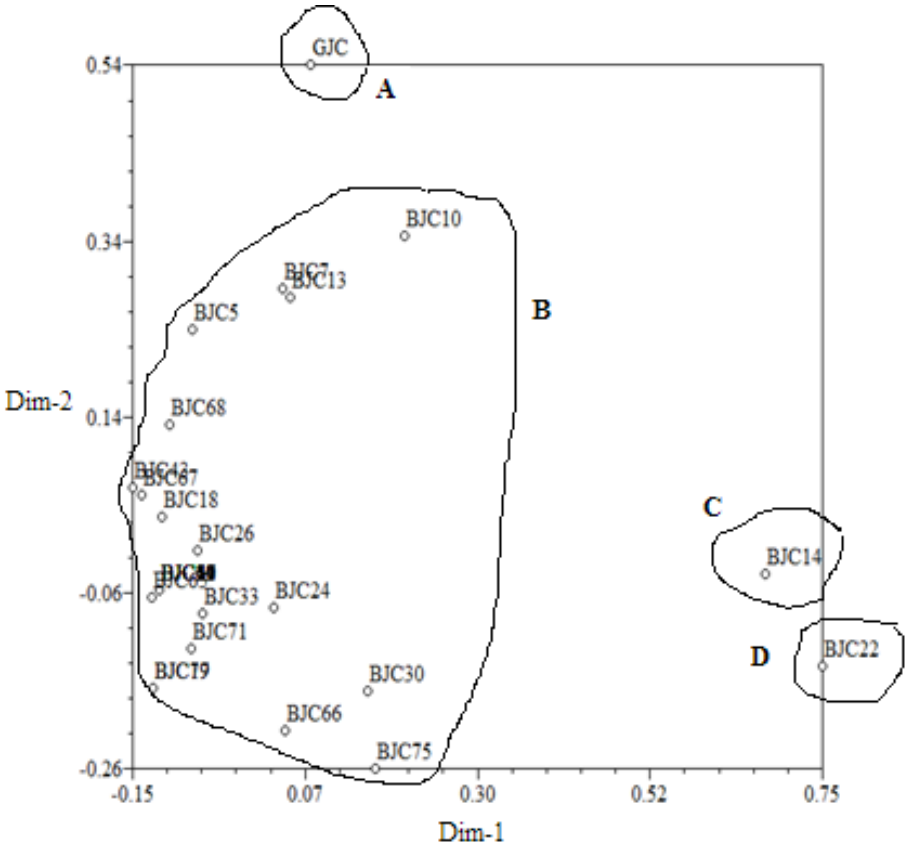

Figure 4. Principal Coordination Analysis (PCoA) plot based on SSR markers.

Table 4. Correlations among relationship estimates based on SSRs and morphological traits with *J. curcas* accessions using matel test.

SSR markers	Morphological markers							
	PBC	SL	SW	NLL	LW	LL	PL	NMFpc
Jcuint018	0.63							
Jatr691	0.56							
Jatr209	0.56							
Jatr684		0.66	0.58					-0.53
Jcuint002				0.56	-0.68	-0.54	-0.55	
Jatr739						-0.51		

PBC, Peduncle base color; SL, Seed length; SW, Seed width; NLL, Number of leaf lobes; LW, Leaf width; LL, Leaf length; PL, Petiole length; NMFpc, Number of male flowers /cluster.

lobes as well correlated positively with molecular marker Jcuint002 (0.56).

The negative correlations were observed between molecular marker Jcuint002 with leaf width (-0.68), leaf length (-0.54) and petiole length (-0.55). The leaf length showed a negative correlation with molecular marker Jatr739 (-0.51). The number of male flowers per cluster also correlated negatively with molecular marker Jatr684 (-0.53).

DISCUSSION

J. curcas is a polymorphic species with variations reported in tree architecture, plant height, number of fruits, seed dimensions (length, width, lateral diameter) and seed weight (Guan et al*.,* 2013). In this study, the highest coefficient of variation was observed in the number of female flower per cluster, number of male flower per cluster, peduncle length, total of flowers, length

of fruit stalk, number of fruits per cluster and seed weight. This indicates a wider variation existing among the accessions for the characters. Such wide variation in the species was not surprising because the characters are predominantly influenced by environment (Ovando-Medina et al*.,* 2011). The trend has been reported previously by several authors for different characters in different population of *J. curcas* (Kaushik et al*.,* 2007; Guan et al*.,* 2013; Saadaoui et al*.,* 2015). In addition, low coefficients of variation were revealed by number of leaf lobes, seed length, and number of seed per fruit and this indicated that a narrow variation existed among accessions for the characters. These traits with low coefficients of variations are more prone to highly hereditary traits, because of their uniformity.

The analysis of variance for quantitative characters also revealed high significant differences (P<0.01) in the thirty accessions. This indicated that substantial amount of genetic variability existed in the accessions. Saadaoui et al*.* (2015) investigated five characters of *J. curcas*, foliar surface area, leaf length, leaf width, petiole length and number of nodes, and all of them revealed high morphological variability (P<0.05) among the eight accessions in Tunisia. Similarly, Guan et al*.* (2013) found that five seed traits (seed weight, seed length, seed width, lateral diameter, seed length and width ratio) were significantly different among eight populations of *J. curcas* in China. The qualitative characters also revealed substantial amount of variability in the accessions. The pigmentation on emerging leaves was in line with what was observed by Pazeto et al*.* (2015) where the young leaves were distributed as red and purple. In a study conducted on 24 accessions of *J. curcas* collected from different zones of India, Kaushik et al*.* (2007) reported the variability in seed traits and oil content. There were significant differences (P<0.05) in seed size, 100-seed weight as well as in oil content between accessions. However, the coefficient of variation was higher for phenotype than genotype, indicating a predominant role of the environment. Similar results were found by Sunil et al*.* (2008a) who observed correlation between morphological characteristics (plant height, collar height and thickness, number of primary branches, petiole length, number of fruits per cluster, pedicel length and seed yield) with the oil content of the seed. Saikia et al*.* (2015) found moderate variation in plant height, stem girth, branches per plant and seed weight.

The clustering based on morphological characters slightly match that of groupings derived through molecular analysis, in that four groupings were produced by both methods, with group A and B having some similar accessions. The four groupings were confirmed by the Principal Coordinated analysis. For both morphological and molecular clustering, the highest group (B) comprised of accessions from the northern and southern parts of the country, suggesting that there is no relation between the place of origin and the clustering for the accessions. This also may be due to similar agro-climatic conditions or seed movement. The morphological markers revealed slightly higher genetic distance estimates ranging from 0.51 to 2.48 while the molecular marker showed relatively smaller genetic distance estimates ranging from 0.71 to 1.00. Cai et al*.* (2010) investigated the genetic diversity of 219 *J. curcas* accessions from China using Simple Sequence Repeat (SSR) markers and revealed a low genetic diversity in the Chinese germplasm. Pamidimarri et al*.* (2009a) isolated SSR markers, investigated the genetic diversity of *J. curcas* accessions from India, and found a narrow genetic diversity in accessions. Also, low polymorphism was found in 64 genotypes from five geographic locations (Brazil, Cape Verde, Cuba, Mozambique and Senegal) using 32 SSR markers (Ricci and Chekhovskiy, 2012). Ambrosi et al*.* (2010) analyzed 26 accessions from different geographical regions (including Mexico, South America, Asia and Africa), using 10 RAPD, 6 ISSR and 10 SSR markers, and low genetic variability was documented not only among accession groups but also among accessions of different geographical origin, with the exception of Mexican landraces. Tanya et al*.* (2011) characterized 26 Mexican, 3 Chinese, 3 Thai and 4 Vietnamese accessions using SSR markers, and five of these loci clearly displayed distinct banding patterns between 26 Mexican accessions (non- toxic) and the 10 Asian accessions (toxic). The slight difference between clustering based on molecular markers and morphological markers may be that most of the characters are controlled by polygenes and are highly influenced by environment (Behera et al., 2012). However, the SSR markers and the morphological markers cluster analysis clearly separated the Ghana accession (GJC) and the Botswana accessions. The characters that separated the Ghana accession from others were seed weight and inflorescence compactness, which suggests that the Ghana accessions could be valuable in the improvement of the genetic materials found in Botswana, as it can be used to improve the seed yield of the local accessions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Antioxidants enhance banana embryogenic cell competence to Agrobacterium mediated transformation

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Gene transfer into the plant cell is a key step towards its successful genetic modification and its efficiency is heavily dependent on plant and bacterial cell biological status and a wide array of physical conditions. Gene transfer efficiencies in East African Highland Banana (EAHB) cell lines remains low compared to other monocotyledonous crops like rice and wheat due to factors such as high oxidative stress. The use of antioxidants is fundamental in influencing gene transfer events during *Agrobacterium*-plant cell co-cultivation. Here we report significant enhancement of gene transfer efficiency in the EAHB cultivar 'Nakinyika' (EA-AAA) by supplementing co-cultivation medium with antioxidants; ascorbic acid (AA), glutathione (GSH), tocopherol (TOC) and silver nitrate (SN). The most enhancing antioxidant by number of blue foci after histochemical assay, as a parameter of gene transfer efficiency, was ascorbic acid (174 cells) at a concentration of 40 mg/L, followed by glutathione (91 cells) and tocopherol (91 cells) both at 50 mg/L. The least enhancement was observed when ascorbic acid (39 cells), silver nitrate (41 and 31 cells) were used at concentrations of 20, 4 and 6 mg/L compared to 72 cells in controls (no anti-oxidants). Regeneration efficiency increased from 29 cells in controls to 46% for SN at 8 mg/L; 43% for GSH at 100 mg/L; 30% for TOC at 75 mg/L; and 48% for AA at 20 mg/L. Polymerase chain reaction (PCR) results using *gusA* specific primers showed that these regenerants were putative transformants and grew normally during regeneration, rooting and multiplication. GSH and TOC significantly enhanced gene transfer efficiency while AA and SN showed significant increases in shoot regeneration compared to controls. The current results show that antioxidants significantly enhance gene transfer and regeneration efficiency in recalcitrant banana cell lines and could significantly enhance the overall transformation efficiencies in cases where numerous transgenic lines are required in a short time.

Key words: Highland bananas, transgenic, browning, transformation efficiency, agrobacterium.

INTRODUCTION

Banana ranks among the 10 most important crops in the tropical and sub-tropical zones of the world (Ortiz and Swennen, 2014). Annual banana production in the world is estimated at 145 million tons of which less than 10%

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enters the commercial market suggesting that the crop is more important as food for local consumption than for export (Brown et al., 2017). In the Great Lakes region (Uganda, Rwanda, Burundi, Democratic Republic of Congo) bananas constitute between 30 - 60% of the daily per capita calorie intake with consumption rates of up to 250 kg per person per (Abele et al., 2007; Komarek, 2010).

Despite its importance, banana production is affected by a number of biotic factors such as pests and diseases and abiotic constraints like declining soil fertility (Wairegi et al., 2010; Robinson and Sauco, 2010). Breeding for host resistance using classical methods remains a tedious endeavour because of the crop's high sterility, polyploidy, linkage drag and long generation times of most of edible cultivars (Wulff et al., 2011; Namukwaya et al., 2012; Brown et al., 2017). Genetic engineering provides a promising alternative strategy for the improvement of commercial banana varieties with resistance to biotic and abiotic stresses (Ortiz and Swennen, 2014). As a pre-requisite for successful genetic manipulation, it is necessary to establish an efficient *in vitro* plant regeneration system and a method to deliver genes at a high frequency suitable for transformation (Barampuram and Zhang, 2011). Agrobacterium tumefaciens mediated transformation is the outstanding method for plant genetic transformation due to several advantages in the T-DNA transfer process, including high efficiency, low-copy number inserts, large DNA segments, low rearrangement rate and low cost (Gelvin, 2012).

Functional genomics studies of crop plants such as banana necessitates a cost effective testing of a huge number of candidate genes or other nucleotide sequences and require high throughput transformation systems (Ortiz and Swennen, 2014). Nevertheless, low transformation efficiency still remains a great challenge in the application of this technology in recalcitrant crops, predominantly monocotyledonous plants, similar to banana, which are not susceptible to *Agrobacterium* spp. Browning or necrosis, is a common phenomen associated with agrobacterium mediated transformation and it greatly lower the efficiency of this technique. Several studies have associated browning to the production of reactive oxgyen species (ROS), as a defense response to agrobacterium infection during transformation. This oxidative stress has been reported to inhibit growth and alter plant metabolic pathways that lead to poor plant regeneration (Dan, 2008). A number of factors including plant growth regulators and antioxidants have been reported to improve genetic transformation efficiency of plants (Dutt et al., 2011). Antioxidants include ascorbic acid that functions by to reducing explant necrosis and hence increase explants viability whereas glutathione and tocopherol reduce hyperhydricity and ROS (Dan, 2008). Ascorbic acid and Silver nitrate added in co-cultivation medium was found to increase sugarcane leaf explant viability from 10 to 90% (Gustavo et al., 1998). Similarly, incorporation of 100 mg/l glutathione and 50 mg/l tocopherol in the co-cultivation medium increased the agrobacterium mediated transformation efficiency of peanut from 3.9 to 14.6% and 10.3%, respectively (Zheng et al., 2005). Similar studies have reported positive results associated with the use of several anti-oxidants in both transformation efficiency and shoot regeneration (Dhekney et al., 2009; Dutt et al., 2011).

In this study, we hypothesised that the use of ascorbic acid, silver nitrate, glutathione and tocopherol as anti-oxidants to enhance transformation and regeneration of banana cultivar 'Nakinyika'.

MATERIALS AND METHODS

General materials

All laboratory reagents used in this study were obtained from scientific companies including Sigma (USA) and Fisher scientific (USA). Agarose was supplied by Roche Diagnostics (USA), while bacterial agar was purchased from Oxoid (Aus). All DNA molecular weight markers, reagents for Polymerase chain reaction (PCR) and restriction enzymes were purchased from Roche Diagnostics (Aus), New England Biolabs (USA), Invitrogen (Aus) or Promega (Aus). All primers used were purchase from Gene Works (Aus). Nucleic acid purification kits were purchased from Roche Diagnostics (Aus), or Promega (Aus).

Plant material

Embryogenic cell suspensions (ECS) of banana cultivar 'Nakinyika' were used. ECS were sub - cultured in cell suspension culture medium (MA2). A settled cell volume of 1 ml of ESC was sub-cultured in 50 ml of liquid MA2 and cultured at reduced density for 5 days to increase cell competence. Five days after sub culturing, ECS were collected in 50 mL falcon tubes and used as starting material for transformation experiments.

Binary vector and Agrobacterium strains

Agrobacterium tumefaciens super virulent strain EHA 105 was used in this study. The pCAMBIA2301 vector (containing a reporter g*usA* gene regulated by the CaMV35S promoter and the kanamycin selection marker gene) was used (Figure 1). The vector was transformed into *Agrobacterium* using chemical treatment (Chuanchuen et al., 2002) and maintained on YEB medium (0.1% yeast extract, 0.5% beef extract, 0.5% peptone, 0.5% sucrose and 0.04% $MgSO_4$) supplemented with kanamycin (100 mg/L). The bacterium was cultured at 28°C with continuous shaking at 150 rpm until the optical density of OD_{600nm} was attained. The bacterial cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C and re-suspended in 25 ml of TMA1 medium supplemented with 300 mM acetosyringone and varying concentrations of the four antioxidant treatments (Table 1). Prior to the co-cultivation, the bacterial suspension was activated at 25°C for 3 h with shaking at 70 rpm until an O. D_{600nm} of 0.6 was reached.

Transformation, selection and regeneration

Banana cells were transformed with *Agrobacterium* strain EHA

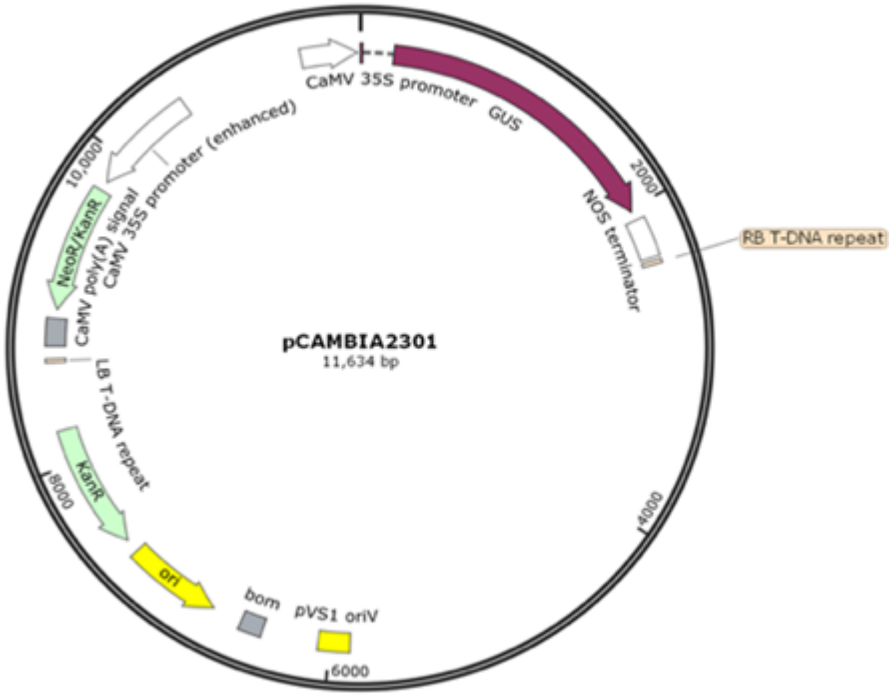

Figure 1. Schematic presentation of T-DNA of binary vector pCAMIBIA2301 used for transformation. It contains aminoglycoside phosphotransferase gene for plant cell selection and the *gusA* gene has a 5' extension with a catalase intron to ensure expression in plants but not bacteria.

Table 1. Media compositions: The different media types used at banana ECS culture, Agrobacterium-mediated transformation and regeneration of plants stages.

Medium type	Medium composition
ECS sub-culture	MA2 (Cell suspension culture medium); Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962) supplemented with 100 mg/L glutamine, 100 mg/L malt extract,1 mg/L2,4-Dichlorophenoxyaceticacid (2,4-D), 45 g/L sucrose, pH 5.3
Infection	TMA1(liquid) - (MS Macronutrients, MS Micronutrients, MS vitamins, 1 mg/L biotin, 100 mg/L malt extract, 100 mg/L glutamine, 230 mg/L proline, 5 g/L polyvinylpyrrolidone-10 (PVP-10), 200 mg/L cysteine,1 mg/L indoleacetic acid (IAA), 1 mg/L naphthaleneacetic acid(NAA),4 mg/L 2,4-D, 85.5 g/L sucrose, pH 5.3) supplemented with 200 mM acetosyringone and different varying concentrations of four antioxidants
Co-cultivation/resting	TMA1 (solid) - (MS Macronutrients, MS Micronutrients, MS vitamins, 1 mg/L biotin, 100 mg/L malt extract, 100 mg/L glutamine, 230 mg/L proline,5 g/L polyvinylpyrrolidone-10 (PVP-10), 200 mg/L cysteine,1 mg/L indoleacetic acid (IAA), 1 mg/L naphthaleneacetic acid(NAA),4 mg/L 2,4-D, 85.5 g/L sucrose, pH 6) supplemented with 300 mM cetosyringone and different varying concentrations of four antioxidants
Selection	MA3; RD1; MA4; Schenk and Hildebrandt (SH)Macronutrients, SH Micronutrients (Schenk and Hildebrandt, 1972), MS vitamins, 100 mg/L glutamine, 100 mg/L malt extract, 1 mg/L biotin, 230 mg/L proline, 45 g/L sucrose, 10 g/L lactose, 0.05 mg/L zeatin,0.1 mg/L kinetin, 0.2 mg/L NAA, 3 g/L gelrite, pH 5.7; supplemented with timentin (200 mg/L) and kanamycin (50 mg/L) with varying concentrations of different antioxidants
Regeneration	(MS salts and vitamins, 100 mg/L myo-inositol, 5 mg/L BAP, 30 g/L sucrose, 3 g/L gel rite, pH 5.8)
Rooting	MS salts and vitamins, 10 mg/L ascorbic acid, 100 mg/L myo-inositol, 1 mg/L indole-3-butyric acid (IBA), 30 g/L sucrose, 3 g/L gelrite, pH 5.8

harbouring pCAMBIA2301 through co-cultivation of the cells the *Agrobacterium* as described by Khanna et al. (2004) and Magambo et al. (2016). Five days after the co-cultivation in the dark at 22°C, the *Agrobacterium*-infected cells were washed and transferred onto selective embryo formation medium (MA3; Table 1), that was supplemented with timentin (200 mg/L) and kanamycin (50 mg/L) to kill off the bacterium. The different antioxidants were added to the selective media at varying concentrations and the culture were

Table 2. Different Antioxidants and their varying concentrations used in the study.

Antioxidants name	Concentrations (mg)		
Ascorbic acid	20	40	60
Glutathione	50	100	150
Tocophel	25	50	75
Silver nitrate	4	6	8

incubated for 3 months with fortnightly transfer onto fresh medium. Putatively transformed embryos were then transferred to semi-solid RD1 medium (Table 1) amended with kanamycin (50 mg/L). Embryos germinating on this medium were transferred to medium MA4 (Table 1). The shoots developed on MA4 were transferred to proliferation medium (Table 1) and after 2 weeks, individual shoots were transferred to rooting medium. The regenerated plants were further used for molecular analysis.

Antioxidants

Antioxidants have been reported in the control of tissue browning and necrosis in plant tissue culture. Previous studies indicated that, tissue browning/ necrosis leading to poor plant regeneration in vitro and successful use of antioxidants is a key in solving the problems in the tissue culture (Tang et al., 2004; Naser et al., 2019). Therefore, using several antioxidants in many plant species such as AA, GSH, TOC and SN (Table 2) and other antioxidant namely, Cysteine, Dithiothreitol (DTT) and Polyvinylpyrrolidene (PVPP) have yielded tremendous results both at transformation and regeneration stages (Mei et al., 2011). The four antioxidants used in this study, each was varied in three different concentrations (Table 2) and each concentration was replicated three times.

Histochemical GUS assays

GUS assays were conducted on infected banana cell suspension after 5 days of co-cultivation with the *Agrobacterium* following the method of (Jefferson et al., 1987). Three samples from each concentration of antioxidant were randomly selected and stained. The frequency of transient GUS expression was determined as the percentage of the number of infected cells that exhibited the blue colour. After the regeneration process, the transformation frequency was determined as the percentage of the number of shoots regenerated against the total number of embryos cultured on selection media (Figure 4).

PCR analysis

Genomic DNA was isolated from 11 randomly selected putative transformed plantlets by means of the CTAB protocol (Gawel and Jarret, 1991; Stewart and Via, 1993) and used PCR analysis. PCR was performed using specific primers for the *gusA* gene, comprising the forward primer 5'TTTAACTATGCCGGGATCCATCGC-3'and reverse primer 5'CCAGTCGAGCATCTCTTCAGCGTA-3'. The PCR master mixes (20 µl) contained 0.5 ng of gDNA or 200 ng of plasmid DNA template, 10 µl of GoTaq Green master mix (Promega) and 5 pmol of each primer. The PCR parameters were as follows, the initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and a final extension of 72°C for 5 min. PCR amplicons were separated on agarose gel and stained in ethidium bromide before visualization under UV light.

Data collection and analysis

Data on stained and regenerated shoots was collected by counting shoots per medium type and presented as images and graphs. The means presented were for three replicates. Analysis of variance (ANOVA) was conducted using XLSTAT software version 9.0 (Addin soft, SARL, 2010). The means were separated using Tukey's HSD test at $P<0.05$ significance threshold of 0.05 and a post-hoc was applied thereafter.

RESULTS

Transformation, selection and regeneration of transgenic banana

Transient GUS expression assays showed that Cultivar "Nakinyika" ECS were successfully transformed. There were more GUS expressions in transformed embryogenic cells that had antioxidants added in the co-cultivation media compared to the controls where antioxidants were not added (Figure 2A and B). Different concentrations of ascorbic acid, glutathione, silver nitrate and tocopherol added to MA3 media resulted into variable numbers of GUS stained cells (Figure 3). Transformation efficiency results indicate that, cultivar 'Nakinyika' ECS were successfully transformed based on GUS expression assays (Figure 2: A (no anti-oxidant) and B (anti-oxidant). In addition, B which had antioxidant added showed more stained cells than A indicating, there was an improvement in the transformation as a result of antioxidants.

Results indicate that antioxidant increased transformation efficiency over the controls as presented in Figure 3. The highest number of stained cells (174 cells) was realized with Ascorbic acid at a concentration of 40 mg/L, followed by Glutathione (91 cells.) at 50 mg/L and Tocopherol (91 cells) at 50 mg/L. The least staining was observed when Ascorbic acid (39 cells), Silver nitrate (41 cells and 31 cells) were used at concentrations of 20, 4 and 6 mg/L, respectively (Figure 3). Therefore, all anti-oxidants can be useful for regeneration mostly the concentrations that gave better response than the control. Comparison of the various anti-oxidant concentrations at both transformation efficiency and regeneration showed

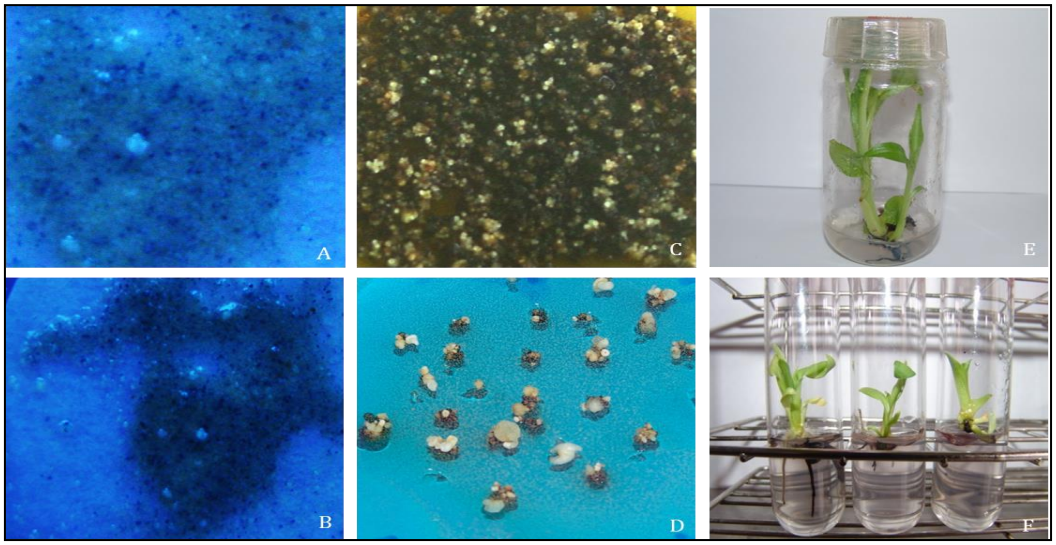

Figure 2. Schematic presentation of A: histochemical GUS assays with no anti-oxidant, B: histochemical GUS assay with anti-oxidant, blue foci represent the transformed ECS of cultivar 'Nakinyika', C: cells on selection medium (MA3), D: embryos germinating on selective medium (RD1) following Agrobacterium-mediate transformation, E: putatively transgenic shots on selective medium, F: transgenic.

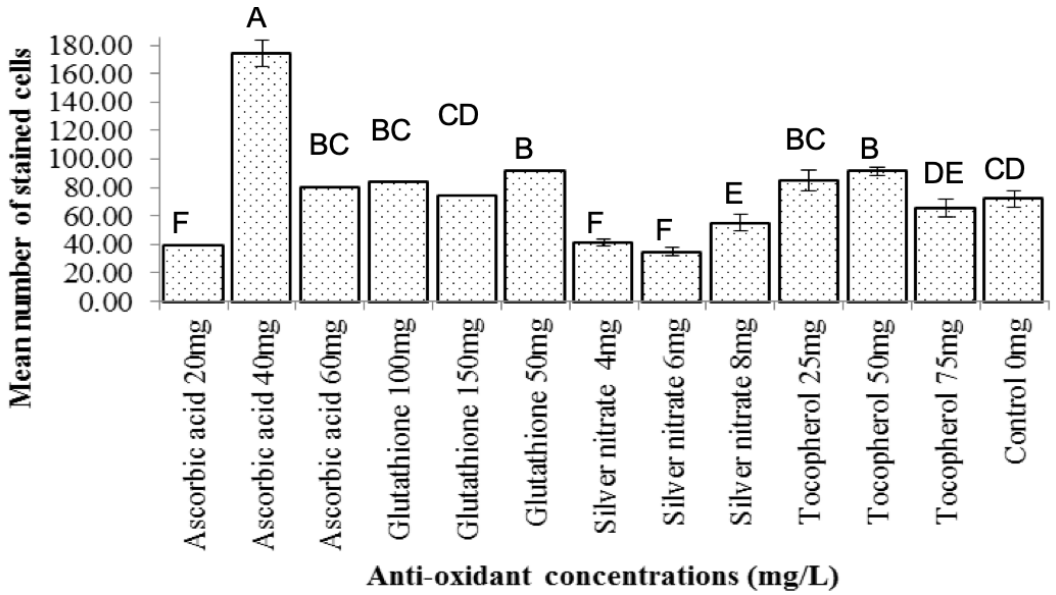

Figure 3. Effects of anti-oxidants on transformation efficiency of banana cultivar 'Nakinyika'. Means with the same letters were not significantly different from each other at p≥0.05%. Results are presented as mean ± standard error. Means are of three replicates.

that the three anti-oxidants (Ascorbic acid, Glutathione and Tocopherol) performed well at co-cultivation level basing on the number of stained cells, than the control of no anti-oxidant except Silver nitrate. At regeneration level, all the four anti-oxidants at the varied concentrations gave better results than the control, based on from the number of shoots obtained. The exception was Glutathione at 50 mg/L whose effect did not differ from the control. However, at both transformation and regeneration levels, ascorbic acid was better than the rest of the antioxidants, followed by Glutathione (Figures 3 and 4). From the data, all antioxidants gave better

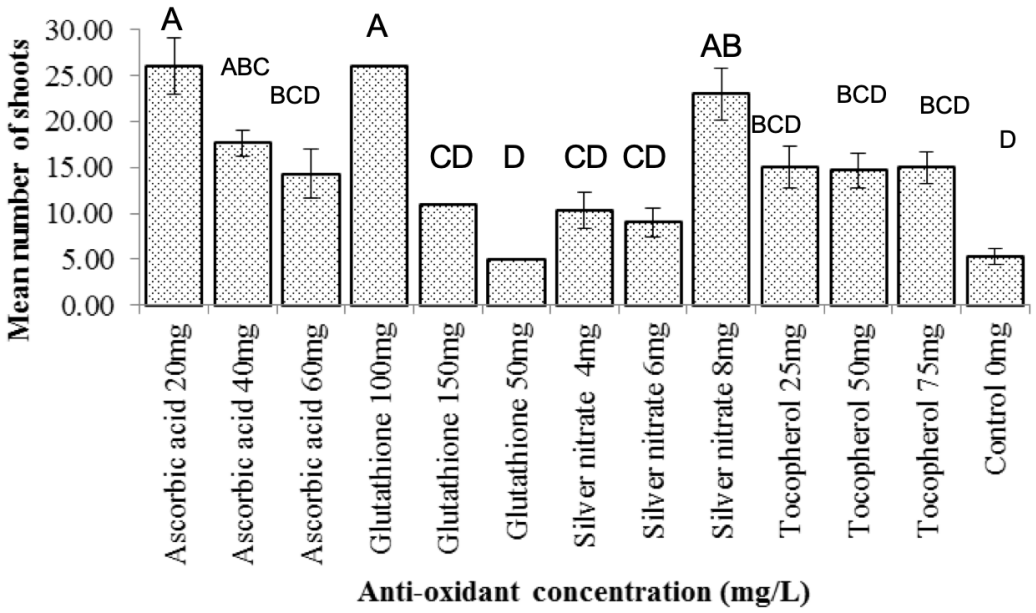

Figure 4. Effects of anti-oxidants on shoot regeneration of banana cultivar 'Nakinyika'Means with the same letters were not significantly different from each other at p≥0.05%. Results are presented as mean ± standard error. Means are of three replicates.

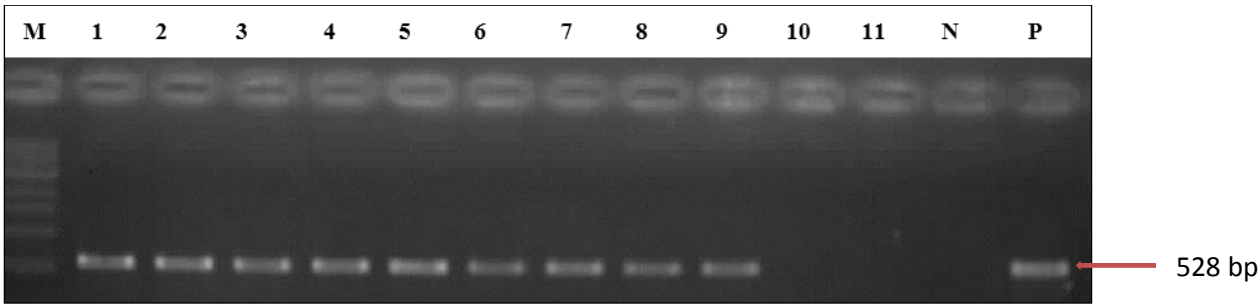

Figure 5. PCR amplification using Gusgene primers Lane M: DNA maker. Lanes 1-11: transgenic plants, lane N: Nontransgenic control P: Plasmid DNA.

responses at shoot regeneration other than at co-cultivation, the best antoxidants being ascorbic Acid at 20 mg, glutathione at 100 mg and silver nitrate at a rate of 8 mg/L (Figure 4).

PCR analysis

PCR analysis using gene specific primers gave the expected 528 bp fragment for coding region of *gusA* gene. Out of eleven putatively transformed plants that were randomly selected for confirmation, 9 lines were PCR positive while two lines did not show any amplification (Figure 5). In comparision, there was no amplfication observed in the non-transformed control.

There were no bands seen from some samples that were anaylsed for the Gus gene.

DISCUSSION

In contrast, browning and necrosis of transformed cells and difficulty to regenerate transgenic plants from the transformed cells are common phenomena in *Agrobacterium*-mediated transformation process in many plant species (Hiei et al., 2014). The problems of browning and necrosis affecting transformation' and occurrence of shoot escapes severely reduce transformation efficiency. *Agrobacterium*-mediated transformation is an injury process and therefore reactive oxygen species (ROS) such as superoxide radical (O-2), hydrogen peroxide (H_2O_2), the hydroxyl radical (OH), and

the peroxyl radical (RO2) are generated causing tissue browning and necrosis (Sharma et al.,2012). Additionally, dead cells release toxins that kill other cells in their proximity, resulting into low regeneration (Lindsey and Gallois, 1990). Therefore, an effective antioxidant is needed to protect the infected cells and improve their survival and successful transformation. Antioxidants act through inihibition of polyphenol oxidase enzymes that catalyse oxidation phenolics into highly reactive quinones that cause browning in injured tissues (Khosroushahi et al., 2011; Sharma et al., 2012; Selvarajan et al., 2018). Previous studies have reported use of various antioxidants in other plant species fore xample, it was observed that, applying 400 mg/1 cysteine in the coculture medium increased both the frequency of transient a-glucuronidase (GUS) expression in target cells of corm by 56% more than the control which yielded only 17% (Frame et al., 2002; Selvarajan et al., 2018). In addition, Toldi et al. (2002) demonstrated that, reduction in hyperhydricity of leaf explants, increase in leaf explant viability, as well as the frequency of transformation from 13% (without glutathione) to 45% in Agrobacterium mediated transformation of a desiccation-tolerant plant and *Craterostigma plantagineum*, was due to the addition of glutathione in the selection medium. Furthermore, previous research reported that, when ascorbic acid, sodium selenite, DL-a-tocopherol, and glutathione were added in a cocultivation medium, there was reduction on ROS production, antioxidant activity, and stable transformation efficiency during peanut Agrobacterium-mediated transformation (Zheng et al., 2005). They found that glutathione, tocopherol, and selenite not only eliminated the formation of H^sub 2^O^sub 2^ produced in wound tissue during preparation of leaf expiants and their cocultivation with A. tumefaciens, but also decreased malondialdehyde (MDA) formation and enhanced the activities of the antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). Furthermore, it was reported that, when cysteine was added incocultivation medium at a rate of 400 mg/L, it increased the Agrobacterium infection in the cotyledonary node of soybean (Olhoft et al., 2001). They further reported that, a combination of cysteine and DTT in the co- cultivation medium significantly increased the transformation efficiency (Olhoft et al., 2003).

Similarly in the present study, Ascorbic acid at 40 mg/L was the most effective antioxidant in transformation. Above this concentration, the antioxidant effects were not different from the other tested antioxidants. Ascorbic acid, Glutathione and Tocopherol were effective at about 50 mg/L at cell level during transformation. The observed ineffectiveness below and above this concentration is likely to be due to insufficient levels and toxicity, respectively. In contrast, the high concentrations were generally not deterrent at the tissue and plant level during regeneration and subsequent culturing.

The effect of silver nitrate on transformation success was not different from the control. Low transformation levels of silver nitrate could be partly linked to its antibacterial effects (Pandian et al., 2010). This toxicity might have reduced the agrobacterium population affecting the transformation process. Overall, the effect of the antioxidants at transformation did not match with the shoot regeneration trend. Cell injury and related oxidation was more limiting at the cell transformation stage. Due to the low cell surface exposure at the subsequent culture procedures of transfers and regeneration there was little oxidation which could be handled even at a lower ascorbic acid level of 10 mg/L. Contrarily to its effect at transformation, silver nitrate incoporated at 8 mg/L resulted into higher shoots recovery. This propmotive effect could be attributed to improvement in callus growth and embryogenesis through inhibition of ethylene as reported in wheat (Cristea et al., 2012).

Ascorbic acid is a common anti-oxidant in banana micro propagation media using the meristem culture where it is used at 10 to 20 mg/L in less and highly browning prone cultivars, respectively. It is suggestive that Ascorbic acid should be used at 40 mg/L during transformation and subsequently reduced to 20 ml/L at the plant regeneration stage. There was a significant anti-oxidant dependency in *Agrobacterium*-mediated banana transformation. Hiei et al. (2014) demonstrated that the increase in transformation frequency was primarily due to the increase in transgene integration efficiency rather than in tissue regeneration efficiency. Silver nitrate at a rate of 8mg, ascorbic acid at 20 mg and glutathione at 100 mg should be useful at cell-bacterium co-cultivation. Similarly, silver nitrate, ascorbic acid and glutathione can be adapted for shoot regeneration. For the PCR, the results confirmed that the GUS gene was successfully incorporated into ‘Nakinyika’ cultivar due to its presence observed in the 9 randomly selected lines. These results correlate well with the assessment of the transformation efficiency after co-cultivation when cells were stained which confirmed higher transformation in relation to the transgene presence. Similarly, the negative results observed in the two samples could be due to the presence of contaminants within the DNA samples or these could have been escapes. Due to cultivar differences in phenolic contents, these antioxidants should be evaluated on other banana genotypes as well.

Conclusion

The results of this study show that, antioxidants significantly enhance gene transfer and regeneration efficiency in recalcitrant banana cell lines and could significantly enhance the overall transformation efficiencies and generation of numerous transgenic lines. This study has established an efficient procedure for the *Agrobacterium*-mediated transformation of banana. Principally, we have developed an optimized co-

cultivation and regeneration conditions through supplementation with the antioxidants ascorbic acid, silver nitrate and glutathione for transgenic banana plants of the 'Nakinyika' cultivar. Our results provide an opportunity for further reproduction of transgenic banana plant cultivars.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ROS, Reactive Oxgyen Species; **RO2,** Peroxyl radical; **OH,** Hydroxyl radical; **H_2O_2,** Hydrgen peroxide; **O-2,** Super oxide radical; **DNA,** Deoxyribo Nucleic Acid; **PCR,** Polymerase chain reaction; **GSH,**Glutathione; **TOC,** Tocopherol; **AA,** Ascorbic acid; **SN,** Silver nitrate; **ECS,** Embryogenic cell suspension; **CTAB,**Centyltrimethyl ammonium bromide; **Mg,** Milligram; **SH,** Schenk and Hildebrandt; **RD1,** Embryo development media; **mL,** Millilitre; **GUS,** ß-glucuronidase; **G,**Gram; **ANOVA,** Analysis of Variance; **NAA,** Napthalene acetic acid; **IAA,** Indole acetic acid.

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Preparation and physicochemical characteristics of cross-linked resistant starch under heat-moisture treatment

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Sweet potato starch was subjected to cross-linking to achieve high content of resistant starch (RS) under heat-moisture treatment and the physicochemical properties of cross-linked sweet potato starches were investigated. The cross-linking agent was sodium trimetaphosphate and sodium tripolyphosphate (STMP/STPP). A maximum RS content (72.45%) was obtained after 10% STMP/STPP (99/1% w/w) at 120°C and pH 11.5 with a 20% moisture level. Through the analysis of ^{31}P (phosphorus) nuclear magnetic resonance (^{31}P NMR) spectroscopy, the RS-72.45% contained distarch monophosphate (DSMP) and monostarch monophosphate (MSMP) was not detected. Fourier-transform infrared spectroscopy (FTIR) presented the cross-linking reaction, confirmed by the comparison of native curves. After cross-linking, in the starches with high moisture treatment (≥25%) a loss of birefringence and surface erosion were observed. X-ray diffraction patterns showed that the crystal type was unchanged but with slight alteration in the relative crystallinity of cross-linked starches. Differential scanning calorimetry (DSC) showed gelatinization temperature increased as the moisture content increased, while gelatinization enthalpy apparently decreased.

Key words: Sweet potato starch, Heat-moisture treatment, ^{31}P Nuclear magnetic resonance spectroscopy, optimization, X-ray difraction (XRD), scanning electron microscopy (SEM), polarized light microscopy.

INTRODUCTION

Generally, RS is divided into physically inaccessible starch (RS_1), native granular starch with the B-type crystal pattern (RS_2), retrograded starch (RS_3), and chemically modified starch (RS_4) typically through esterification and cross-linking. It can be added to baked and extruded food to improve its processing technology and product quality, such as improving the brittleness and expansibility of the product. As a type of high RS, cross-linked starch has increasingly drawn broad interest for functional properties and health benefits (Hung and Morita, 2004; Yeo and Seib, 2009; Miller et al., 2011). Many researches have been conducted on cross-linking reagents of $POCl_3$,

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sodium tripolyphosphate (STPP), epichlorohydrin (EPI), sodium trimetaphosphate (STMP), $NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 12H_2O$ (Lim and Seib, 1993; Hirsch and Kokini, 2002; Woo and Seib, 2002; Zhang and Wang, 2009), being capable of forming either ether or ester inter-molecular linkages between hydroxyl groups on starch molecules (Rutenberg and Solarek, 1984). Normally, STMP and STPP are regarded as appropriate cross-linking agents to prepare food-grade RS (Ashwar et al., 2017). This starch is low in toxicity with no reports on it adverse effects on humans (Woo and Seib, 1997); just by comparison, both $POCl_3$ and EPI are toxic and flammable (MSDS, 1996; Woo and Seib, 1997), while $NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 12H_2O$ show low effect for cross-linking. Regarding the cross-linking reaction condition, there are many reports such as slurry reaction condition, semi-moist reaction condition (Woo and Seib, 2002), ultra high pressure (Hwang et al., 2009; Kim et al., 2012), supercritical fluid extrusion (Landerito and Wang, 2005; Manoi and Rizvi, 2010).

Apart from heat-moisture treatment been defined as a physical modification that involves incubation of starch granules at low moisture level (< 35% water (w/w)) during a certain period of time, at a temperature above the glass transition temperature but below the gelatinization temperature (Jacobs and Delcour, 1998), no information exists regarding it as a condition for cross-linking reaction except Sang and Seib (2006) who reported that simultaneous heat-moisture treatment and phos-phorylation of high-amylose corn starch achieved high RS with good boiling-stability for food use. The paper mainly studied the effect of RS content under the heat-moisture condition.

Accordingly, in this research, the cross-linked sweet potato starch with high RS_4 content was prepared under the condition of heat-moisture treatment. The modification conditions were optimized using single factor experiment. The objective of this study is to establish optimum conditions for the production of starches with the highest RS_4 content. Moreover, the structure of the cross-linked starch was characterized by FT-IR and ^{31}P NMR and the physicochemical properties were investigated.

MATERIALS AND METHODS

Sweet potato starch was obtained from Quxian national grain storage, Sichuan, China. Resistant starch assay kit was purchased from Megazyme International Ireland Limited, including pancreatic α-amylase, amyloglucosidase and glucose-oxidase-peroxidase-aminoantipyrine (GOPOD) reagent enzymes. STPP, STMP, sodium hydroxide, hydrochloric acid and other chemicals were analytical grade reagents.

Preparation of cross-linked resistant starch (RS_4)

Sweet potato starches were cross-linked by the method of heat-moisture treatment. Sweet potato starch (50 g, dry basis) was stirred for 60 min at ambient temperature in water (75 mL) containing sodium sulfate (5 g), and the mixed reagents STMP/STPP (99/1) was different levels (1, 2, 4, 8, 10, and 12% (w/w), based on dry basis). The mixture was adjusted by adding 1 M NaOH solution to pH (10, 10.5, 11, 11.5 and 12). Then dried to suitable moisture content (15, 20, 25 and 30%), at high temperature (100, 110, 120, 130 and 140°C) reaction for 4 h. After cooling to room temperature, the reaction mixture was dispersed in 100 mL distilled water, the suspensions were neutralized to pH 6.0 with 0.1 M HCl solution, washed with distilled water (300mL×7), dried at 40°C for 24 h in an oven, and sifted through a 100 mesh sieve.

Determination of phosphorus content

Phosphorus content was colorimetrically determined by the reaction with ammonium molybdate according to the method of Smith and Caruso (1964).

Determination of resistant starch content

Resistant starch content was analysed using resistant starch assay kit based on AOAC (2002.02) and McCleary et al. (2018). Starch sample (100 mg, db) was equilibrated horizontally in a shaking water bath with 4.0 mL enzyme mixture (pancreatic α-amylase, 10 mg/mL; amyloglucosidase, 3 U/mL) for 16 h (37°C, 200 strokes/min). Afterwards, 4.0 mL ethanol (99% v/v) was used to terminate the reaction and the residue obtained was washed with ethanol (50% v/v) twice, and treated with KOH solution in an ice bath for 20 min (4 M, 2 mL) to solubilize the RS. The RS solution obtained was added 8 mL of 1.2 M sodium acetate buffer (pH 3.8). After incubation with amyloglucosidase (0.1 mL, 3300 U/mL) at 50 for 30 min, the samples were centrifuged at 1500×g for 10 min. 3 mL GOPOD was added to aliquots (0.1 mL) of the supernatant, and the mixture was incubated at 50°C for 20 min. Absorbance was measured using a spectrophotometer at 510 nm.

^{31}P NMR spectroscopy

Purified phosphorylated starch was digested by the procedure of Sang et al., (2007). Cross-linked starch (1.0 g, db) was suspended in calcium chloride solution (2.0 mM, pH 8.2). Heat-stable α-amylase solution (100 µL, 3000 Ceralpha U/mL) was added, and the suspension was heated in a boiling water bath for 30 min with vigorous stirring. The digest was cooled and its pH was readjusted from 7.5 to 8.2. Then α-amylase solution (100 µL) was added and the digestion step was repeated. After cooling, the digest was adjusted to pH 4.5 by adding 1 M hydrochloric acid. Glucoamylase (200 µL) was added, and the digest warmed to 60°C and allowed to digest for 1 h. The digest was cooled, centrifuged, and the supernatant freeze-dried.

The freeze-dried digest of a phosphorylated starch (α,□-dextrin) was dissolved in deuterium oxide (1.0 mL) containing 0.02% sodium azide as preservative, and the resulting solution was adjusted to pH 8.0 ± 0.1. ^{31}P NMR spectra were acquired on NMR spectrometer (Varian INOVA-400, Bruker, Germany), operating at 400 MHz for ^{1}H and 202.34 MHz for ^{31}P respectively, with a 3 mm NMR probe. The ^{31}P NMR experiments were performed at 25°C using a delay of 6 s between pulses (pulse width 15.0 µs), sweep width of 12730 Hz and 400 transients for each spectrum. ^{31}P chemical shifts were referenced with respect to 85% H_3PO_4.

Scanning electron microscopy (SEM)

Starch granules were mounted directly onto aluminum stubs using

double-sided adhesive tape, and then coated with 20 nm gold under vacuum. Images of starch granules were obtained with a field emission SEM (EVO 18, Zeiss, Germany) at an acceleration potential of 10 kV and magnification of ×3000.

Polarized light microscopy

Birefringence of starch granules were observed under an optical microscope (Model BH-2, Olympus, Japan). All samples were dispersed in solution (glycerine/deionised water; 1:1 v/v) and the images were recorded at 500× magnification under polarized light.

X-ray diffraction (XRD)

X-ray patterns were performed using X-ray diffractometer (D8 Advance, Bruker, Germany). Starch samples were equilibrated in a saturated relative humidity chamber for 24 h at ambient temperature. The operating conditions were 40 kV and 40 mA with Cu □-radiation, the samples were scanned in the range of 4-35°(2θ). Relative crystallinity was calculated as the ratio of the areas of crystalline and amorphous regions of X-ray diffractograms (Nara and Komiya, 1983).

Swelling factor (SF)

SF of native and cross-linked starches (2%, w/w) was measured according to the method of Tester and Morrison (1990). The mixture was heated at 80°C for 30 min, centrifuged (3500×g, 15 min) and the supernatant was removed to aluminum box and weighted at 105°C. The SP is reported as the ratio of the volume of swollen granules to the volume of dry starch.

Differential scanning calorimetry

Thermal characteristics were measured with a Perkin Elmer differential scanning calorimetry (DSC) 8000 (Norwalk, CT, USA) instrument. Starch (4.5 mg, db) was weighed in a gold DSC pan adding 10.5 µL distilled water. To ensure complete equilibration of water and sample, pans were kept at ambient temperature for about 12 h. The pan was heated from 30 to 150°C at 5°C/min; an empty pan was used as a reference. The DSC software was applied to analyze onset temperature (To), peak temperature (Tp), conclusion temperature (Tc), and gelatinization enthalpy (ΔH).

FT-IR spectroscopy

During the cross-linked, amplitudes of hydroxyl groups peaks of starch molecules differed as could be confirmed by FTIR. All FT-IR patterns were recorded on a Vector 33 spectrometer (Bruker, Germany) using an attenuated total reflection method. FT-IR samples were prepared by mixing the starch with KBr and compressed to the sheet. Each spectrum was recorded over the wavenumber range from 4000 to 400 cm^{-1} with a spectral resolution of 4 cm^{-1} by 64 scans at room temperature. For each spectrum, the FT-IR spectra were adjusted by subtraction of the KBr spectra (background).

Statistical analysis

The data reported are averages of triplicate observations. Mean values and standard deviations were analyzed and reported using the Origin Program 8.0 (Origin Lab Company, USA). Analysis of variance with a significance level of 5% was done and Duncan's test applied to determine differences between means using the commercial statistical package (SPSS 17.0).

RESULTS AND DISCUSSION

Optimization of reaction conditions

In the case of STMP/STPP, the reaction was located by the phosphorus (P) level boundary of less than 0.4% and the resistant starch (RS) boundary at its highest level. In this study, reaction conditions including cross-linking agents, pH, moisture content and reaction temperature in the process of preparation resistant starch was analyzed, and it was found that RS content could be greatly increased by cross-linking reaction under heat-moisture treatment.

A comparison among samples showed that increased RS content (from 18.25 to 76.60%) was directly correlated with increased amount of cross-linking agent (from 1 to 12%) under pH 11.5, 25% moisture, 120°C condition (Figure 1). Consequently, 12% cross-linking agent was eliminated on account of phosphorus content beyond the edible denatured starch prescribed maximum of 0.4%. The 10% cross-linking agent was appropriate.

The reaction pH is another important factor in the production of RS. Effect of pH on the RS content under 10% cross-linked agent, 25% moisture, 120°C condition is shown in the Figure 2. Appropriate pH would facilitate molecular hydroxyl activation, which could increase the reactivity so that STMP/STPP nucleophilic attacks to starch molecular. The RS yield increased gradually as the reaction pH increased from 10 to 11.5, reaching a maximum of 68.10% at pH 11.5. However, a marked reduction (68.10 to 50.21%) was observed when the pH increased from 11.5 to 12, indicating that excessive alkaline which probably destroyed the starch granule structure was adverse to formation of resistant starch.

For determining optimum moisture content at pH 11.5, 25% moisture and 120℃, some different moisture contents were used. As shown in Figure 3, the result presented that the 20% moisture content generated the greatest increase in RS content (72.45%). Under the condition of higher moisture (more than 20%), RS content tended to decrease for the two possible reasons: one is hydroxyl groups of water competed for the phosphorylating agent with the hydroxyls of starch, which resulted in decrease of RS. Another is over moisture lead to swelling of starch which hindered the preparation of RS.

Regarding the reaction temperature, 120°C was favorable for production of RS under 10% cross-linked agent, pH 11.5 and 20% moisture (Figure 4). When the temperature was higher than 120°C, RS content straightly decreased. The reason to that was probably because temperature at over 120°C provides enough energy to break the intra-molecular structure, resulting

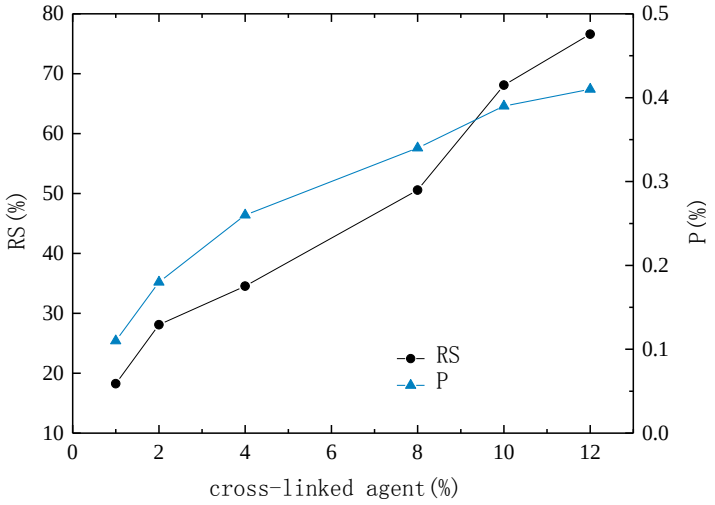

Figure 1. Effect of cross-linked agent amount on the RS content under the pH 11.5, 25% moisture and 120°C condition.

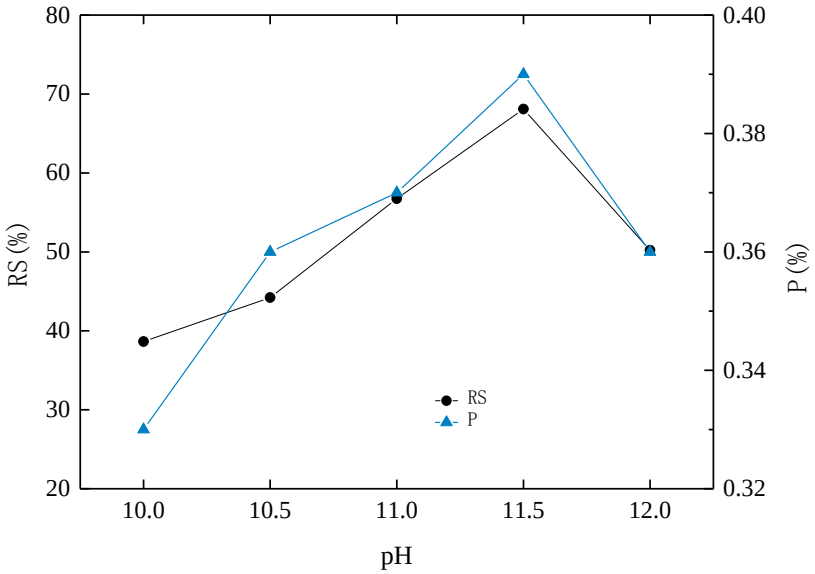

Figure 2. Effect of pH on the RS content under 10% cross-linked agent, 25% moisture and 120°C condition.

in partly gelatinization of starch granules.

Based on the results with all factors (amount of cross-linking agent, pH, moisture content and reaction temperature), sweet potato starch was cross-linked with 10% STMP/STPP (99:1) in 20% moisture, pH 11.5 and 120℃, produced the highest contents of type 4 resistant starch (RS_4).

^{31}P NMR spectrum of RS-72.45%

The key feature of ^{31}P NMR spectrum is the ability to identify product of crosslinking reaction. According to the results of Sang et al., (2007); wheat starch cross-linked with STMP in alkaline slurry was found to contain DSMP ester, whereas with STPP reacted to produce two kinds of products, including MSMP and DSMP. In this study, it was observed that in case of heat-moisture treatment phosphorylated sweet potato starch (RS-72.45%) with 0.39% P prepared by roasting the starch with STMP/STPP (99/1) at an initial pH 11.5 was shown to favor the formation of DSMP ester. The ^{31}P NMR spectrum (Figure 5) of the α,γ-dextrin of RS-72.45% showed strong signals for DSMP at δ 0 to + 1 ppm.

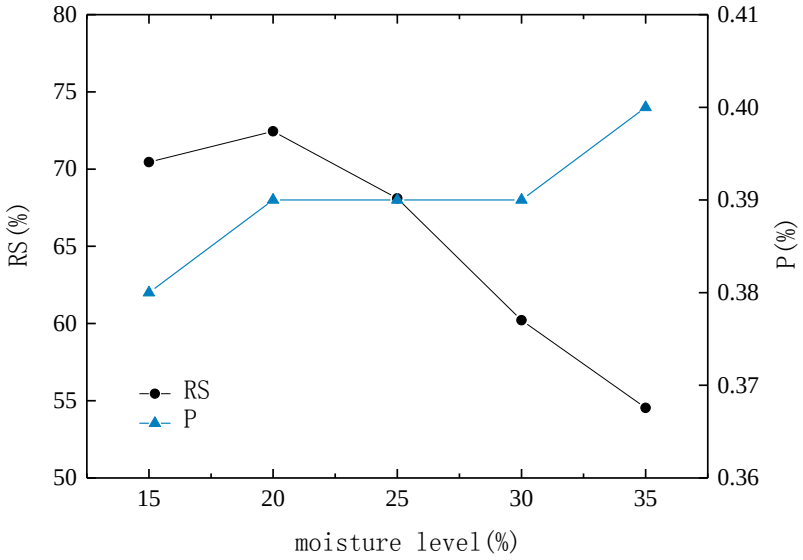

Figure 3. Effect of moisture level on the RS content under 10% cross-linked agent, pH 11.5 and 120°C, condition.

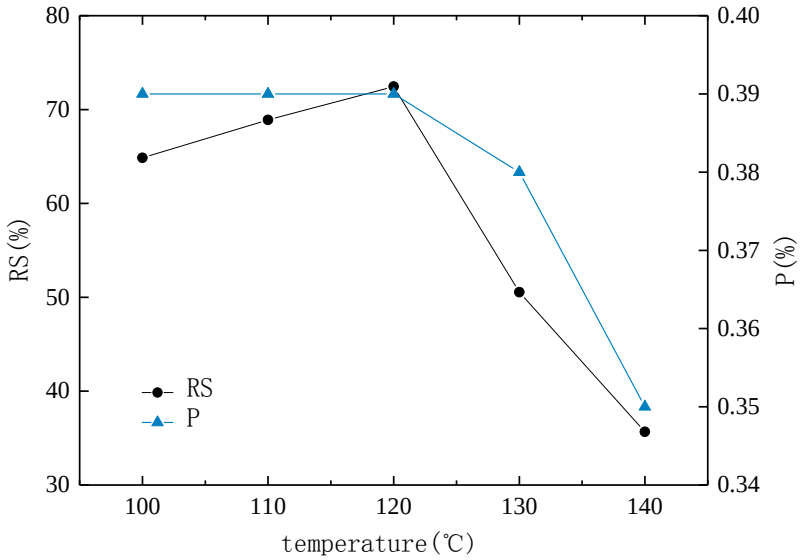

Figure 4. Effect of temperature on the RS content under 10% cross-linked agent, pH 11.5 and 20% moisture.

Surprisingly, MSMP linkages (δ 3.5-5.0 ppm) were not observed in the digest of the total reaction mixure. A similar outcome that MSMP structures were lost in the nonsoluble portion of the digest was reported by Sang et al., (2007) who explained the monophosphate groups associated with STMP perhaps gave strong localized chelation of calcium ion and denatured the α-amylase. The high content RS of CLM-20 (the cross-linked reaction under the 20% moisture) could ascribe the formation of DSMP which played a key role in making the phosphorylated starch resistant to α-amylase and amyloglucosidase digestion (Sang et al., 2010).

SEM and microscopy

While scanning electron microscope is superior to detect the granule shape and surface characteristics of starch, polarized light microscopy gives comprehensive view to image the average radial orientation of helical structures.

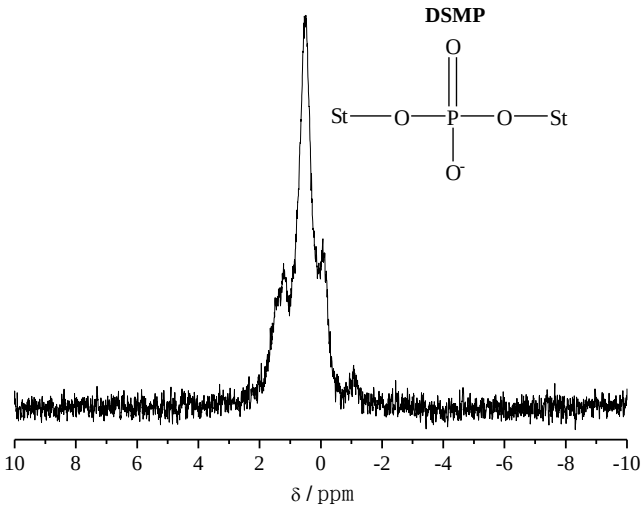

Figure 5. ^{31}P NMR spectra of α, γ-dextrins prepared from RS-72.45% starch. No signal was observed beyond the regions of 10 and -10 ppm.

The SEM images and birefringence patterns of native and cross-linked sweet potato starches are clearly shown in Figure 6. Native (Figure 6A) and CLM-20 starch granules (Figure 6B) had similar morphology; they were spherical or polygonal in shape with smooth surfaces, whereas CLM-30 and CLM-35 starch granules exhibited surface erosion with slight fragments and concaves. These characteristics in the surface of cross-linked starch granules indicate different heat-moisture treatments consequently led to different structural changes.

Similarly, the intensity of birefringence of CLM-20 (Figure 6F) starch almost remained unchanged compared with native starch (Figure 6E). However, birefringence loss occurred in the CLM-30 (Figure 6G) and CLM-35 (Figure 6H) starches (started at the hilum or botanical center of the granules and spreaded gradually to the periphery), the extent of this loss increased with increase in the moisture content treatment. This conclusion was in agreement with the investigation of Chung et al., (2009), who demonstrated that HMT with high moisture content could facilitate double helices move, which lead to a loss of radial orientation. Moreover, the weaker birefringence intensity and the lower RS content suggested that resistance to enzyme of cross-linked starch correlated with starch granule structure integrity. As shown in Figure 6, all the gelatinization starts at the hilum or botanical center of the granules and spreads rapidly to the periphery (de Castro et al., 2018; Ye et al., 2018).

XRD

The X-ray diffraction pattern and relative crystallinity of native and cross-linked starches are shown in Figure 7. There was no apparent difference between the native and modified starches in the X-ray diffraction pattern. All the phosphorylated starches showed similar diffraction patterns with peaks at 2θ of around 5.6, 15, 17, 18 and 23º compared to native starch, which are typical characteristics of C-type starch (Zobel, 1988). Similar results were observed for corn starch whereby unchanged diffraction pattern was speculated, and the cross-linking reaction mainly took place in the amorphous regions of starch granule and did not change the crystalline patterns of starches (Hoover and Sosulski, 1986; Koo et al., 2010).

Relative crystallinities of all the cross-linked starches except CLM-35 slightly increased (crystallinity variation 0.9-6.3%), which indicated that covalent bonds introduced in the process of cross-linking were much stronger than hydrogen bonds which fastened interaction of starch molecular. However, the decrease in relative crystallinity of CLM-35 compared with native starch was perhaps due to the damage of crystal structure in high moisture reaction, which could be proved from the result of birefringence (Figure 6). In other words, cross-linked starches with higher moisture levels reaction showed larger reductions in relative crystallinity.

DSC

Gelatinization parameters of native and cross-linked sweet potato starches (Table 1) are presented. The result showed that the gelatinization temperature was increased, while the enthalpy of all the cross-linked starches compared with native starch was decreased. The differences in gelatinization temperatures among the starches can be attributed to the two aspects. On the one

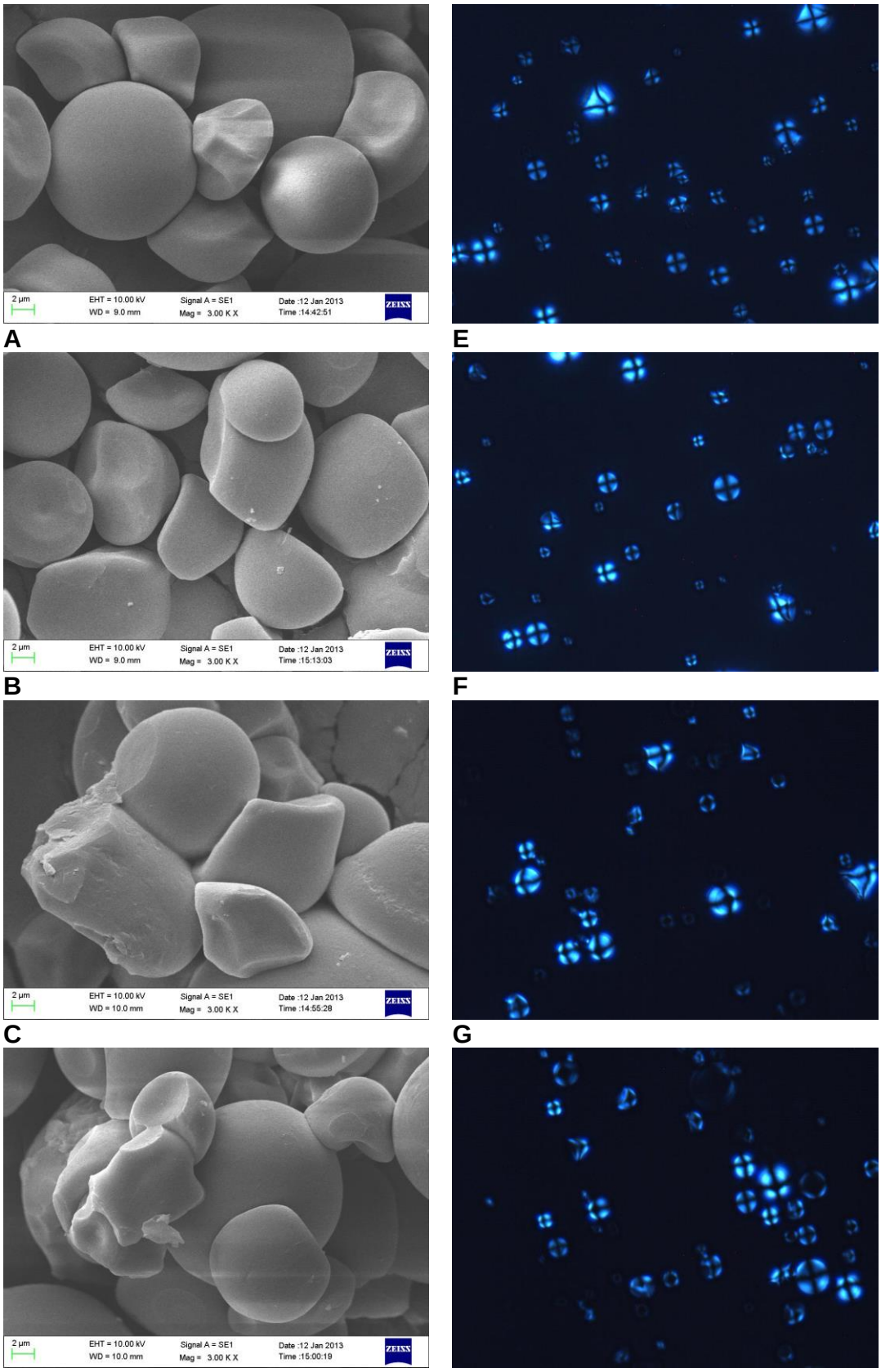

Figure 6. Native, CLM-20, CLM-30, CLM-35 of sweet potato starch visualized by SEM (A, B, C, and D respectively), at 3000× magnification; and native, CLM-20, CLM-30, CLM-35 of sweet potato starch viewed by polarized light microscopy (E, F, G and H respectively), at 500× magnification.

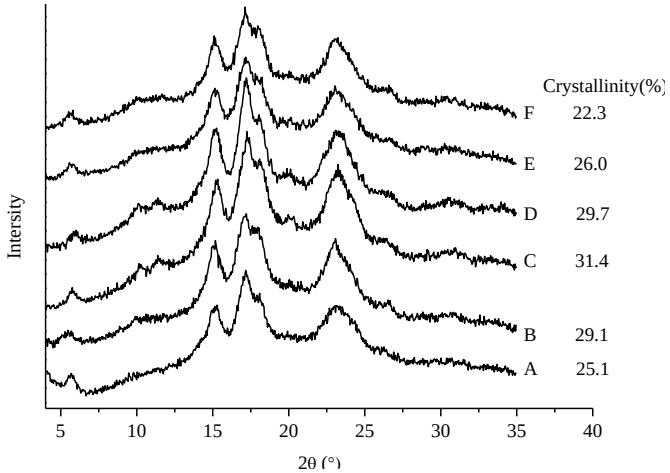

Figure 7. X-ray diffraction patterns (XRD) and relative crystallinity of native and cross-linked sweet potato starches prepared under 10% cross-linked agent, pH 11.5 and 120°C condition and different moisture contents. (A) Native starch, (B) CLM-15, (C) CLM-20, (D) CLM-25, (E) CLM-30, (F) CLM-35.

Table 1. Thermal characteristics of native and cross-linked starches.

Sample	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g^{-1})	SF (g/g)
Native starch	60.05 ± 0.41^{a}	76.38 ± 0.56^{a}	85.08 ± 0.43^{a}	14.89 ± 0.22^{e}	17.31 ± 0.92^{d}
CLM-15	68.01 ± 1.12^{b}	81.30 ± 0.21^{b}	90.97 ± 0.14^{c}	12.43 ± 0.53^{d}	5.21 ± 0.37^{b}
CLM-20	74.65 ± 0.33^{d}	84.42 ± 1.45^{d}	87.74 ± 0.18^{b}	8.11 ± 0.27^{c}	3.38 ± 0.42^{a}
CLM-25	73.36 ± 0.36^{c}	83.12 ± 0.39^{c}	91.31 ± 0.49^{cd}	7.27 ± 0.51^{b}	4.21 ± 0.33^{ab}
CLM-30	75.01 ± 0.53^{de}	84.21 ± 0.20^{cd}	91.59 ± 0.43^{cd}	6.01 ± 0.47^{a}	4.82 ± 0.24^{b}
CLM-35	76.00 ± 0.36^{e}	84.48 ± 0.17^{d}	92.02 ± 0.36^{d}	5.41 ± 0.32^{a}	6.33 ± 0.71^{c}

Values are given as mean ± standard deviation. Different letters indicate significantly different ($p < 0.05$) when analyzed by Duncan's New Multiple Range Test.

hand, introduction of cross-linking covalent groups into the starch by STMP could reinforce the interchain association tensioning the starch structure, which facilitated less water penetration into the starch granules with a consequent decrease in swelling (Table 1), leading to an increase in the gelatinization temperature. On the other hand, increase in T_o, T_p, and T_c was due to interaction between amylose-amylose and/or amylose-amylopectin chains, and to the formation of additional complexes between amylose and starch lipids (Hoover and Vasanthan, 1994).

In general, the great reduction ΔH in all the cross-linked starches is most probably due to the melting of imperfect amylopectin-based crystals, with potential contributions from both crystal packing and helix melting enthalpies (Lopez-Rubio et al., 2008). That decline in enthalpy of gelatinization probably resulted from partial unwinding of double-helices during the modification reaction at 120°C, sufficient moisture and Ph initial 11.5 (Sang and Seib, 2006). Starch cross-linked with high moisture markedly weakened its bonding forces and structurally weaker complex may require less energy to complete the thermal transition. Cross-linked starches treated with higher moisture levels showed larger reductions in gelatinization enthalpy than those treated at low moisture levels. The decreased ΔH values following the cross-linked sweet potato starches reflect the loss of double helices and some crystallites. The conclusion was confirmed by the NMR spectrum.

FT-IR spectroscopy

Figure 8 showed the FT-IR spectra of native starch and RS-72.45%. The characteristic peak position in the infrared spectrum of sweet potato starch is mainly: 3440 cm^{-1} is O-H stretching vibration, 2931 cm^{-1} is C-H stretching vibration, 1642 cm^{-1} is the bending vibration of H_2O, and 1017 cm^{-1} is the stretching vibration of C-O connected with the hydroxyl group of C_6 primary alcohol.

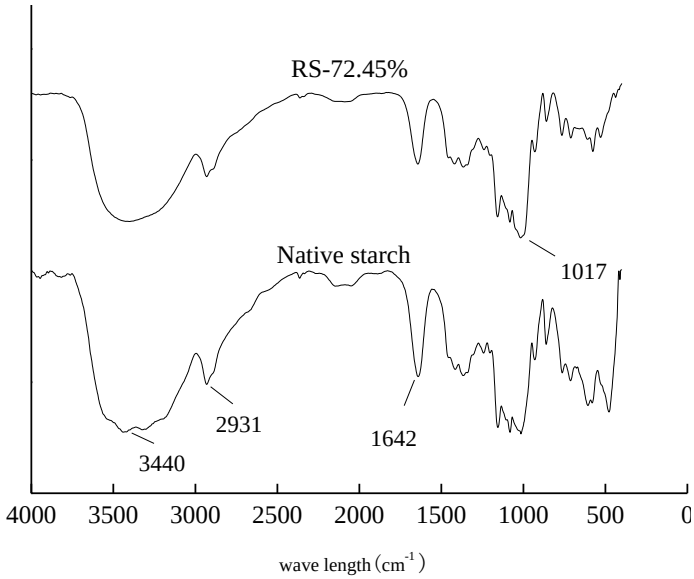

Figure 8. FT-IR spectra of native starch and RS-72.45%.

The peak of RS-72.45% was obviously changed, compared with the native starch. The absorption peak of RS-72.45% was enhanced at 1017 cm^{-1}. The reason may be that the wave length of 995 to 1050 cm^{-1} was stretching vibration of P-O-C, corresponding to the C_6-O-H stretching vibration band in starch, which indicated that C_6-O-P was formed in the reaction process.

However, the peaks of RS-72.45% at 3440 cm^{-1}, 2931 cm^{-1} and 1642 cm^{-1} were weaker than the native starch, which indicated that the stretching vibration of O-H、C-H and bending vibration of H_2O was weak dealing with the cross-linking and heat-moisture. The essence of the crosslinking reaction of starch molecules with multidimensional space mesh structure was formed by alcohol hydroxyl and crosslinking covalent bond, with hydrogen bond was replaced by the cross-linking of covalent bond. The cross-linking reaction was confirmed by the comparison of infrared spectrum curves.

Conclusion

RS_4 was prepared with mix reagents STMP/STPP (99/1%, w/w) under heat-moisture treatment. The highest content of RS (72.45%) was obtained under the following condition: 10% STMP/STPP, pH 11.5, 20% moisture level, and temperature of 120°C. ^{31}P nuclear magnetic resonance spectroscopy showed that RS-72.45% was DSMP. According to the single factor experiment and ^{31}P nuclear magnetic resonance analysis, the resistance to digest of cross-linked starch was related to the starch granule structure integrity and the space steric hindrance of DSMP. The reason may be that the interaction of amylase of glycosidic bond was limited. It suggested that the birefringence and fragments of cross-linked starch were weakened under the excessive moisture reaction (especially CLM-35). The X-ray diffraction result also showed that the relative crystallinity of CLM-35 decreased compared to native starch. The DSC demonstrated that cross-linked starches under heat-moisture treatment had better heat stability than native starch. The FT-IR confirmed that the cross-linking reaction occurred by the comparison of infrared spectrum curves.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests..

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Comparative study of fatty acid composition and nervonic acid contents of four tropicals plants: *Ricinodendron heudelotii, Cyperus esculentus, Citrullus colocynthis* and *Irvingia gabonensis* from Côte d'Ivoire

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This work was carried out to compare the potential applications of *Ricinodendron heudelotii*, *Cyperus esculentus*, *Citrullus colocynthis* and *Irvingia gabonensis* oil seeds by investigating their physicochemical parameters. Physicochemical parameters of the extracted oils were respectively as follow: Refractive index (1.49; 1.46; 1.45 and 1.44), acid value (1.35±0.02; 1.87 ± 0.45; 13.85 ± 0.02 and 39.04 ± 0.34 mg KOH/g), peroxide value (37.98 ± 0.01; 86.16 ± 0.32; 48.77 ± 0.22 and 2.34 ± 0.31 meq O_2/kg), iodine value (142.12 ± 0.34; 75.46 ± 0.35; 120.45 ± 0.29 and 07.42 ± 0.3 g I_2/100 g), saponification value (154.67 ± 0.28; 175.47 ± 0.30; 175.64 ± 0.28 and 176.03 ± 0.50 mg KOH/g), unsaponifiable matter (1.88 ± 0.04; 0.88 ± 0.03; 0.74 ± 0.03 and 1.26 ± 0.05%), vitamin A (0.00 ± 0.00; 2.78 ± 0.07; 3.66 ± 0.04 and 7.17 ± 0.32 µg/g), cholesterol (0.00 mg/g) for *R. heudelotii*, *C. esculentus*, *C. colocynthis* and *I. gabonensis.* P-anisidine value (301.17 ± 0.98; 24.47 ± 0.03; 29.04 ± 0.03 and 0.39 ± 0.00). *R. heudelotii* and *C. colocynthis* seeds showed relatively high content of linoleic acid (about 26.61 and 53.09% of total fatty acids). The nervonic acid content of the *R. heudelotii* seed is 45.24 ± 0.02 of total fatty acids. All these interesting characteristics should arouse attention for the usage of these oils seeds in food, pharmaceutical, cosmetic industries and biodiesel. These results suggest that they can be good for table, cooking and frying oils. The high linoleic acid level makes them good oils for the fight against cardiovascular illnesses. In addition, the high content of nervonic acid in *R. heudelotii* oil could be used in cases of diseases involving demyelination, such as adrenoleukodystrophy and multiple sclerosis where there is a considerable reduction in the levels of nervonic acid in the sphingolipids.

Key words: Acid nervonic, fatty acids, oils seeds.

INTRODUCTION

The need to maintain good health is the driving force in the search for alternative oil seeds of spices with high medicinal and nutritional potentials. Spices, depending on the part of the plant being used can be classified into

fruits, seed, leaves or floral parts and bulbs used to season food due to their distinctive flavor and aroma (Betti et al., 2009), as well as for therapeutic purposes. Many oils and fats for human consumption or for industrial purposes are derived from plants. Indeed, seeds constitute essential oil reserves of nutritional, industrial and pharmaceutical importance (Combe and Boue-Vaysse, 2004). Extracted oils from plant seeds are mainly composed of triacylglycerols (95 to 98%) which are esters of glycerol and complex mixtures (2 to 5%) of minor compounds (Aluyor et al., 2009). Those minor compounds include fat soluble vitamins, pigments such as chlorophylls and carotenoids, phenolic compounds, phospholipids, mono and diacylglycerols and free fatty acids (Kamal-Eldin, 2013). The fatty acids composition determine the physical properties, stability, and nutritional value of lipids (Hedren et al., 2002). This nutritional value is linked to essential fatty acids (EFA) that are polyunsatured fatty acids (PUFA). These compounds are essential for the human nutrition because, they are unable to be physiologically synthesized. In this respect, diet must cover organism needs (Naudet et al., 1992). Among PUFA, the most important families are the well-known ω_3 (n-3) and ω_6 (n-6) ones (Alais and Linden, 2009). These families are similar as they both comprise a precursor, namely linoleic acid (LA) for the ω_6 and linolenic acid (ALA) for the ω_3 (Dubois et al., 2007). EFA are used as substrates to synthesize a number of biologically active compounds such as steroid hormones, prostaglandins and leukotrienes (Nukhet et al., 2001). A part from the leukotrienes and prostaglandins structuring, the ratio of n-6/n-3 fatty acids has an essential effect on cardiovascular health (Wijendran and Hayes, 2004). Also, PUFA are essential for highly excitable membranes such as the brain and nervous tissues because of their role in membrane fluidity (Anhwange et al., 2010). An ω_6 fatty acid deficiency is characterized by growth retardation in children, skin lesions, dry scaly dermatitis and reproductive failures (Anhwange et al., 2010). However, cognitive development and visual acuity may be impaired in children receiving inadequate intakes of ω_3 fatty acids (Nagakura et al., 2000). In view of the cardinal role of EFA in human health and diseases, characterization of the fatty acids composition of oils has become a current focus of lipid research (Savag et al., 1999; Anwer et al., 2006). It is from this perspective that a number of non-conventional oilseeds from several plants of sub-Saharan Africa such as *Raphia sese, Raphi laurentii*, *Canarium schweinfurthii, Dacroydes edulis, Coula edulis, Balanites eagyptiaca, Vitellaria paradoxa, Telfairia occidentalis, Pentaclethra macrophylla, Parkia Africana, Cucumis melo, Tetracarpidium conophorum* and *Irvingia gabonensis* have been investigated (Kapseu et al., 2007). These works have revealed the indisputable potentialities of most of these unexploited oils for food, pharmaceutical or cosmetic applications. Therefore, to contribute to non conventional oils promotion, we focused our attention on oilseeds of four tropical plants, namely *Ricinodendron heudelotii, Cyperus esculentus, Citrullus colocynthis,* and *I. gabonensis*.

These species are annual tree can grow between 20 to 50 m in height with a straight trunk that has a diameter of about 2.7 m for *R. heudelotii* and *I. gabonensis* (Kapseu et al., 2007). *C. esculentus* and *C. colocynthis* plants which produce seeds of about 1 and 3 to 5 mm length, respectively (Mbaye et al., 2001; Mahadevan et al., 2009). In most countries of tropical Africa and particularly in Côte d'Ivoire, leaves of these plants are widely consumed as green vegetables due to their richness in polysaccharides, vitamins and minerals (Leung et al., 1968) but, their seeds are underexploited. Vegetable oil is for the most part used as food and feed, but it is increasingly used as a biofuel and as feedstock by the chemical industry (Dyer et al., 2008). Expanding its usage for non food applications will require a substantial increase in the total production of vegetable oil. This increase has the potential to be met by increasing the oil content in presently used oil crops or introducing new high-oil-yielding crops. Much progress has been made in understanding how plants produce and accumulate oils. The specific enzymes involved in the metabolic pathway leading to triacylglycerols (TAGs) stored in the oil bodies, as well as the pathway that supplies the precursors generated from imported sucrose, are to a large extent known (Voelker and Kinney, 2001; Rawsthorne, 2002). However, we still have a poor understanding in key areas such as factors important for regulating the flux of photosynthates into storage compartments, the synthesis of fatty acids, or the level of oil content in storage tissues. Hence, research in these areas is of great importance to enable a substantial increase in vegetable oil production. Today the level of knowledge on oilseeds remains insufficient therefore, the aim of this work was to contribute to these tropical plants promotion by investigating their oilseeds properties in order to explore and discuss their nutritional and industrial potentiality.

MATERIALS AND METHODS

Plant materials

R. heudelotii, *C. esculentus*, *C. colocynthis* and *I. gabonensis* seeds were collected from Abidjan district (Côte d'Ivoire) in January 2014. Seeds were rinsed thoroughly with distilled water to remove dirt and dried at 40°C for 24 h in an electric oven (Memmert, Germany) according to Achu (2006).

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Chemicals

Analytical grade solvents, standards, reagents and culture media were used to perform analysis. Solvents (n-hexane, chloroform, acetic acid, diethyl-ether, ethanol, methanol and n-heptane) put up were from Prolabo (France). Standards such as fatty acids, cholesterol, vitamin A, erucic acid and trifluoroacetic acid (TFA) were from Sigma-Aldrich (Germany). Wijs reagent was from Prolabo (France). The standard of nervonic acid has been provided by Merck (Germany).

Oils seeds extraction

Oils were extracted from 50 g crushed seeds (Laboratory crusher, Culatti, France) with 300 ml of n-hexane (40 to 60°C) in a Soxhlet extractor. Then the solvent was removed (vacum-packed) at 40°C with a rotary evaporator (Heidolph, Hei-Vap, Germany). The extracted lipid was weighed to determine the oil content of the seed. Crude oils were stored at 4°C in air tight brown sterile glass bottles (AFNOR ISO 734-1, 2006) until further use for physicochemical analysis.

Physicochemical analysis

Specific gravity and refractive index

Specific gravity and refractive index of oils seeds were determined at 20°C following the ISO 6320:2000(F) method by using a refractometer (Mettler RE 50), respectively. Viscosity of oils seeds was determined at 25°C by using a viscometer tube Anton SVM 3000 (AFNOR, 2000).

Acid, peroxide, iodine, saponification and p-anisidine values

Acid, peroxide, iodine, saponification and p-anisidine values were determined following the AFNOR (2000) methods. pH value of oil samples was determined at 25°C according to Afane et al. (1997) by using a pH meter (Hanna, Hi 8915 ATC, Spain). 2 ml of each oil sample were dissolved in 15 ml of n-hexane. The pH-meter electrode was standardized with buffer solutions (pH 4.0 and 7.0) and then, immersed into the sample to record pH value. The determination of the p-anisidine number is based on the principle that in acetic acid medium, p-anisidine reacts with the conjugated aldehydes derived from lipid oxidation to form yellow compounds which absorb at 350 nm. We determined this index by the method described by Doleschall (2002). The p-anisidine index is a reliable indicator of lipid oxidative rancidity (Van Der Merwe, 2004).

Unsaponifiable matter, vitamin A and vitamin E

Unsaponifiable matter content of oil samples was determined following the IUPAC (1979) method. The vitamin A and E was determined by High Performance Liquid Chromatography (HPLC) according to ISO 14565:2000(F). The sample is saponified with ethanolic potassium hydroxide solution and vitamin A is extracted into petroleum ether. The petroleum ether is removed by evaporation and the residue dissolved in methanol. The vitamin A concentration of the methanol extract is determined by reverse phase liquid chromatography under conditions giving a single peak for all the isomers of vitamin A. The sample injection is at 325 nm for the vitamin A and 292 nm for vitamin E. The flow rate is set at 0.25 ml/min at 70°C.

Solid fat content (SFC)

The solids content was determined by nuclear magnetic resonance (ISO 8292: 1992 (F)). The solid content of a fatty phase at different temperatures is an important element for the knowledge of the rheological properties of fat. This method applies to fatty substances with pronounced polymorphisms. The preparation of test samples at specified temperatures makes it possible to measure magnetic decay signals emitted by liquid and solid fat protons by magnetic resonance with calculation and automatic display of the solid fat content.

Cholesterol content

Cholesterol content was determined by using the enzymatic colorimetric method (Meiattini, 1978; Naito, 1988). About 100 mg of unsaponifiable fraction was dissolved in 1 ml of hexane. Then, 10 µl of mixture was added to 1 ml of the enzymatic reagent and the whole mixture was allowed to stand for 10 min at room temperature. The absorbance was measured at 505 nm using a spectrophotometer (T80+, PG instruments, England) against a blank. A standard cholesterol solution was tested as reference following the same procedure.

Determination of fatty acid composition (FAC) by gaz

FAC of the oils samples was determined by GC analysis according to AFNOR ISO 5509 (2000). The fatty acids were converted to their methyl esters (FAMEs) as described by AFNOR (ISO 5509-2000). About 0.4 g of oil sample was mixed with 5 ml of isooctane and 5 ml of a methanolic solution of potassium hydroxide (2N). The whole mixture was shaken up for 30 s and allowed to settle for 5 min. The top layer containing the FAMEs was used for gas chromatography (GC) analysis. FAMEs solution (1 µl) containing the internal standard (erucic acid) was injected into a gas chromatograph (Perkin Elmer, Clarus 580) equipped with a flame ionization detector (FID) and a capillary column Rt-2560 biscyanopropyl polysiloxane (100 m × 0.25 mm i.d. × 0.2 µm). The carrier gas was hydrogen and the flow rate was adjusted to 1.2 ml/min. Temperatures of detector and injector were 260°C. The initial column temperature was fixed to 140°C and programmed to increase by 4°C per min intervals until 240°C and, kept for 15 min at this temperature. The fatty acid methyl esters peaks were identified by comparing their retention times with those of standards. After adjusting areas with the internal standard (erucic acid), the yield of each fatty acid was calculated as follow: area of the fatty acid/areas of total fatty acids in the oil sample × 100 (%).

Statistical analysis

In the experiment, each test for the sample was analyzed in triplicate. The data were expressed as means ± standard deviation (SD). Differences between means were analysed by analysis of variance (one way ANOVA) using GraphPad Prism 5(Analyst soft Inc) software. Statistical significance was measured at $p < 0.05$.

RESULTS

Oil yield

The oil content of *R. heudelotii* seeds (54.27 ± 1.73), *I. gabonensis* seeds (64.98 ± 0.03), *C. esculentus* (20.18 ±

Table 1. Extraction yield and physicochemical properties of *R. heudelotii, I. gabonensis, C. esculentus* and *C. colocynthis* seeds.

Parameter	Oilseed			
	RH	IG	CE	CC
Extraction yield	54.27±1.73[a]	64.98±0.00[b]	20.18±0.66[c]	51.70±0.00[d]
Specific gravity at 25°C	0.94±0.00[a]	0.85±0.00[a]	0.88±0.00[a]	0.88±0.00[a]
Refractive index at 25°C	1.49±0.00[a]	1.44±0.00[a]	1.46±0.00[a]	1.46±0.00[a]
Dynamic viscosity (mPa.s)	596.88±0.40[a]	502.20±0.23[b]	519.81±0.33[c]	517.85±0.01[c]
kinetic viscosity (cm^2/s)	632.85±0.08[a]	547.38±0.23[b]	554.99±0.33[c]	553.03±0.01[c]
pH	5.50±0.01[a]	5.71±0.00[a]	4.45±0.01[c]	5.22±0.00[a]
Unsaponifiable matter (%)	1.88±0.00[a]	1.26±0.00[a]	0.88±0.00[a]	0.77±0.00[a]
Cholesterol (mg/g)	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
SMP (Sleep Melting Point) (°C)	0.00±0.00[a]	38.13±0.08[b]	9.75±0.03[c]	0.00±0.00[a]
SFC (Solid Fat Contain) at 0°C	65.26±0.02[a]	70.42±0.24[b]	63.22±0.02[c]	61.22±0.02[d]
SFC (Solid Fat Contain) at 5°C	64.19±0.18[a]	57.72±0.35[b]	54.19±0.02[c]	54.35±0.02[d]
SFC (Solid Fat Contain) at 10°C	41.20±0.00[a]	38.12±0.02[b]	31.19±0.00[c]	29.50±0.00[d]
SFC (Solid Fat Contain) at 15°C	19.13±0.02[a]	35.15±0.08[b]	10.13±0.02[c]	12.64±0.01[d]
SFC (Solid Fat Contain) at 20°C	0.18±0.00[a]	34.67±0.04[b]	0.10±0.00[a]	0.20±0.00[a]
SFC (Solid Fat Contain) at 25°C	0.02±0.00[a]	30.41±0.23[b]	0.00±0.00[a]	0.00±0.00[a]
SFC (Solid Fat Contain) at 30°C	0.02±0.00[a]	25.25±0.03[b]	0.00±0.00[a]	0.00±0.00[a]
SFC (Solid Fat Contain) at 35°C	0.02±0.00[a]	17.92±0.09[b]	0.00±0.00[a]	0.00±0.00[a]
SFC (Solid Fat Contain) at 40°C	0.00±0.00[a]	10.58±0.04[b]	0.00±0.00[a]	0.00±0.00[a]

[a,b,c,d] Means in line with no common superscript differ significantly ($p < 0.05$).

0.66) and *C. colocynthis* (51.07 ±0.7). These four oilseeds can be classified in descending order of their oil content*: I. gabonensis*, *R. heudelotii, C. colocynthis* and *C. esculentus* (Table 1).

Physicochemicals characteristics

There was no significant difference ($p < 0.05$) between most of the physicochemical parameters of the four seed oils except for dynamic viscosity (59.75 ± 0.39 for *R. heudelotii*, 50.22 ± 0.23 for *I. gabonensis*, 52.02 ± 0.23 for *C. esculentus* and 51.79 ± 0.005 for *C. colocynthis* mPa.s), iodine values (142.12 ± 0.34 for *R. heudelotii*, 7.42 ± 0.29 for *I. gabonensis*, 75.46 ± 0.35 for *C. esculentus* and 120.45 ± 0.29 for *C. colocynthis* I_2/100 g), vitamin A (0.00 ± 0.00 for *R. heudelotii*, 7.17 ± 0.32 for *I. gabonensis*, 2.48 ± 0.07 for *C. esculentus* and 3.67 ± 0.04 for *C. colocynthis* µg/g), and p-anisidine value (301.17 ± 0.98 for *R. heudelotii*, 0.39 ± 0.00 for *I. gabonensis*, 24.47 ± 0.03 for *C. esculentus* and 29.09 ± 0.03 for C. colocynthis respectively. vitamin E (α-tocophérol 26.63 ± 0.44 and α-tocotriénol 94367.75 ±1.91 10^3 for *R. heudelotii*, α-tocophérol 00.00 ± 0.00 and α-tocotriénol 19625.75 ± 1.13 10^3 for *I. gabonensis*, α-tocophérol 71.67 ± 0.47 and α-tocotriénol 00.00 ± 0.00 for *C. esculentus*, α-tocophérol 00.00 ± 0.00 and α-tocotriénol 19625.153 ± 1.90 10^3 for *C. colocynthis* ppm), and p-anisidine value (301.17 ± 0.98 for *R. heudoletii*, 0.39 ± 0.00 for *I. gabonensis*, 24.47 ± 0.03 for *C. esculentus* and 29.09 ± 0.03 for *C. colocynthis* respectively (Table 1). The values of specific gravity were 0.86 ± 0.01 while those of refractive index were about 1.46 ± 0.00. Quality parameters such as free fatty acids (FFA) and peroxide value (PV) were closed to 1.5% and 85 meq O_2/kg, respectively. Oils extracted from *R. heudelotii, C. esculentus, C. colocynthis,* and *I. gabonensis* seeds were cholesterol free and their respective unsaponifiable matter contents were less than 1%. The saponification values of the four oilseeds were about 150 to 176 mg KOH/g (Table 1).

Solid fat content (SFC)

The different solid fat content obtained show a higher value for *I. gabonensis*. Indeed, this oil remains practically solid until 20°C. The oil of *R. heudelotii*, *C. esculentus* and *C. colocynthis* are practically fluid at room temperature (Figure 1). The saponification values of the two oilseeds were about 150 to 180 mg KOH/g (Table 2).

Characterization of FAC

Data presented in Table 3 show the FAC of the different oils analyzed. Total saturated fatty acid were in the range of 93.26% (IG) to 25.53% (CC). In these oils, the major saturated fatty acids were myristic (C14 :0) and lauric (C12 :0) in the oil of IG. Chromatographic profiles of the

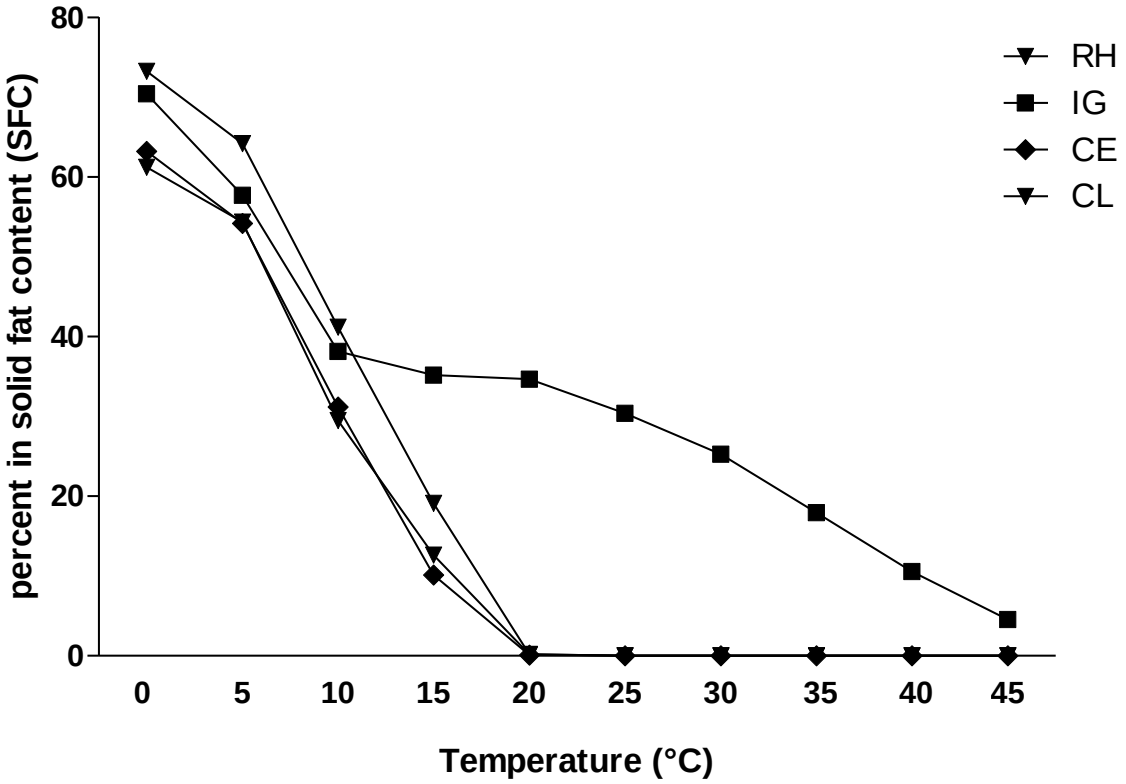

Figure 1. Evolution curve of the solid fat content (SFC) as a function of the temperature of the four extracted oil samples. Percent change in solid as a function of tempereature.

Table 2. Chemical parameters of four oils.

Parameter	Oilseed			
	RH	IG	CE	CC
Acidity ((%)	0.68±0.01[a]	14.92±0.13[b]	0.70±0.00[a]	6.95±0.01[d]
Acid value (mg KOH/g)	1.35±0.02[a]	39.05±0.34[b]	1.88±0.45[a]	13.85±0.02[d]
Saponification value (mg KOH/g)	154.67±1.82[a]	176.03±0.50[b]	175.47±0.30[c]	175.64±3.04[d]
Ester value	153.32±1.80[a]	136.98±0.84[b]	174.12±0.32[c]	161.79±3.05[d]
Iodine value (g I_2/100 g)	142.12±0.34[a]	7.42±0.29[b]	75.46±0.35[c]	120.45±0.29[d]
Peroxide value (meq O_2/kg)	37.98±2.03[a]	2.34±0.32[b]	86.16±1.06[c]	48.77±0.22[d]
Paraanisidin value	301.17±0.98[a]	0.39±0.00[b]	24.47±0.03[c]	29.04±0.03[d]
Totox	640.32±1.83[a]	3.13±0.32[b]	135.09±0.99[c]	106.86±0.21[d]
Vitamine A (µg/g)	nd	7.17±0.32[b]	2.48±0.07[a]	3.66±0.04[a]

Mean ± SD (n=3), significant differences in the same row are shown by different letter ($p < 0.05$).

fatty acid composition and their relative amounts in the different oilseeds are shown in Figures 2 and 3. Proportion of fatty acids from the oilseeds studied highlighted the presence of six main compounds namely palmitic, stearic, oleic, linoleic, linolenic and nervonic acids in the oils of *R. heudelotii*, *C. Colocynthis* and *C. esculentus*. On the other hand, in *I. gabonensis* oil, we find the presence of saturated fatty acid, namely lauric, myristic and palmitic acid. On average, these fatty acids were approximately 93.24, 96.29 and 97.35% of the total fatty acids in *R. heudelotii, C. esculentus*, and *C. colocynthis* respectively (Table 3). The four oilseeds were mainly composed of saturated fatty acids (SFA) (approximately 12 to 25% of total fatty acids) for *R. heudelotii, C. esculentus*, and *C. colocynthis* and unsaturated fatty acids (UFA) (approximately 75 to 80%).

Table 3. Fatty acids composition (% of methyl fatty acids).

Fatty acids	RH	IG	CE	CC
Lauric acid C12:0	0.42±0.00^{a}	33.42±0.14^{b}	0.08±0.01^{a}	0.06±0.00^{a}
Myristic acid C14:0	0.61±0.00^{a}	51.80±0.19^{b}	0.23±0.01^{a}	0.11±0.01^{a}
Palmitic acid C16:0	5.24±0.01^{a}	6.36±0.07^{b}	15.18±0.02^{c}	16.16±0.14^{d}
Stéaric acid C18:0	6.53±0.00^{a}	1.00±0.01^{b}	5.22±0.01^{c}	8.99±0.05^{d}
Arachidic acid C20:0	0.15±0.00^{a}	0.00±0.00^{a}	0.66±0.01^{a}	0.43±0.00^{a}
Behenic acid C22:0	0.10±0.00^{a}	0.67±0.02^{a}	0.12±0.01^{a}	0.09±0.00^{a}
Lignoceric acid C24:0	0.03±0.00^{a}	0.00±0.00^{a}	0.22±0.01^{a}	0.13±0.00^{a}
SFA	12.92±0.02^{a}	93.26±0.44^{b}	21.04±0.05^{c}	25.53±0.20^{d}
Palmitoleic acid C16:1	0.02±0.00^{a}	1.41±0.01^{b}	0.25±0.00^{a}	0.07±0.01^{a}
Oleic acid C18:1	9.24±0.00^{a}	2.87±0.07^{b}	66.01±0.06^{c}	19.10±0.12^{d}
Erucic acid C22:1	0.04±0.03^{a}	1.98±0.78^{b}	0.14±0.01^{a}	0.16±0.01^{a}
Nervonic acid C24:1	45.24±0.02^{a}	0.87±0.06^{b}	0.46±0.02^{b}	0.31±0.00^{b}
MUFA	54.55±0.06^{a}	7.12±0.92^{b}	66.86±0.09^{c}	19.64±0.13^{d}
Linoleic acid C18:2	26.61±0.02^{a}	1.61±0.25^{b}	9.94±0.01^{c}	53.10±0.68^{d}
Linolenic acid C18:3	0.40±0.00^{a}	0.91±0.00^{a}	0.04±0.02^{a}	0.07±0.03^{a}
Arachidic acid C20:4	0.01±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
PUFA	27.06±0.05^{a}	4.50±1.04^{b}	10.12±0.04^{c}	53.33±0.72^{d}
Total UFA	81.61±0.11^{a}	11.62±1.96^{b}	76.97±0.12^{c}	72.97±0.86^{d}

Mean ± SD (n=3), significant differences in the same row are shown by different letter ($p < 0.05$) SFA, Saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acids ; UFA, Unsaturated fatty acid.

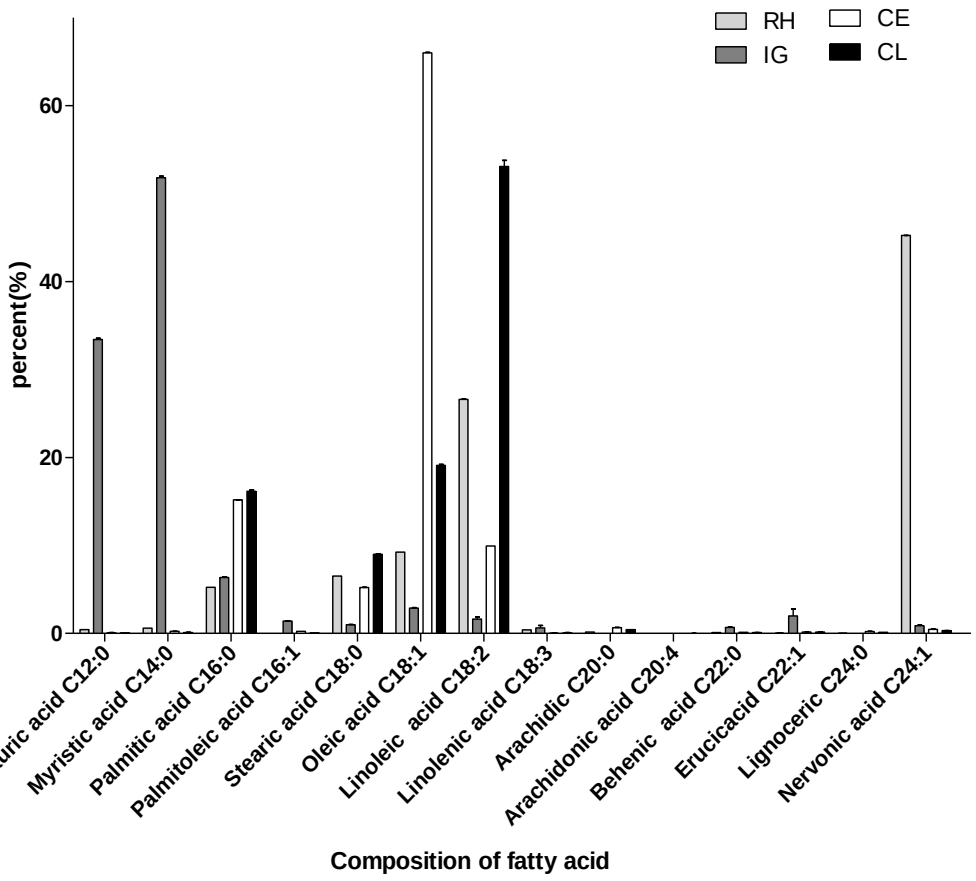

Figure 2. Fatty acids composition (% of methyl fatty acids).

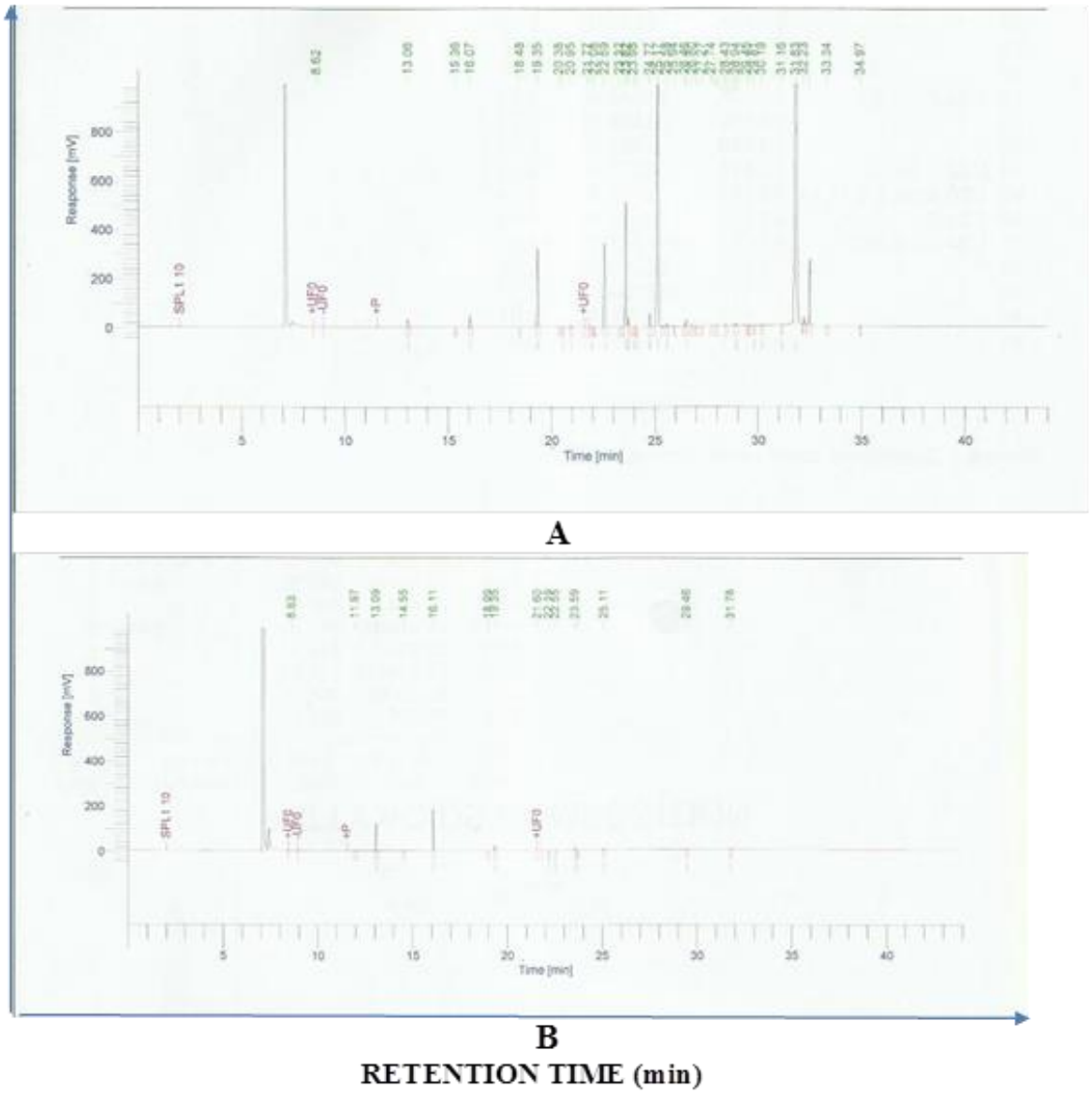

Figure 3. Gas chromatograms of fatty acids constituents of *R. heudelotii* (A) and *I. gabonensis* (B) oilseeds. Experiments were performed in triplicate by gas chromatographic analysis (GC-FID) of fatty acids methyl esters derivedfrom *R. heudelotii* and *I. gabonensis* oilseeds. Data represents mean ± SD.

The monounsaturated fatty acid (MUFA) consisted of oleic acid with a high content in *C. esculentus* oil of 66.01% and a rate of 19.10 in *C. colocynthis* (Table 3). These oilseeds were also composed of polyunsaturated fatty acids (PUFA) which was essentially linoleic acid with a value of 53.10% in *C. colocynthis* and 26.61% (Table 3). Nervonic acid accounts for the bulk of MUFA in *R. heudelotii* oil, 45.24%. The total unsaturated acid content in the four samples analyzed was significantly different. This content is higher in the *R. heudelotii* oil with a value of 81.61%, followed by *C. esculentus* is 76.97% and *C. colocynthis* with a content of 72.97%. These three oils generally remain fluid at room temperature. The density of the oils analyzed showed that the oil of *R. heudelotii* at the highest density is 0.94 g / ml. The oils of *I. gabonensis*, *C. esculentus* and *C. colocynthis* have similar densities respectively 0.85±0.00, 0.88±0.00 and 0.88±0.00 (Figure 4).

Analytical characteristics of tocopherols and tocotrienols

Data presented in Table 4 show the tocopherol and tocotienol contents (ppm) of the different oils analyzed. The range of total content of vitamin E was from 19695 to 110893 ppm and was higher than that found in some

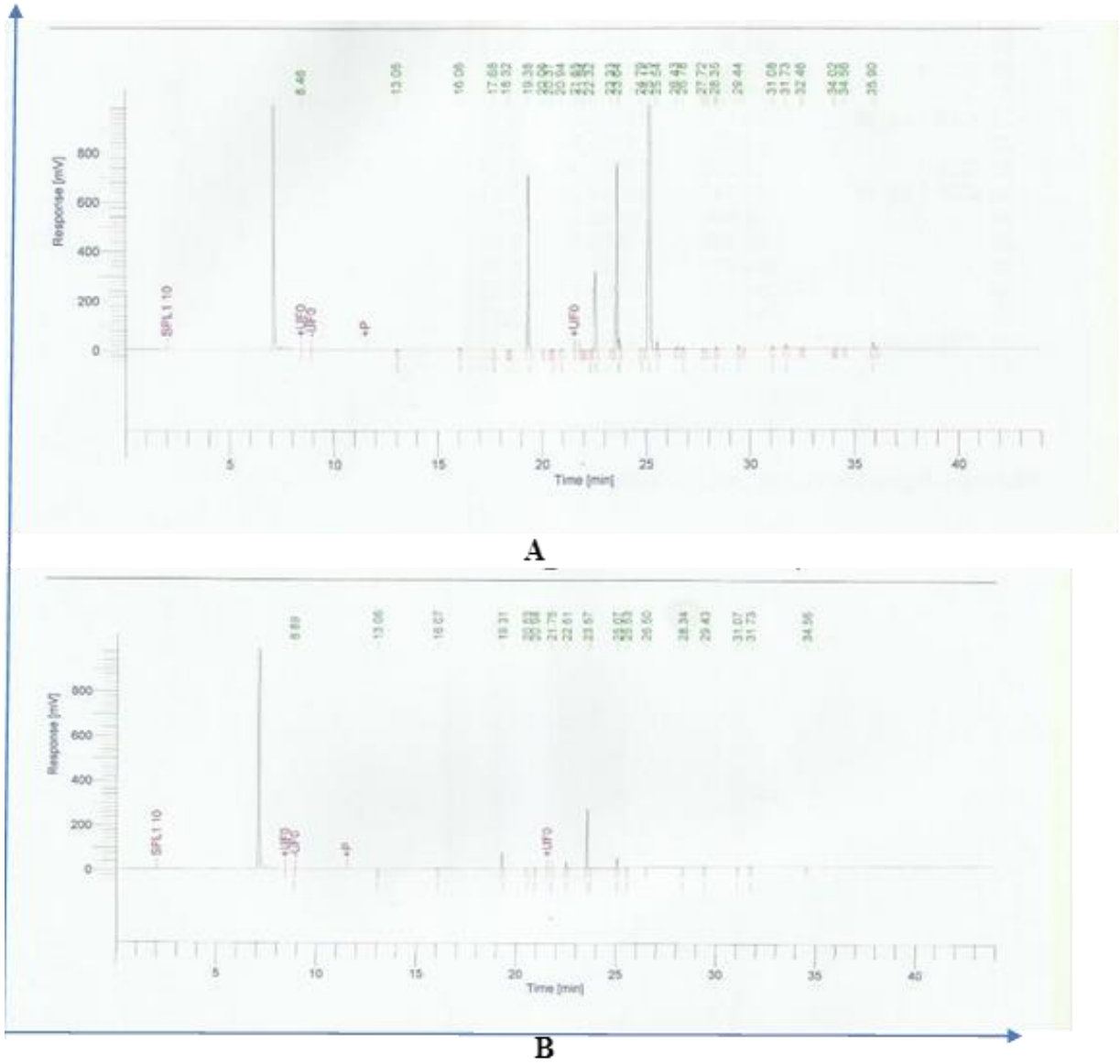

Figure 4. Gas chromatograms of fatty acids constituents of *C. colocynthis* (A) and *C. esculentus* (B) oilseeds. Experiments were performed in triplicate by gas chromatographic analysis (GC-FID) of fatty acids methyl esters derivedfrom *C. colocynthis* (A) and *C. esculentus* (B). Data represents mean ± SD.

conventional oils such as palm oils reported by Monde (2011). Tocopherols and tocotrienol are major antioxydants of vegetables oils, and the main ones of these oils. Of these four oils the richest vitamin E is that of *R. heudelotii* with a content of 110893,29 ppm. *I. gabonensis* oil is the poorest vitamin E oil with a value of 19674,72 ppm. These results are significantly higher than those found by Anhwange et al. (2010).

Vitamine A content

Figure 5 shows the different chrommatograms obtained during the analysis of vitamins A in the different oils. It turns out that vitamin A was not detected in the *R. heudelotii* oil. In addition, the oil rich in vitamin A is oil of *I. gabonensis*.

DISCUSSION

In regard to the oil content, *R. heudelotii, C. esculentus, I. gabonensis* and *C. colocynthis* seeds are lipidrich than most of the conventional oilseeds such as cotton (13%), soybean (14%) and palm fruit (20%) (Nzikou et al., 2011; Kapseu et al., 2005) and can be used as an alternative source of oil for lipid industries (Nzikou et al., 2009). The result shows also higher oil content compared to earlier

Table 4. Changes in the tocotrienol and tocopherol of differents oils studied (ppm).

Vitamin E	RH	IG	CE	CC
α-Tocopherol (ppm)	26.63±0.44[a]	0.00±0.00[b]	71.67±0.47[c]	0.00±0.00[a]
β-Tocopherol (ppm)	80.61±0.04[a]	12.01±2.30[b]	11.70±0.62[c]	32.37±1.64[d]
δ-Tocopherol (ppm)	286.03±1.14[a]	36.45±4.65[b]	0.00±0.00[c]	36.45±4.65[b]
ϒ-Tocopherol (ppm)	16.77±1.24[a]	1.11±0.10[b]	22881.91±1527.11[c]	1.14±0.09[b]
α-Tocotrienol (ppm)	94367.75±1911.54[a]	1962.15±1131.53[b]	0.00±0.00[c]	19625.15±1131.53[b]
β-Tocotrienol (ppm)	nd	nd	nd	nd
δ-Tocotrienol (ppm)	16115.51±85.93[a]	nd	nd	Nd
ϒ-Tocotrienol (ppm)	0.00±0.00[a]	nd	nd	Nd
Vitamin E (ppm)	110893.29±1827.36[a]	19674.72±1133.99[b]	22965.28±1526.14[c]	19695.11±1137.58[b]

nd, not detected. Mean ± SD (n=3), significant differences in the same row are shown by different letter ($p < 0.05$).

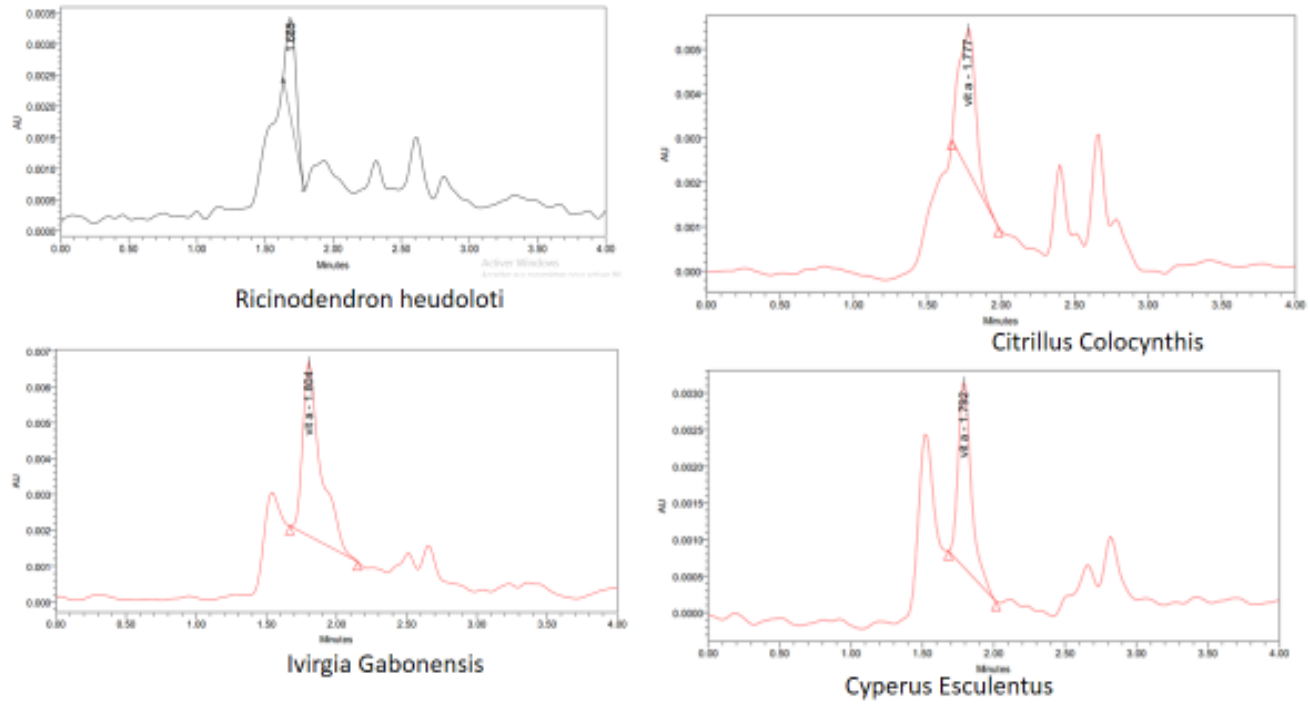

Figure 5. Chromatogram of vitamin A.

reports (El-Adawy and Khalil, 1994; Nzikou et al., 2011). The oil content of *R. heudolotii* seeds did not shows much variation compared to previous report (Ogunka-Nnoka and Bravil, 2013). However, this oil content was lower than that (42.2%) reported by Silou et al. (1999). These variations between oil yields in seeds could be attributed to their cultivation climate, ripening stage, harvesting time and the extraction method employed (Egbekun and Ehieze, 1997). The specific gravity of *C. esculentus, C. colocynthis* and *I. gabonensis* oilseeds are lower than those reported for most conventional oilseeds which are about 0.9 (Codex, 1993). Among the four oils analyzed alone, the specific gravity of *R. heudelotii* oil is close to the norm of the Codex Alimentarius, which are about 0.9. In addition, the viscosity dynamic values of both of the oilseeds were in the range (550 to 650 mPas) of most vegetable oils (Besbes et al., 2004). These results corroborate the fluid state of the studied oils at ambient temperature and this physical characteristic could be suitable for skin care products preparation (Reiger, 1989; Dhellot et al., 2006). Thirdly, these oils of *C. esculentus, R. heudelotti* and *C. colocynthis* oilseeds contain mostly polyunsaturated fatty acids, which easily undergo oxidation, raising peroxide values in these seeds. They are lower than those of *R. heudelotii* oils (19-114) (Aboubakar et al., 2000). Most of our values are lower than 15 m.equiv.g of O_2/kg of oil (the maximum level for cold pressed and virgin oils, Codex Alimentarius, 1999),

showing that these oils are good edible oils. The relatively high peroxide values of *C. esculentus, R. heudolotti and C. lanatus* oilseeds indicate that they are less liable to oxidative rancidity at ambient temperature (DeMan, 1992). Therefore, these oilseeds could be suitable in combination with antioxidants for cosmetic formulations (Judde, 2004). In addition, the studied oilseeds could be recommended for soap making and in the manufacture of lather shaving creams due to their relatively higher saponification values (Wolf, 1968; Eka, 1980). Also, the unsaponifiable matter contents of *R. heudelotii* and *I. gabonensis* oilseeds are higher than those reported for other potential cosmetic oils such as cotton seed oil (0.52%), peanut oil (0.33%) and palm kernel oil (0.22%) (Kapseu and Parmentier, 1997). This lipid fraction is a good source of stabilizers and provides essential moisture to skin (Helme, 1990). The refractive indexes of the three oilseeds *R. heudelotii*, *C. esculentus*, *C. colocynthis* are within the range of those reported for edible oils (Rossell, 1991). The study indicates that seed oils of both plants contain lower FFA and so, they can be recommended for salads seasoning and can be stored for longer period without deterioration (Anwar et al., 2007; Matos et al., 2009). The iodine values are approximately the same as those of other oils such as soybean (120 to 143 g I_2/100 g) and sunflower (110 to 143 g I 2/100 g) oils (Codex, 1993). However, these values are higher than those reported by Kapseu and Parmentier (1997) for other non-conventional oilseeds such as *Coula edulis* (90-95 gI_2/100 g), *Dacroydes edulis* (60 to 80 g I_2/100 g) and *Canarium schweinfurthii* (71.1 to 94.9 g I_2/100 g). In view of the results above, the studied oilseeds could be categorized as semi-drying oils which consist predominately in polyunsatured fatty acids (Anhwange et al., 2010). Therefore, these oils could be nutritionally beneficial to patients suffering from most of the lipid disorders (Njoku et al., 2001). PUFA amounts of *R. heudelotii* and *H. colocynthis* oilseeds are higher than those reported for most of the nonconventional oilseeds as sheabutter (6.9%), avocado (15.5%), *D. edulis* (25.2%) and *Canarium schweinfurthii* (28.8%) (Chalon et al., 2001). The higher content of total PUFA observed in the studied oilseeds may confer flexibility, fluidity and selective permeability to cellular membranes and may also be beneficial for reducing cardiovascular disease risk (Das, 2006). The linoleneic acid content of *R. heudelotii* and *C. colocynthis* oilseeds are higher than those reported for sea buckthorn seed oil (28.8%) and raspberry seed oil (29.1%) which are considered as new sources of linoleneic oils and could be used in human diet for antiinflammatory, anti-thrombotic, anti-hypertensive and antiarrhythmic actions (Kamal-Eldin, 2006; Nzikou et al., 2007).

Oilseed was higher than that (1.57%) reported by Nzikou et al. (2011) for the same specie. Due to their linoleic acid content which were less than 17%, the studied oils could be used in human diet to decrease plasma LDLcholesterol also called “bad cholesterol” (Winjendran and Hayes, 2004; Mhanhmad et al., 2011). As regards palmitic and stearic acids, which are the main saturated fatty acids of the two oilseeds, previous studies have shown that they are free from deleterious effect on plasma cholesterol (Khosala and Sundram, 1996; Hunter et al., 2000). In addition, they are often used in food industries to provide texture and softness to products (Dubois et al., 2007). Vitamin A contents of *I.gabonensis, C. esculentus* and *C. colocynthis* oilseeds were lower than that reported (1 mg/g) for palm oil (Codex Alimentarius, 1993). The consumption of these oilseeds could cover infants (0 to 6 months) needs, which are estimated at 0.375 mg per day for vitamin A (FAO, 2001). In addition, the studied oilseeds were cholesterol free and this property is advantageous for using these oils for human nutrition without fearing increase in plasma LDL-cholesterol which is positively correlated to cardiovascular diseases (Dubois et al., 2007; Maki et al., 2011).

The determination of the solids content was performed on all four oil samples. These results show that the SFC values decrease as a function of temperature with a variable dispersion according to the sample. In addition, the three oils *R. heudelotii*, *C. esculentus* and *C. colocynthis* remain fluid and free of solids from 20°C. This result is in agreement with some conventional oil such as rapeseed and sunflower oil (Fokou et al., 2004). The characteristics of a ready-to-use plastic grease depend both on the composition of the mixture and on the thermal and mechanical treatments it has undergone. Among all the parameters likely to influence the rheological parameters, the composition of the fatty phase is both the most important and the one on which it is easier to act. This qualitative and quantitative composition of the fatty phase, in effect primarily affects any temperature on the solid / liquid ratio. *I. gabonensis* oil which remains solid at temperatures above 20°C can be used as concrete fat and gives it the rheological property of being used in margarine and cosmetics.

The nervonic acid content was measured. *R. heudelotii* oil has a significant content of nervonic acid with more than 45%. Nervonic acid (cis-tetracos-15-enoic acid acid, 24:1) is a mono-unsaturated omega-9 fatty acid with very long chain length (24 carbon). Nervonic acid plays a role in the biosynthesis of neuronal myelin and is found in the sphingolipids of the white matter of the human brain (Orengo, 2014). In diseases involving demyelination, such as adrenoleukodystrophy (ALD) and multiple sclerosis (MS), there is a considerable reduction in the levels of nervonic acid in sphingolipids. Nervonic acid has been studied as a raw material in the pharmaceutical industry for the production of drugs used in the symptomatic treatment of MS and ALD, of which rho oil is a good source, like oil. Camelina and new prototypes of brassicaceae (vegetable cabbage, rapeseed, watercress, horseradish, black mustard) that produce seed oil

(possibly rapeseed oil or camelina) with a high enriched content of nervonic acid for human and animal targets related to health. In micronutritional counseling one could propose a supplementation based on oil of *R. heudelotii* (Orengo, 2014).

Conclusion

It could be concluded in view of the results of this investigation that *R. heudelotii, I. gabonensis, C. esculentus* and *C. colocynthis* seeds may be developed for oil production. The oilseeds of these tropical plants are predisposed to human consumption due to their low content in FFA and peroxide. Saponification values and physical properties of these oils make them suitable in cosmetic industries for skin care products as soaps. Oils extracted from *R. heudelotii* and *C. colocynthis* seeds are good source of EFA predominantly composed of linolenic acid. The fatty acids profile make them more nutritionally balanced than most of the conventional advisable oils. Linoleic and linolenic acid content confer to these oils a number of nutritional, cosmetic and dietetic properties.

In view of all these potentialities and qualities, *R. heudelotii, I. gabonensis, C. esculentus* and *C. colocynthis* seeds may be considered as new sources of non-conventional oils which could be use in pharmaceutical, cosmetic and food industries. This study could be improved by investigating the effects of the four oilseeds before using them as supplements in food, pharmaceutical and cosmetic industries.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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