African Journal of Biotechnology

Volume 15 Number 25, 22 June 2016

ISSN 1684-5315

Academic Journals

ABOUT AJB

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

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

Contact Us

Editorial Office: ajb@academicjournals.org

Help Desk: helpdesk@academicjournals.org

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

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

African Journal of Biotechnology

Table of Content: Volume 15 Number 25 22 June, 2016

ARTICLES

African Journal of Biotechnology

Table of Content: Volume 15 Number 25 22 June, 2016

ARTICLES

academicJournals

Vol. 15(25), pp. 1276-1284, 22 June, 2016
DOI: 10.5897/AJB2016.15350
Article Number: A455EDE59035
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Review

Electrospun fibers for wound healing and treatment of hyperglycemia: A review

Soares J. M. D.[1], Silva J. C.[2], Almeida J. R. G. S.[1], Quintans-Júnior L. J.[2] and de Oliveira H. P.[1]*

[1]Postgraduate Program in Natural Resources of the Semiarid, Federal University of Sao Francisco Valley, Petrolina, PE, Brazil.
[2]Department of Physiology, Federal University of Sergipe, São Cristóvão, SE, Brazil.

Received 20 March 2016, Accepted 3 June, 2016

The use of electrospun fibers in diabetic wound healing assays represents an extremely important topic for investigation. Electrospun fibers have been applied in the immobilization of drugs, allowing sustained and controlled release of active materials. This review aimed to summarize the state-of-art in the application of electrospun fibers on diabetes, hyperglycemic and diabetic ulcers treatment. Regarding the diabetes control and treatment, electrospinning technique contributes to application of wound healing (*in vitro* and *in vivo* experiments). The glycemic control is favored due to controlled release which has been sustained and improved. The data suggest that the development of encapsulated drugs in electrospun fibers has a promising application in the treatment of diabetes and related complications.

Key words: Electrospun fibers, nanofibers, electrospinning technique, anti-diabetic drugs, hypoglycemic drugs, wound healing.

INTRODUCTION

Diabetes mellitus is a metabolic disease characterized by insufficiency in insulin secretion, insulin action, or both (ADA, 2014) that results in hyperglycemia. The International Diabetes Federation (IDF) estimates that in 2040, 10% of adult population will be diabetics. Considering the South and Central America, this number increases to 65% (IDF, 2015).

Numerous pathogenic processes are consequence of diabetes, due to the several pathologies involving heart, blood vessels, eyes, kidneys and nerves (ADA, 2014; IDF, 2015; Chen et al., 2015). In addition, diabetic wound healing has been considered one of major complications of diabetes mellitus (Siersma et al, 2014) which results in peripheral neuropathy and/or large vessel disease complicated by trauma (Singh et al., 2005; Boulton, 2008).

The glycemic control is critically important for minimization in the impacts of diabetes and corresponding complications. Recent studies have demonstrated that new therapies can be used in the control of blood

*Corresponding author. Email: helinando.o@univasf.edu.br. Tel: +55(74)21027645.

glucose and the diabetic complications, such as wound healing (Schneider et al., 2009; Arnolds and Heise, 2007; Moura et al, 2014; Nishimura et al., 2012; ITA, 2015).

Electrospinning technique is an extensively studied technology for nanofiber production (FORMHALS, 1934) recently applied in biomedical issues (Yu et al., 2014; Ding et al., 2014) with focus in the development of methods for diabetes treatment and its complications, such as diabetic ulcers (Xie et al., 2013). The action of electrospun fibers is improved due to the unique properties such as high surface area, high loading and simultaneous delivery for diverse therapies and reasonable cost-effectiveness that provided expansion in the use of fibers for drug delivery (Hu et al., 2014). Natural and synthetic polymers can be used in the electrospinning, including soluble and spinnable polymers in water, biocompatible and biodegradable polymers (Agarwal et al., 2009).

Drug-loaded electrospun fibers have been considered as promising strategies to achieve bioactive wound dressings for chronic non-healing wounds (Schneider et al, 2009) and for controlled release in the gastrointestinal tract. The nanoscale multi-agent delivery system has been applied in the drug loading (Yang et al., 2014); thereby the present systematic review summarizes the current studies about the application of electrospun fibers in the treatment of diabetes, hyperglycemic and diabetic ulcers.

Previous work reveals the potential of application of optimized structures for drug-loaded electrospun fibers production (Araujo et al, 2016; da Costa et al, 2015). Corresponding association with natural products (such as *Morus nigra* (Almeida et al, 2011)) represent an important topic of research with the aim of developing new nanostructured systems for application as prototype of wound dressings for non-healing wounds.

DIABETES CONTROL

As consequence of preponderance of individuals with elevation in the plasma glucose that remains untreated and/or undiagnosed (Buysschaert et al, 2015), there is a global trend of increasing diabetes cases. Numerous studies have described new therapies related to the control of glucose, insulin levels and other diabetic disorders (Nacht et al., 2015; Yong et al., 2015; Yang et al., 2016; Du et al., 2016; Chen et al., 2016)

This development requires the use of *in vivo* or *in vitro* models. The diabetes induction in animal models is typically induced by two mechanisms *viz.* alloxan and streptozotocin (Lenzen, 2008). However, streptozotocin-induced diabetes is a more stable model for experimental diabetes and it can be used for both shorter and longer experimental studies (Radenković et al., 2016). Kuricová et al. (2016) recently demonstrated that is possible to simulate *in vitro* culture conditions mimicking human glycemic variability. The nanotechnology in diabetes research has potential to treat the diabetics complications and to improve the quality of life of the patients (Disanto et al., 2015). Studies have demonstrated that electrospun nanofibers can be used as glucose sensors due to the different mechanisms (Senthamizhan et al., 2016; Ramasamy et al., 2015). In addition, the incorporation of nanostructures promotes the enhanced insulin production in response to glucose stimulation, which enables the development of new diagnostic test for drug development (Blackstone et al, 2014).

PROCESS PARAMETERS FOR ELECTROSPINNING

Relative to electrospun fibers production, the control in the process parameters is a critical parameter in order to obtaining nanofibers with desired morphologies and diameters. The polymer molecular weight, solution concentration and solvent type also affect the diameters and morphologies of the fibers (Cui et al, 2007). According to Jang et al. (2015) pivotal parameters such as polymer concentration, molecular weight, input voltage and gap distance affects the production of nanofibers. Therefore, there was the broad range in the characteristic fiber diameters and fiber morphologies in different studies due to the use of different process parameters, polymers and solvents.

The definition of appropriate polymer represents a key component for development of sustained release electrospun fibers (Chou et al., 2015). Polymers used in these studies are all biocompatible and may be employed for applications involving the development of control drug delivery systems (Li et al., 2000; Zong et al., 2003, Yao et al., 2003; Li et al., 2003; Zhou et al., 2003) (Table 1).

LOADED AND CONTROLLED RELEASE OF DRUGS

The encapsulation efficiency contributes to improve the absorption and prolonged release of components (corresponding data is summarized on Table 1). In all reported data, there were enhancements on resulting release. Sustaining drug release from electrospun nanofibers shows regular release kinetics that depend upon material surface chemistry, drug loading, and processing parameters (Chou et al., 2015).

In these studies, the electrospun fibers show improved activity of the substances followed by controlled release of drugs and/or substances. The profile of *in vitro* assays shows sustained and controlled release of drugs, substances and natural product (Lee et al., 2015, 2014; He et al., 2014; Sharma et al., 2013; Merrell et al., 2009; Yang et al., 2011, 2012; Choi et al., 2008), while for different systems the drug release is order of hours (Sharma et al., 2013), days (He et al., 2014; Merrell et al., 2009; Choi et al., 2008) or weeks (Lee et al., 2015, 2014;

Table 1. Electrospun fibers: production and characterization.

Authors, year	Polymer	Solvent	Process parameters			Characterization			Mechanical properties		Encapsulation / entrapment efficiency
			Voltage applied to polymer solutions(kV)	Flow rate	Work distance (cm)	SEM	TEM	AFM	Tensile strength	Elongation at breakage	
Lee et al., 2014	PLGA	HFIP	17	3.6 mL/h	12	Yes	No	No	Yes	Yes	-
Lee et al., 2015	Collagen and PLGA	HFIP	17	3.6 ml/h	12	Yes	No	No	Yes	Yes	-
Merrell et al., 2009	PCL	Methanol and chloroform	25	2mL/h	10	Yes	No	No	No	No	-
Choi; Leong and Yoo, 2008	PCL and PCL	Methanol and chloroform	15	-	15	No	No	No	No	No	-
Yang et al., 2011	PELA	Chloroform	19	1.6 ml/h	-	Yes	No	No	No	No	79.2 ±14.8%
Yang et al., 2012	PELA	Chloroform	20	1.6 mL/h	-	Yes	No	No	No	No	87.7 ± 16.5 and 79.3 ± 11.6%
He et al., 2014	PGC and PCL	HFP	30	3 mL/h	25	Yes	No	No	No	No	-
Sharma et al. 2013	PVA and NaAlg	Deionized water	15	0.5 ml min^{-1}	15	Yes	No	Yes	Yes	No	99%

PLGA, Poly-D-L-lactide-glycolide; PCL, Poly(ε-Caprolactone; PEG, Poly(ethyleneglycol; PELA,Poly(ethylene glycol)-poly(DL-lactide; PGC,Protected Graft Copolymer; PVA,Poly(vinyl alcohol; NaAlg,sodium alginate; HFIP,1,1,1,3,3,3-hexafluoro-2-Propanol; HFP,1,1,1,3,3,3-Hexafluoro-2-propanol); SEM, Scanning Electron Microscopy; TEM – Transmission Electron Microscopy; AFM – Atomic Force Microscopy.

Yang et al., 2011, 2012).

WOUND HEALING ACTIVITY

The absence of reepithelialization requires the development of acute or chronic non-healing wounds, in which the use of electrospun nano-fibers accelerate wound healing rates in diabetics rats (Lee et al., 2015, 2014; Merrell et al., 2009; Yang et al., 2011, 2012; Choi et al., 2008) from sustained release of substances that increase the re-epithelialization and regeneration of the skin by various mechanisms which depend on the substance/drug, the polymer and the process parameters applied in the production of the fiber (Table 2).

On the other side, the metformin is used in the treatment of type 2 diabetes. It represents the first-line pharmacological therapy associated with low weight gain and a reduced amount of hypoglycemic occurrence (UKPDS, 1998). In spite of this application, no trials about the effect of metformin on wound healing in diabetic patients (Salazar et al., 2015) were performed. Studies have demonstrated that metformin acts in wound healing around oral implants (Inouye et al., 2014) and promotes nerve regeneration after sciatic nerve injuries in diabetes mellitus, providing its therapeutic values for peripheral nerve injury repair in diabetes mellitus (Ma et al., 2015).

The use of metformin-loaded electrospun fibers

Table 2. Glycemic control and healing diabetic wounds provided by electrospun fibers.

Authors, year, country	Substance / natural product	Animals	Concentration	Release of pharmaceutical	Design	Parameters assessed	Results and conclusions
Lee et al. 2014 China	Metformin	Sprague–Dawley rats	40 mg	*In vivo* and *In vitro* by HPLC	Wound repair assay	- Wound healing test - Histological examination	- The drug eluting membranes continuously released metformin for three weeks. The wound closure in group metformin-eluting PLGA membrane was visibly faster than those in groups - There were keratinocytes migrated and proliferated fastest without significant inflammatory response surrounding skin. Additionally, the groups treated with nanofibers exhibited more stratum corneum in the wound.
Lee et al. 2015 China	Glucophage	Sprague–Dawley rats	40 mg	*In vivo* and *In vitro* by HPLC	Wound healing assay	- Wound healing test - Histological examination	- In vitro release the glucophage-loaded membranes continuously released drug for three weeks. On all days of evaluation wound closure in collagen/PLGA with glucophage-loaded group had proceeded visibly faster. - The nanofibrous scaffolds demonstrated highly integration into the surrounding skin without any significant inflammatory response and the use of collagen in the nanofibers increased the collagen content compared the others and demonstrated complete epithelialization with the thinning of epidermis at the wound region
Merrell et al. 2009 USA, China	Curcumin	Male C57/B6 mice	3 and 17%wt/wt	*In vitro* by cell culture media using fluorometer	Wound healing assay	- *In vitro* anti-Inflammatory property - *In vivo* diabetic mouse wound closure	- The fibers with a higher content of curcumin released an amount of significantly higher after 12 h, and so three days later - Curcumin loaded PCL nanofibers showed reduction pro-inflammatory response in mouse peritoneal macrophages *in vitro* and enhanced the rate of wound closure *in vivo*
Choi; Leong and Yoo 2008 Republic of Korea and USA	rhEGF	Female C57BL/6 mice	0.0045 nmol/mg of rhEGF to the amine groups	---	Wound healing assay	- Degree wound healing animal models - Immunohistochemical staining	- The rhEGF nanofiber accelerated wound healing rates at the initial stage of the healing process. This accelerated wound healing rates at the initial stage of the healing process because of the degradation and inactivation of EGF on the nanofiber and EGF was required only at the initial state of the wound healing process - In vitro release the bFGF/PELA fibrous mats

Table 2. Contd.

Yang et al. 2011 China	bFGF	Male SD rats	12.5 mg	*In vitro* by immersion in solution using the method of ELISA assay	Skin regeneration assay	- Macroscopic evaluation of wound healing - Histological and immunohistochemical evaluation	occurred for 25 days. The burst release was detected during initial 12 h, followed with a constant fast release for 15 days, and then a slow release for another 10 days. - The regenerated skin of bFGF/PELA group was similar with normal skin, enhances the collagen expression, had no inflammatory cell remaining and showed similar structure with normal skin with hair growth and the shriveling of the epithelium
Yang et al. 2012 China	pbFGF and PEI	Male SD rats	-	*In vitro* by immersion in solution using the detection with Hoechst 33258 dye	Wound healing assay	- Evaluation of Wound Healing Process	- The release profile occurred in two phases: an initial burst release followed by a sustained phase for 4 weeks. - The wounds were almost closed after 3 week treatment with polyplex-infiltrated in fibers content PEG with molecular weights of 2 kDa and at week 4 the epidermic cells were fully differentiated, basal cells were closely arranged, and the horny layer and large amounts of hair, sebum could be clearly observed and the histological sections showed similar structure with normal skin 4 weeks after operation
He et al., 2014 China and USA	Pioglitazone	C57BL/6 mice	12 mg/ml of pioglitazone by polymer solution	*In vitro* kinetics of drug release was measured by a HPLC	Drug-eluting scaffold	- The insulin production by transplanted islets - Blood glucose concentrations;	- The *in vitro* kinetic study showed that pioglitazone saw a sustained release from DES for up to 4 days. - The stimulation of scaffolds with pioglitazone increase the secretion of insulin and thus yielded an accelerated elimination of hyperglycemia compared to non-stimulated subjects and the glucose level in the scaffold pioglitazone group returned to physiological level by 60 min whereas the glucose level in the control group remained abnormally high - The *in vitro* release of insulin on the nanofibers showed the sustained and controlled release profile. The drug was released as primarily diffusion based for the initial period of 2 to 3 h beyond which it is based on the erosion of the polymer until the10th hour when the maximum amount of drug is released

Table 2. Contd.

Sharma et al. 2013 India	Insulin	Male Wistar rats	-	*In vitro* by immersion in solution using the spectrophotometer	Delivery anti-diabetic drug assay	- *In vitro* release - *In vivo* evaluation of the drug loaded	- The insulin loaded in a PVA–NaAlg nanofiber had a significant decrease in the blood glucose level and the effect lasted for an average period of 10 h

HPLC, High-performance liquid chromatography; bFGF, basic fibroblast growth factor; rhEGF, recombinant human epidermal growth factor; pbFGF, polyplexes of basic fibroblast growth factor-encoding plasmid; PEI, poly(ethylene imine.

2015, 2014). Furthermore, the use of collagen associated with metformin in membranes fibrous increases the content of collagen I, suggesting that nanofibrous containing metformin and collagen can be effective tissue-engineering scaffold for regenerating skin around diabetic wounds (Lee et al., 2015).

Curcumin possesses modulating effects on wound healing (AKBIK et al., 2014). The effect of its incorporation in the nanofibers returned wound closure in order of 80% along 10 days demonstrating the potential of curcumin-loaded nanofibers in the elevation of wound closure rate applied in a diabetic mouse (Merrell et al., 2009). Studies performed with curcumin in its raw state returned no significant response of material (Kant et al, 2015, 2014).

These studies show that the curcumin can be a new agent for accelerating diabetic wound healing in humans and animals since they show potential in the treatment of cutaneous wounds with corresponding improvement in cutaneous wound healing in acute phase diabetic rats (Kant et al., 2015) and still the topical application in the acceleration of cutaneous wound healing, with corresponding decrease in the persistence of the inflammatory state in acute diabetic rats (Kant et al., 2014).

Recent study reported that encapsulation of curcumin circumvents conventional difficulties related with curcumin administration, enabling delivery of this therapeutic substance and allowing its use as a novel topical agent for burn wound infection and cutaneous injuries (Krausz et al., 2015). Thus, studies with the encapsulation of curcumin in fibers have a promising future for the reason that electrospun fibers can improve the delivery of the therapeutic substance.

The inflammatory response, formation of granulation tissue, reepithelialization, matrix formation and wound closure are provided and regulated by an equally complex signaling network involving numerous growth factors, cytokines and chemokines (Barrientos et al., 2008). Growth factors are important substances that have the ability to modulate cell behavior and guide tissue repair and renewal (Mitchell et al, 2016). Currently, the release of growth factors has great potential for development of new therapies which favors the healing and skin regeneration (Gainza et al., 2015). Articles suggest that electrospinning process can produce materials containing growth factors that allows the creation of multi-functionalized bioactive nanomaterials to chronic non-healing wounds and that can serve as skin tissue engineering scaffolds for wound healing (Schneider et al, 2009; Bertoncelj et al, 2014; Wang et al, 2016).

The growth factors, such as basic fibroblast growth factor and recombinant human epidermal growth factor, immobilized or incorporated have healing activity in diabetic animals (Choi et al., 2008; Yang et al., 2011, 2012, Sun et al., 2014). Recombinant human epidermal growth factor (rhEGF) immobilized on the electrospun nanofibers for treating diabetic ulcers in female mice demonstrated enhanced keratinocytic expression of human primary keratinocytes in comparison to rhEGF in solution, accelerating wound healing rates at the initial stage of the healing process (Choi et al., 2008). Basic fibroblast growth factor (bFGF) incorporated into electrospun ultrafine fibers indicated that bFGF-loaded fibrous mats (*in vitro* investigations) enhances cell adhesion, proliferation, and secretion of extracellular matrix and enhanced collagen deposition and ECM remodeling. The arrangement and components of collagen fibers were similar to normal tissues (Yang et al. 2011). Polyplexes of basic fibroblast growth factor-encoding plasmid (pbFGF) with poly(ethylene imine) incorporates into electrospun fibers showed a sustained release of 4 weeks and demonstrated significant higher wound recovery rate with collagen deposition and maturation, complete reepithelialization and skin appendage regeneration (Yang et al. 2012).

Hyperglycemia control

The hyperglycemic is other problem related with the diabetes and all of its complications. The use of electrospun nanofibers allows a sustained and controlled release profile of drugs that can reduce blood glucose. Insulin is a hormone synthesized in the pancreas which plays a critical role in intermediary metabolism. It is the key hormone for regulation of blood glucose and, generally, normoglycaemia is maintained by the balanced interplay between insulin action and insulin secretion (Stumvoll et al., 2005). Recent trials have demonstrated progress in technologies for insulin delivery *in vivo* and/or *in vitro* (Zhang et al., 2011; Nishimura et al., 2012; Ling and Chen, 2013; Balducci et al., 2014). The poor oral bioavailability and invasive drug delivery methods represent important barriers to be overcome in this topic.

To our knowledge, there are no studies about the encapsulation of insulin using electrospun fibers. A unique study reports that it is possible to encapsulate insulin on nanofibers. The sublingual route based on composite nanofiber diffusion along transmucosal patch can delivers the insulin in a sustained and controlled manner by controlling the morphology of the composite nanofiber patch (Sharma et al., 2013). This study also shows that the insulin cannot be easily released from the composite nanofiber patch due to the crosslinking between the drug molecule and polymeric chains unless polymeric nanofibers are degraded.

He et al. (2014) reported that scaffold with pioglitazone was able to locally deliver the drug to transplanted islets; hence, there was increased insulin secretion and a lower blood glucose level in diabetic mice. Furthermore, the decrease of hyperglycemic also aided in the recovery of renal functions compromised by the diabetes.

Thus, the encapsulation by electrospun fibers allows for better bioavailability of drugs, facilitates its sustained action and act as an adjuvant for recovery of complications caused by diabetes. In this direction, it is clear that studies are required in order to apply this technology and ensure its applicability *in vivo*.

The development of long-acting drug formulations has been explored in many applications in order to overcome challenges with adherence and emerging drug resistance (Chou et al., 2015) with the use of electrospun fibers, which has been considered a promising source for the treatment of diabetes and its complications, such as diabetic wounds.

CONCLUSIONS AND FUTURE PERSPECTIVES

The recent studies summarized in this article have showed advances in the treatment of diabetes and opens a discussion to expand the understanding of the use of electrospun fibers for treatment of chronic diseases associated with high cost to public health and quality of life of patients. We have highlighted in this review the future trends for treatment of hyperglycemic and diabetic wound healing diabetics relates with use of electrospun fibers. The electrospinning is a promising technique with increasing interest in health area. The electrospun nanofiber has ability to load or immobilized drugs/ substances, promotes a controlled and sustained release of the drug or substance. Besides that, the nanofibers can be explored for less vulnerable drugs to the attack by enzymes, providing improved stability. In diabetes, electrospinning technique has shown excellent results in relation to wound healing in *in vitro* and *in vivo* experiments, due to fast decrease in the area of the wound associated with better healing. In spite of few trials about the action of electrospun fibers in glycemic control, there are strong indications that *in vitro* and *in vivo* release has been improved and sustained. Further studies are needed to prove the effectiveness and efficiency of drug encapsulated and the associated risks and side effects of this encapsulation.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENT

This work was supported by Brazilian Funding Agency FACEPE.

REFERENCES

Agarwal S, Greiner A, Wendorff JH (2009). Electrospinning of Manmade and Biopolymer Nanofibers-Progress in Techniques, Materials, and Applications. Adv. Funct. Mater. 19:2863-2879.

Akbik D, Ghadiri M, Chrzanowski W, Rohanizadeh R (2014). Curcumin as a wound healing agent. Life Sci. 116(1):1-7.

Almeida JRGS, Guimarães AL, Oliveira AP, Araujo, ECC, Silva FS, Neves LF, Oliveira RA, Sa PGS, Quintans-Junior LJ (2011). Evaluation of hypoglycemic potential and pre-clinical toxicology of *Morus nigra* L. (Moraceae). Acta Farmaceutica Bonaerense, 30:96-100.

ADA (American Diabetes Association) (2014). Diagnosis and classification of diabetes mellitus. Diabetes Care 37(1):81-90.

Araujo ES, Nascimento MLF, de Oliveira HP (2016) Electrospinning of Polymeric Fibres: An Unconventional View on the Influence of Surface Tension on Fibre Diameter. Fib. Text. East. Eur. 24: 22-29.

Arnolds S, Heise T (2007). Inhaled insulin. Best Pract. Res. Clin. Endocrinol. Metab. 21(4):555-571.

Balducci AG, Cagnani S, Sonvico F, Rossi A, Barata P, Colombo G, Colombo P, Buttini F (2014). Pure insulin highly respirable powders for inhalation. Eur. J. Pharm. Sci. 51:110-117.

Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M (2008). Growth factors and cytokines in wound healing. Wound Repair Regen. 16(5):585-601.

Blackstone BN, Palmer AF, Rilo HR, Powell HM (2014). Scaffold architecture controls insulinoma clustering, viability, and insulin production. Tissue Eng. Part A. 20(13-14):1784-1793.

Bertoncelj V, Pelipenko J, Kristl J, Jeras M, Cukjati M, Kocbek P (2014). Development and bioevaluation of nanofibers with blood- derived growth factors for dermal wound healing. Euro. J. Pharm. Biopharm. 88(1):64-74.

Boulton AJ (2008). The diabetic foot: grand overview, epidemiology and pathogenesis. Diabetes Metab. Res. Rev. 24(1):3-6.
Buysschaert M, Medina JL, Bergman M, Shah A, Lonier J (2015). Prediabetes and associated disorders. Endocrine 48:371-393.
Chen H, Jia P, Kang H, Zhang H, Liu Y, Yang P, Yan Y, Zuo G, Guo L, Jiang M, Qi J, Liu Y, Cui W, Santos HA, Deng L (2016). Upregulating Hif-1α by Hydrogel Nanofibrous Scaffolds for Rapidly Recruiting Angiogenesis Relative Cells in Diabetic Wound. Adv. Healthc. Mater. 5(8):907-918.
Chen Y, Sloan FA, Yashkin AP (2015). Adherence to diabetes guidelines for screening, physical activity and medication and onset of complications and death. J. Diabetes Complications 29(8):1228-1233.
Choi JS, Leong KW, Yoo HS (2008). In vivo wound healing of diabetic ulcers using electrospun nanofibers immobilized with human epidermal growth factor (EGF). Biomaterials. 29(5):587-596.
Chou SF, Carson D, Woodrow KA (2015). Current strategies for sustaining drug release from electrospun nanofibers. J. Control. Release 220:584-591.
Cui W, Li X, Zhou S, Weng J (2007). Investigation on Process Parameters of Electrospinning System through Orthogonal Experimental Design. J. Appl. Polym. Sci. 103(1):3105-3112.
da Costa FPC, Araujo ES, NASCIMENTO MLF, de Oliveira HP (2015) Electrospun Fibers of Enteric Polymer for Controlled Drug Delivery. Int. J. Polym. Sci. Article ID 902365:1-8.
Ding F, Deng H, Du Y, Shi X, Wang Q (2014). Emerging chitin and chitosan nanofibrous materials for biomedical applications. Nanoscale 6(16):9477-9493.
DiSanto RM, Subramanian V, Gu Z (2015). Recent advances in nanotechnology for diabetes treatment. Wiley Interdiscip. Rev. Nanomed Nanobiotechnol. 7(4):548-564.
Du Y, Zhang W, Wang ML (2016). Sensing of salivary glucose using nano-structured biosensors. Biosensors 6(1): 1-14.
Formhals A. US patent 1,975,504, 1934.
Gainza G, Villullas S, Pedraz JL, Hernandez RM, Igartua M (2015). Advances in drug delivery systems (DDSs) to release growth factors for wound healing and skin regeneration. Nanomedicine. 11(6):1551-1573.
He Q, Zhang X, Han B, Xu J, Tang K, Fu Z, Yin H (2014). A synergistic therapeutic scheme for hyperglycemia and nephrotic disorders in diabetes. Theranostics. 4(5):556-564.
Hu X, Liu S, Zhou G, Huang Y, Xie Z, Jing X (2014). Electrospinning of polymeric nanofibers for drug delivery applications. J. Control. Release 185:12-21.
Inouye KA, Bisch FC, Elsalanty ME, Zakhary I, Khashaba RM, Borke JL (2014). Effect of metformin on periimplant wound healing in a rat model of type 2 diabetes. Implant Dent. 23(3):319-327.
International Diabetes Federation IDF (2015). IDF Atlas, 7thed. Brussels, Belgium: International Diabetes Federation.
Ita K (2015). Transdermal delivery of drugs with microneedles: Strategies and Outcomes. J. Drug Deliv. Sci. Technol. 29:16-23.
Jang S, Kim Y, Oh JH (2015). Influence of Processing Conditions and Material Properties on Electrohydrodynamic Direct Patterning of a Polymer Solution. J. Electron. Mater. 45(4):1-8.
Kant V, Gopal A, Kumar D, Pathak NN, Ram M, Jangir BL, Tandan SK, Kumar D (2015). Curcumin-induced angiogenesis hastens wound healing in diabetic rats. J. Surg. Res. 193(2): 978-988.
Kant V, Gopal A, Pathak NN, Kumar P, Tandan SK, Kumar D (2014). Antioxidant and anti-inflammatory potential of curcumin accelerated the cutaneous wound healing in streptozotocin-induced diabetic rats. Int. Immunopharmacol. 20(2):322-330.
Krausz AE, Adler BL, Cabral V, Navati M, Doerner J, Charafeddine RA, Chandra D, Liang H, Gunther L, Clendaniel A, Friedman JM, Nosanchuk JD, Friedman AJ (2015). Curcumin-encapsulated nanoparticles as innovative antimicrobial and wound healing agente. Nanomedicine. 11(1):195-206.
Kuricová K, Pácal L, Šoupal S, Prázný M, Kaňková K (2016). Effect of glucose variability on pathways associated with glucotoxicity in diabetes: Evaluation of a novel in vitro experimental approach. Diabetes Res. Clin. Pract. 114:1-8.
Lee CH, Chang SH, Chen WJ, Hung KC, Lin YH, Liu SJ, Hsieh MJ, Pang JH, Juang JH (2015). Augmentation of diabetic wound healing and enhancement of collagen content using nanofibrous glucophage-loaded collagen/PLGA scaffold membranes. J. Colloid. Interface Sci. 439:88-97.
Lee CH, Hsieh MJ, Chang SH, Lin YH, Liu SJ, Lin TY, Hung KC, Pang JH, Juang JH (2014). Enhancement of diabetic wound repair using biodegradable nanofibrous metformin-eluting membranes*: in vitro* and *in vivo*. ACS Appl. Mater. Interfaces 6(6):3979-3986.
Lenzen S (2008). The mechanisms of alloxan- and streptozotocin-induced diabetes. Diabetologia. 51(2):216-226.
Li WJ, Danielson KG, Alexander PG, Tuan RS (2003). Biological response of chondrocytes cultured in three-dimensional nanofibrous poly(epsilon-caprolactone) scaffolds. J. Biomed. Mater. Res. A. 67(4):1105-1114.
Li X, Deng X, Yuan M, Xiong C, Huang Z, Zhang Y, Jia W (2000). In Vitro Degradation and Release Profiles of Poly-DLLactide-Poly(ethylene glycol) Microspheres with Entrapped Proteins. J. Appl. Polym. Sci. 78:140-148.
Ling MH, Chen MC (2013). Dissolving polymer microneedle patches for rapid and efficient transdermal delivery of insulin to diabetic rats. Acta Biomater. 9(11):8952-8961.
Ma J, Liu J, Yu H, Chen Y, Wang Q, Xiang L (2015). Beneficial Effect of Metformin on Nerve Regeneration and Functional Recovery After Sciatic Nerve Crush Injury in Diabetic Rats. Neurochem. Res. 41(5):1130-1137.
Merrell JG, McLaughlin SW, Tie L, Laurencin CT, Chen AF, Nair LS (2009). Curcumin Loaded Poly(ε-Caprolactone) Nanofibers: Diabetic Wound Dressing with Antioxidant and Anti-inflammatory Properties. Clin. Exp. Pharmacol. Physiol. 36(12):1149-1156.
Mitchell AC, Briquez PS, Hubbell JA, Cochran JR (2016). Engineering growth factors for regenerative medicine applications.
Moura LI, Dias AM, Suesca E, Casadiegos S, Leal EC, Fontanilla MR, Carvalho L, de Sousa HC, Carvalho E (2014). Neurotensin-loaded collagen dressings reduce inflammation and improve wound healing in diabetic mice. Biochim. Biophys. Acta. 1842(1):32-43.
Nacht B, Larndorfer C, Sax S, Borisov SM, Hajnsek M, Sinner F, List-Kratochvil EJW, Klimant I (2015). Integrated catheter system for continuous glucose measurement and simultaneous insulin infusion. Biosen. Bioelectron. 64:102-110.
Nishimura A, Hayakawa T, Yamamoto Y, Hamori M, Tabata K, Seto K, Shibata N (2012). Controlled release of insulin from self-assembling nanofiber hydrogel, PuraMatrix™: Application for the subcutaneous injection in rats. Eur. J. Pharm. Sci. 45(1-2):1-7.
Radenković M, Stojanović M, Prostran M (2016). Experimental diabetes induced by alloxan and streptozotocin: The current state of the art. J. Pharmacol. Toxicol. Methods 78:13-31.
Ramasamy R, Ramachandran K, Philip GG, Ramachandran R, Therese HA, Kumar GG (2015). Design and development of Co3O4/NiO composite nanofibers for the application of highly sensitive and selective non-enzymatic glucose sensors. RSC ADVANCES 5(93): 76538-76547
Salazar JJ, Ennis WJ, Koh TJ (2015). Diabetes Medications: Impact on Inflammation and Wound Healing. J. Diabetes Complications (15): 1056-8727.
Schneider A, Wang XY, Kaplan DL, Garlick JA, Egles C (2009). Biofunctionalized electrospun silk mats as a topical bioactive dressing for accelerated wound healing. Acta Biomater. 5(7):2570-2578.
Sharma A, Gupta A, Rath G, Goyal A, Mathura RB, Dhakate SR (2013). Electrospun composite nanofiber-based transmucosal patch for anti-diabetic drug delivery. J. Mater. Chem. B 1:3410-3418.
Siersma V, Thorsen H, Holstein PE, Kars M, Apelqvist J, Jude EB, Piaggesi A, Bakker K, Edmonds M, Jirkovská A, Mauricio D, Ragnarson Tennvall G, Reike H, Spraul M, Uccioli L, Urbancic V, van Acker K, van Baal J, Schaper NC (2014). Health-Related Quality of Life Predicts Major Amputation and Death, but Not Healing, in People With Diabetes Presenting With Foot Ulcers: The EurodialeStudy. Diabetes Care 37(3):694-700.
Singh N, Armstrong DG, Lipsky BA (2005). Preventing Foot Ulcers in Patients With Diabetes. J. Am. Med. Assoc. 293(2):217-28.
Senthamizhan A, Balusamy B, Uyar T (2016). Glucose sensors based on electrospun nanofibers: a review. Anal. Bioanal. Chem. 408:1285-1306.
Stumvoll M, Goldstein BJ, van Haeften TW (2005). Type 2 diabetes:

principles of pathogenesis and therapy. Lancet. 365(9467):1333-1346.
Sun X, Cheng L, Zhao J, Jin R, Sun B, Shi Y, Zhang Lu, Zhang Y, Cui W (2014). bFGF-grafted electrospun fibrous scaffolds via poly(dopamine) for skin wound healing. J. Mater. Chem. B. 2:3636-3645.
UK Prospective Diabetes Study UKPDS. (1998). Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). Lancet. 352(9131):854-865.
Wang Z, Qian Y, Li L, Pan L, Njunge LW, Dong L, Yang L (2016). Evaluation of emulsion electrospun polycaprolactone/hyaluronan/ epidermal growth factor nanofibrous scaffolds for wound healing. J. Biomater. Appl. 30(6):686-698.
Xie Z, Paras CB, Weng H, Punnakitikashem P, Su LC, Vu K, Tang L, Yang J, Nguyen KT (2013). Dual growth factor releasing multi-functional nanofibers for wound Healing. Acta biomater. 9(12):9351-9359.
Yang G, Wang J, Li L, Ding S, Zhou S (2014). Electrospun Micelles/Drug-Loaded Nanofibers for Time-Programmed Multi-Agent Release. Macromol. Biosci. 14(7):965-976.
Yang Y, Xia T, Chen F, Wei W, Liu C, He S, Li X (2012). Electrospun Fibers with Plasmid bFGF Polyplex Loadings Promote Skin Wound Healing in Diabetic Rats. Mol. Pharm. 9(1):48-58.
Yang Y, Xia T, Zhi W, Wei L, Weng J, Zhang C, Li X (2011). Promotion of skin regeneration in diabetic rats by electrospun core-sheath fibers loaded with basic fibroblast growth factor. Biomaterials. 32(18):4243-4254.
Yang J, Tang S, Cheke RA (2016). The regulatory system for diabetes mellitus: Modeling rates of glucose infusions and insulin injections Commun Nonlinear Sci Numer Simul. 37:305-325.
Yao L, Haas TW, Guiseppi-Elie A, Bowlin GL, Simpson DG, Wnek GE (2003). Electrospinning and Stabilization of Fully Hydrolyzed Poly(Vinyl Alcohol) Fibers. Chem. Mater. 15(9):860-1864.
Yong C, Wang Z, Zhang X, Shi X, Ni Z, Fu H, Ding G, Fu Z, Yin H (2014). The therapeutic effect of monocyte chemoattractant protein-1 delivered by an electrospun scaffold for hyperglycemia and nephrotic disorders. Int J Nanomedicine. 17(9):985-993.
Yu H, Jia Y, Yao C, Lu Y (2014). PCL/PEG core/sheath fibers with controlled drug release rate fabricated on the basis of a novel combined technique. Int. J. Pharm. 469(1):17-22.
Zhang Y, Wei W, Lv P, Wang L, Ma G (2011). Preparation and evaluation of alginate-chitosan microspheres for oral delivery of insulin. Eur. J. Pharm. Biopharm. 77(1):11-19.
Zhou S, Deng X, Yang H (2003). Biodegradable poly(ε-caprolactone)-poly(ethylene glycol) block copolymers: characterization and their use as drug carriers for a controlled delivery system. Biomaterials 24(20):3563-3570.
Zong X, Ran S, Kim KS, Fang D, Hsiao BS, Chu B (2003). Structure and Morphology Changes during in Vitro Degradation of Electrospun Poly(glycolide-co-lactide) Nanofiber Membrane. Biomacromolecules 4(2):416-423.

academicJournals

Vol. 15(25), pp. 1285-1291, 22 June, 2016
DOI: 10.5897/AJB2016.15229
Article Number: 71DCCFA59039
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Predominance of N-acetyl transferase 2 slow acetylator alleles in children less than ten years experiencing adverse treatment events following artemisinin-based combination therapy in North and South West Regions of Cameroon

Jean P. C. Kengne[1,2], Akindeh M. Nji[1,2], Innocent M. Ali[1,2,3], Olivia A. Achonduh[1,2], Ekollo M. Aristid[1,2] and Wilfred F. Mbacham[1,2,4]*

[1]The Biotechnology Center, University of Yaounde I, Yaounde, Cameroon.
[2]Department of Biochemistry, University of Yaounde I, Yaounde, Cameroon.
[3]Department of Biochemistry, University of Dschang, Dschang, Cameroon.
[4]Department of Biochemistry and Physiology, Faculty of Medicine, University of Yaounde I, Cameroon.

Received 18 January, 2016; Accepted 10 May, 2016

The human *N*-acetyltransferase II (NAT2) gene may vary between individuals resulting in variability in the incidence of adverse drug reactions. We set out in this *adhoc* analysis to determine the distribution of allele frequencies of *NAT2* gene variants among children less than ten years treated with artemisinin-based combinations in Cameroon. Children with uncomplicated malaria were deparasitized with artemisinin based combination therapy (artesunate-amodiaquine, dihydroartemisinin-piperaquine and artemether-lumefantrine) and followed up for 42 days and adverse events recorded. Blood was spotted on filter paper prior to drug administration for DNA extraction by chelex method. Standard nested PCR followed by restriction enzyme analysis with *KpnI*, *TaqI*, and *BamHI* for detection of polymorphisms in the *NAT2* was performed. Allelic frequencies and acetylator phenotypes were compared between participants with or without adverse drug events. The prevalence of slow, intermediate and fast acetylators was 54, 34 and 12% respectively. Significant clustering of mutant alleles (*NAT2*5, NAT2*6* and *NAT2*7)* occurred among those who experienced skin rash and tiredness (OR = 5.765, P = 0.046) (OR = 13.280, P = 0.020). No significant difference was observed between fast and slow acetylators with respect to the development of other recorded adverse events. Overall, producers of the slow acetylator alleles were more likely to develop any adverse events (OR = 8.286, P = 0.017) during the study period. Mutant alleles of the wild type NAT2*4 associated with the slow acetylator phenotype were the most predominant NAT2 allelic type and individuals with the phenotype were more likely to develop adverse events to ACTs.

Key words: *N*-Acetyltransferase 2, Artemisinin based combination therapy, adverse events, slow acetylators, allele.

INTRODUCTION

African countries on recommendation by WHO have increasingly adopted artemisinin-based combinations therapies (ACTs) as first line drugs against uncomplicated malaria. In Cameroon, artesunate-

amodiaquine (ASAQ) was adopted as the first line drug against uncomplicated malaria in 2006. Amodiaquine an accompanying drug in these combinations has been associated with adverse drug reactions leading to reduced adherence to this drug (Adjei et al., 2009; AlKadi, 2007). In the light of increase in use of these ACTs, the science and activities relating to the detection, evaluation, understanding and prevention of adverse reactions to medicines or any other medicine-related problems is necessary for evaluating safety risks (WHO, 2002). Pharmacovigilance is important in the resource-constrained settings because patients may present different profiles of adverse events due to genetic, co-morbidity, and other drug interactions (Pirmohammed et al., 2007).

Drug action on the one hand, depends on how drugs are metabolized and how differences in activity of metabolizing enzymes could significantly contribute to the efficacy of these drugs (Ladero, 2008). Mutations in a gene coding for a drug metabolizing enzyme could result in an enzyme with normal, slow, or fast activity (Agundez, 2008). It has been demonstrated that only 30 to 60% of common drug therapy is successful and that adverse drug reactions could cause 7% of all hospital admissions, 4% of withdrawal from new medical entities, and this can cost society an amount equal to the cost of drug treatment itself (Agundez, 2008). The human *N*-acetyltransferase (NAT) is an expression product of a single intronless protein-coding exon which is 870 bp open reading frame located in chromosome 8p22 and encoding 290 amino acids. It is one of the important phase II drug metabolizing enzymes which catalyses the conjugation or *N*-acetylation of arylamine derived therapeutic drugs and carcinogenic compounds (Agundez, 2008). Two functional polymorphic variants of the NAT gene exist; the NAT I and NAT II (Sim et al., 2008). Genetic polymorphisms in the *NAT2* locus influence individual variation in cancer susceptibility, responses to environmental toxins, and the effectiveness of prescribed medications (Ingelman-Sundberg, 2005; Lee et al., 2010). The *NAT2* gene polymorphisms may vary between individuals and ethnicities and results in variability in the incidence of adverse drug reactions (Kang et al., 2009). Several single nucleotide polymorphisms (more than 15 point mutations) exist on the *NAT2* gene that have given rise to at least 50 allelic variants today (Vatsis et al., 1995). A database for nomenclature of the growing number of different alleles or haplotypes of NAT polymorphisms exist (http://nat.mbg.duth.gr/). Polymorphisms in the wild type, (known as NAT2*4) give rise to combinations of 1-4 nucleotide substitutions in characteristic positions in the 870 bp coding sequence of the gene. Thus, wild type homozygotes are designated as NAT2*4/NAT2*4 made of two wild type alleles (Vatsis et al., 1995). It has been demonstrated that some single nucleotide polymorphisms (SNPs) in the *NAT2* gene may cause structural protein changes or stability or reduced enzyme quantity (Sim et al., 2008; Walraven et al., 2008). Consequently, segregated gene variants in humans give rise to acetylation phenotypes classified as “rapid” (homozygotes of the NAT2*4 polymorphism), intermediate” (heterozygotes of the NAT2*4 polymorphism) and “slow” acetylation phenotypes (Boukouvala, 2005) (homozygotes of the mutant NAT2*5, NAT2*6, NAT 2*7 alleles) depending on the rate of enzyme activity (Chen et al., 2006; Chen et al., 2007). The effects of genetic polymorphism in the NAT2 gene on N-acetylation activity led to investigations of NAT2 SNPs as genetic markers for therapeutic response with resultant adverse reactions to drugs (Furet et al., 2002). Association studies have linked NAT2 polymorphisms and the acetylation status to individual differences in adverse events (Huang et al., 2002), as well as to development of bladder (Hein, 2002) and colon cancers (Garcia-Closas et al., 2005), rheumatoid arthritis (Kumagai et al., 2004) and systemic lupus erythematosus (Rychlik-Sych et al., 2006). The CYP2A6 and CYP2C8 genes are associated with the metabolism of ACTs. Several authors who analyzed the NAT2 gene also analyzed the CYP2A6 and CYP2C8 genes and have these to be correlated with predominantly slow metabolizers status (Gupta et al., 2013). This evidence indicates that when individuals are slow gene CYP2A6 and CYP2C8 they also present with the same “slow” phenotype for the NAT2 gene and could be at risk of developing adverse events. Achonduh et al. (2013), recently showed that NAT2 slow acetylators alleles clustered with individuals experiencing adverse to ASAQ in a study in the Northwest Cameroon. Therequencies of common NAT2 alleles were investigated to assess the distribution of these alleles among patients responding to ACTs in Cameroon. The aim of this study was thus to determine the genotypic frequencies of common *NAT2* genotypes and investigate their association with adverse events in response to ACT treatment in children less 120 months in Cameroon.

MATERIALS AND METHODS

Study area

The study was conducted at two geographically distinct sites, that fall within two of the three major geo-ecological zones of Cameroon as described by MARA maps (Kleinschmidt et al., 2001) namely; Mutengene is situated at coordinates 04°01’N, 09°11’E. The climate is equatorial with a rainfall of 10,000 mm per annum and a

*Corresponding author. E-mail: wfmbacham@yahoo.com. Tel: +237 677579180.

temperature average of 25°C. The vegetation is semi-mangrove and tropical wet forest. The study site is limited to the south and south-east by the sea and to the north and north east by mount Cameroon, an active volcano that is 4,100 m above sea level. The population works predominantly on palm and rubber plantations or estates that are owned by the agro-industrial Cameroon Development Corporation (CDC).

Garoua is in the north of Cameroon and lies at the coordinates, 06°24'N, 10°46'E. Garoua serves as a river port in years when the rainfall is abundant. Situated in the river Benue basin, it receives an average annual rainfall of 380 mm. It has about 4 months of rainy season. The mean temperatures for most of the year are about 31^{o}C and the vegetation is guinea-savannah. The population is predominantly of the Fula ethnic group and comprises of cattle grazers. A few have taken to trading in small provision merchandise with neighbouring Nigeria.

Screening and enrollment

Eligible children aged 6 to 120 months with acute uncomplicated falciparum malaria were screened at the outpatient department and informed consent obtained from parents or guardians. Patients meeting the inclusion criteria were randomized to trial allocation and pre-treatment investigations (clinical and laboratory assessments) conducted. Criteria for inclusion were; children of either gender, suffering from acute uncomplicated *P. falciparum* malaria confirmed by microscopy, or presenting with fever (axillary temperature ≥ 37.5°C) or having a history of fever in the preceding 24 h; ability to ingest tablets orally; willingness and ability to attend the clinic on stipulated follow-up days. A presentation was made of the anticipated risks and benefits, the discomfort to which the subjects were exposed, as well as the right to interrupt the participation at any time on their own free-will. A total of 696 patients were enrolled for the study and were registered into the three arms of the study at the 2 study sites.

ACT Administration and follow-up

Children were randomized to receive artesunate-amodiaquine, dihydroartemisinin-piperaquine and artemether-lumefantrine in the ratio of 2:2:1 respectively.. The first dose of the antimalarials was administered in the hospital and the rest of the tablets were administered at home, according to the prescription. The medication was administered with water after a meal. Follow up of children was ensured by community health workers (CHW) who visited participants daily in the first three days and reported any complaints from the mother. They also visited these mothers with complaints on other days besides the protocol stipulated days (D7, D14, D28, D35 and D42 after first dose). Adverse events following ingestion of the drugs were recorded on a case report form designed to capture these events. They included but were not limited to any signs and symptoms suggestive of malaria or toxicity, date of onset, duration of symptoms, severity of malaria or other treatment administered and patient outcome. Participants were followed up for 42 days post ACT-administration.

Sample collection and DNA extraction

Finger-prick blood was collected and spotted on filter paper at inclusion and during follow up on days D7, D14, D21, D28, D35& D42 for genomic. Blood spots on the filter paper were excised with a sterile pair of surgical scissors. DNA was extracted from the dried blood spots by bioling in Chelex-100 in buffered Tris-EDTA as previously described (Plowe et al., 1995). The DNA was stored in a Tris-EDTA buffer at -20°C until analysis and allelic discrimination analysis.

Genotyping single nucleotide polymorphisms in NAT2 gene

The most common alleles in Africa population were investigated. They contained following mutations: C481T (*rs1799929*, silent mutation, amino acid change L161L), G590A (*rs1799930*, amino acid change R197Q), A803G (rs1208, amino acid change K268R) and G857A (*rs1799931*, amino acid change G286E). The primers used to amplify the gene were: *NAT2* (+) *5'-GCCTCAGGTGCCTTGCATTT-3'* and *NAT2 (-) 5'-CGTGAGGGTAGAGAGGATAT-3'*. The amplification was carried out using a T3 thermal cycler (Biometra, UK). Each PCR cycle was performed in a total volume of 25 µl containing: nuclease free water, 10Xthermopol buffer, 10 mMdNTPs (200 µM of each deoxyribonucleotide), 20 pmol primer and 5 U/µL Taq polymerase and 3 ng of gDNA. After initial denaturation at 95°C for 5 min, 30 cycles of amplification were carried out with denaturation at 95°C for 50 s, annealing at 55°C for 50 s and extension at 72°C for 50 s, followed by a final extension at 72°C for 5 min. To confirm the presence of *NAT2* alleles, PCR products were electrophoresed on a 2% agarose gel and polymorphisms determined by restriction endonuclease digestion of amplified gene fragments as described (Chen et al., 2007). The amplicons were digested under conditions stipulated for the restriction enzymes *Kpn*I and *BamH*I (New England Biolabs, USA) (37°C for 16 h while *Taq I* digested at 65°C for 16 h). The digestion reaction was followed by inactivation at 80°C for 20 min. Digested and undigested fragments of each sample were electrophoresed on a 2% agarose gel stained with Ethidium Bromide and the pattern of migration analysed by UV trans-illumination. Different alleles and combinations of alleles of the NAT2 gene were determined for each sample according to the migration pattern and information indicated in Table 5.

Classification of acetylator genotype

NAT2 acetylator genotypes were produced according to previously published data (Yokogawa et al., 2001). Homozygotes (*NAT2*4/NAT2*4*) or heterozygotes (*NAT2*4/NAT2*5*, *NAT2*4/NAT2*6* and *NAT2*4/NAT2*7* combinations) for the dominant *NAT2*4*wildtype allele were classified as fast acetylator genotypes, while homozygotes of the mutant alleles (*NAT2*5, NAT2*6* and *NAT2*7*) were classified as slow acetylator genotypes.

Data analysis

Data was entered on an SPSS version 16.0 (SPSS Inc., USA) statistical software. Frequency of the NAT2 allele and acetylator genotypes in the study population was performed. The association between NAT2 acetylator genotype and adverse events of ACTs in the study population was assessed by binary logistic regression analysis. The odd ratios (ORs) and 95% confidence interval (CIs) were also determined and a *P*-value < 0.05 was considered as statistically significant.

Ethical clearance

Ethical approval was obtained for the study from the National Ethics Committee for Health Research on human subjects in Cameroon and the Ethics Review Committee of the World Health Organisation (WHO-ERC).

RESULTS

Characteristics of study population

Of the 696 participants recruited in the study, the mean

Table 1. Baseline characteristics of the study population.

Variable	Min	Max	Mean	Std. Deviation
Age (months)	6	120	56.41	33.605
Weight (kg)	6.0	43.0	17.185	7.1618
Haemoglobin (g/dl)	5.5	116.0	10.162	4.7411
Parasitaemia	1040	100000	27698.16	29843.326
Temperature (°C)	35.00	40.80	37.97	1.17

Table 2. Adverse events classification, grading and causality for participants on ACT treatment in Garoua and Mutengene.

Adverse event	Percentage of participants showing event	Severity grading (% participants)				Causality with respect to test drug(% participants)				
		Mild	Moderate	Severe	Life-threatening	No related	Unlikely	Possible	Probable	Most probable
Abdominalpain	3.4	2.2	1	0.2	0	0.7	0.2	0.6	0.6	1.3
Anorexia	3	2.3	0.7	0	0	0.2	0	0.4	0.1	1.4
Asthemie	0.4	0.2	0.3	0	0	0	0	0	0.2	0.3
Cough	5.7	3.8	1.4	0.2	0	1.2	0.6	0.57	1.4	0.7
Diarrhea	3	1.9	1	0	0	0.7	0.3	0.4	0.4	0.4
Tiredness	1.9	1.3	0.4	0	0.2	0	0	0.4	0.3	1.2
Fever	1.6	0.4	0.7	0.3	0.2	0.3	0	0.3	0	1
Skin rash	6.3	3	2.7	0.6	0	0.3	0.9	1.2	0.6	2.6
Vomiting	9.5	6.2	2.7	0.3	0.2	0	0.6	4.2	1.2	2.9

age was 56.41 months. Average hemoglobin was 10.162 g/dL and a geometric mean parasitaemia of 27698.16. The mean temperature was 37.97 with an average weight of 17.185 kg (Table 1).

Adverse reactions following ACTs administration and association with NAT2 alleles

Adverse events reported during the study were asthenia, diarrhea, skin rash, anorexia, abdominal pain, cough, fever, tiredness and vomiting with severity ranging from mild to life-threatening. The causality of adverse events was ranged from no related to most probable with respect to test drug (Table 2). The frequencies of *NAT2* genotypes and phenotypes in the study population are shown in Figures 1 and 2. Relative to fast acetylators, there was an association between NAT2 slow acetylators and susceptibility to develop skin rash (OR = 5.765, P = 0.046) and tiredness (OR = 13.280, P = 0.020) (Table 3). However, no significant difference was observed between fast and slow acetylators and the development of adverse events. Generally, slow acetylators were more likely (OR = 8.286, P = 0.017) to develop any of the adverse events compared to fast acetylators (Table 4).

DISCUSSION

Information gathered on the distribution of genetic polymorphism in populations is essential to understand population wide differences in response to treatment and disease risk. The profiles of the NAT2 genotypes/phenotypes in children less than 10 years were determined and

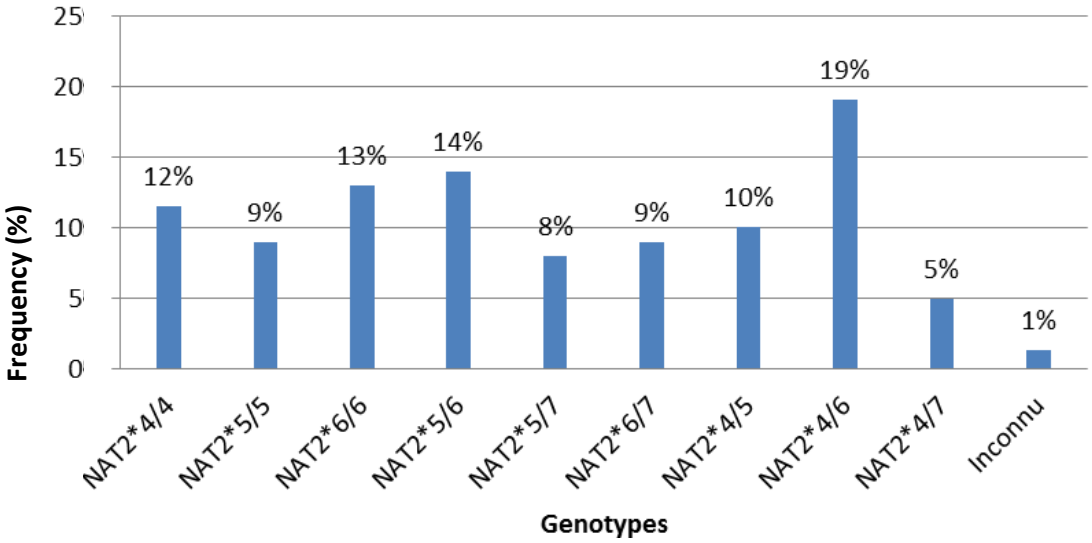

Figure 1. Frequency of NAT2 genotypes in the study population. NAT2*4/4 -fast acetylators; NAT2*5/5, NAT2*6/6, NAT2*5/6, NAT2*5/7, NAT2*6/7- Slow acetylators and NAT2*4/5, NAT2*4/6, NAT2*4/7- Intermediate acetylators.

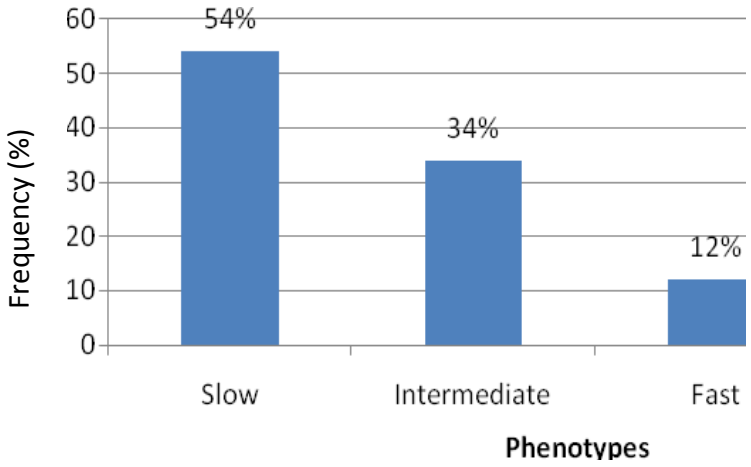

Figure 2. Frequency of NAT2 phenotypes in the study population.

their association with adverse reactions to ACTs as fixed dose combination following deparasitization analysed. Functional genetic polymorphisms of xenobiotic metabolizing enzymes have been associated with therapeutic response differences (Wilkinson, 2005) and disease risk susceptibility (Wilkinson, 2005; Nebert, 1997). In this study, it was observed that the slow acetylator phenotype was an independent risk factor for susceptibility (OR = 6.974, P = 0.017) to any adverse drug event, that is, asthenia, diarrhea, skin rash, anorexia, abdominal pain, cough, fever, tiredness and vomiting compared to those with fast acetylator phenotypes. This could be due to slow elimination of drugs from the system resulting in prolonged exposure and subsequent adverse events. Relative to other adverse events, tiredness and skin rash were observed to be significantly associated with slow acetylator phenotype. The question has been raised why African would preferentially maintain a mutation that places them at a disadvantage of adverse events during therapy. It is probable that over many centuries the pathophysiology allowed for minimal elimination of herbal drugs that accumulated in the body at sub-optimal concentration so that infections could be cleared by the presence of these active ingredients. Selectively the African population with slow metabolism is not exposed to drugs administered at high enough concentrations to usher in adverse events by preferential drug retention.

Table 3. Association between NAT2 phenotype and all adverse events.

Adverse event	Phenotype	Yes	No	OR	95% IC	P-value
Skin rash	Fast	6	14	1.902	0.690 - 5.247	0.166
	Slow	118	105	5.765	1.487 - 8.739	0.046*
Abdominal pain	Fast	2	18	1.069	0.232 - 4.928	0.587
	Slow	21	202	0.728	0.306 - 1.731	0.309
Anorexia	Fast	1	19	0.534	0.068 - 4.203	0.465
	Slow	20	203	0.711	0.288 - 1.754	0.303
Cough	Fast	5	15	1.790	0.611 - 5.244	0.216
	Slow	35	187	0.764	0.386 - 1.509	0.273
Diarrhea	Fast	1	19	0.534	0.068 - 4.203	0.465
	Slow	20	203	0.573	0.228 - 1.436	0.165
Tiredness	Fast	0	20	0.942	0.911 - 0.973	0.318
	Slow	78	145	13.289	4.686 - 15.646	0.020*
Fever	Fast	1	19	1.121	0.136 - 9.233	0.619
	Slow	10	213	2.713	0.702 - 10.482	0.116
Vomiting	Fast	4	16	0.649	0.209 - 2.018	0.323
	Slow	62	160	1.469	0.830 - 2.599	0.119

OR, Odd ratio; CI, confidence interval; *, significant correlation.

Table 4. Association between NAT2 phenotype of participants with or without adverse event.

Phenotype	Adverse event				
	Yes	No	OR	95% IC	P-value
Fast	30	63	0,555	0.327 - 0.943	0.466
Slow	309	390	8,286	5.713 - 11.331	0.017

OR, Odd ratio; CI, confidence interval; *, significant correlation.

Table 5. Restriction sites and lengths of the nucleotide fragments produced by the digestion of the 535 base pair (bp) amplification products with *Kpn I*, *Bam HI* and *Taq I* enzymes.

NAT2 alleles	Position (mutation)	Restriction enzymes	Recognition motif	Fragmentpattern (bp); alleles (wt)	Fragment pattern (bp); point mutation
NAT2*5	481 (C→T)	*Kpn I*	G-GTAC'C	483;52	535
NAT2*6	590 (G→A)	*Taq I*	T'CG-A	205;170;160	330;205
NAT2*7	857 (G→A)	*Bam HI*	G'GATC-C	428;107	535

A, Adenine; C, Cytosine; G, Guanine; NAT2, N-acetyltransferase 2; T Thymine; wt, Wild type

Results presented herein demonstrate a higher prevalence of *NAT2* slow acetylators in this population with the like likelihood to develop adverse events. These findings indicate that *NAT2* polymorphism may be considered a good genetic marker for safety assessment of inter-population differences when populations are exposed to drug such as when assessing the safety profile of newly developed drugs in phase 3 and 4 clinical

trials in Africa.

Conflict of Interests

The authors have not declared any conflict of interests.

Abbreviations

NAT2, *N*-Acetyltransferase II; **PCR,** polymerase chain reaction; **ACTs,** artemisinin-based combinations therapies; **ASAQ,** artesunate-amodiaquine; **WHO,** world health organisation; **CYP,** cytochrome P450 enzymes; **EDTA,** ethylene diamine tetraacetic acid; **DNA,** deoxyribonucleic acid.

REFERENCES

Achonduh OA, AtoghoTB, Mbulli IA, Chedjou JP, Achu M, Nji AM, Fokou E, Kamgue E, Veyee V, Karana O, Sahfe D, Mbacham WF (2013). Adverse events clustering with NAT2 slow metabolisers following deparasitization in children in Bangolan, NWR Cameroon. J. Life Sci. 7(7):742.

Adjei GO, Goka BQ, Rodrigues OP, Høgberg LC, Alifrangis M, Kurtzhals JA (2009). Amodiaquine-associated adverse effects after inadvertent overdose and after a standard therapeutic dose. Ghana Med. J. 43(3):135-138.

Agundez JA (2008). Polymorphisms of human N-acetyltransferases and cancer risk. Curr. Drug Metabol. 9(6):520-531.

AlKadi HO (2007). Antimalarial drug toxicity: A review. Chemotherapy 53(6):385-391.

Boukouvala BSE (2005). Structural analysis of the genes for human arylamine N-acetyltransferases and characterization of alternative transcripts. Basic Clin. Pharmacol. Toxicol. 96(5):343-351.

Chen B, Zhang WX, Cai WM (2006). The influence of various genotypes on the metabolic activity of NAT2 in a Chinese population. Eur. J. Clin. Pharmacol. 62(5):355-359.

Chen M, Xia B, Chen B, Guo Q, Li J, Ye M, Hu Z (2007). N-acetyltransferase 2 slow acetylator genotype associated with adverse effects of sulphasalazine in the treatment of inflammatory bowel disease. Can. J. Gastroenterol. 21(3):155-158.

Furet Y, Bechtel Y, Le Guellec C, Bechtel PR, Autret-Leca E, Paintaud G (2002). Clinical relevance of N-acetyltransferase type 2 (NAT2) genetic polymorphism. Therapie 57(5):427-431.

Garcia-Closas M, Malats N, Silverman D, Dosemeci M, Kogevinas M, Hein DW, Tardón A, Serra C, Carrato A, García-Closas R, Lloreta J (2005). NAT2 slow acetylation, GSTM1 null genotype, and risk of bladder cancer: results from the Spanish Bladder Cancer Study and meta-analyses. Lancet 366(9486):649-659.

Gupta VH, Amarapurkar DN, Singh M, Sasi P, Joshi JM, Baijal R, Ramegowda PH, Amarapurkar AD, Joshi K, Wangikar PP (2013). Association of N-acetyltransferase 2 and cytochrome P450 2E1 gene polymorphisms with antituberculosis drug-induced hepatotoxicity in Western India. J. Gastroenterol. Hepatol. 28(8):1368-1374.

Hein DW (2002). Molecular genetics and function of NAT1 and NAT2: role in aromatic amine metabolism and carcinogenesis. Mutat. Res. 506-507:65-77.

Huang YS, Chern HD, Su WJ, Wu JC, Lai SL, Yang SY, Chang FY, Lee SD (2002). Polymorphism of the N-acetyltransferase 2 gene as a susceptibility risk factor for antituberculosis drug-induced hepatitis. Hepatology 35(4):883-889.

Ingelman-Sundberg M (2005). Genetic polymorphisms of cytochrome P450 2D6 (CYP2D6): clinical consequences, evolutionary aspects and functional diversity. Pharmacogen. J. 5(1):6-13.

Kang TS, Jin SK, Lee JE, Woo SW, Roh J (2009). Comparison of genetic polymorphisms of the NAT2 gene between Korean and four other ethnic groups. J. Clin. Pharm. Ther. 34(6):709-718.

Kleinschmidt I, Omumbo J, Briet O, Van De Giesen N, Sogoba N, Mensah NK, Windmeijer P, Moussa M, Teuscher T (2001). An empirical malaria distribution map for West Africa. Trop. Med. Int. Health 6(10):779-786.

Kumagai S, Komada F, Kita T, Morinobu A, Ozaki S, Ishida H, Sano H, Matsubara T, Okumura K (2004). N-acetyltransferase 2 genotype-related efficacy of sulfasalazine in patients with rheumatoid arthritis. Pharm. Res. 21(2):324-329.

Ladero JM (2008). Influence of polymorphic N-acetyltransferases on non-malignant spontaneous disorders and on response to drugs. Curr. Drug Metabol. 9(6):532-537.

Lee SW, Chung L, Huang HH, Chuang TY, Liou YH, Wu L (2010). NAT2 and CYP2E1 polymorphisms and susceptibility to first-line anti-tuberculosis drug-induced hepatitis. Int. J. Tuberc. Lung Dis. 14(5):622-626.

Nebert DW (1997). Polymorphisms in drug-metabolizing enzymes: what is their clinical relevance and why do they exist. Am. J. Hum. Genet. 60(2):265-271.

Pirmohammed MAK, Dodoo ANO, Winstanley P (2007). Pharmacovigilance in developing countries: Requires collaboration between stakeholders to develop novel models of funding. Br. Med. J. 335(7618):462-462.

Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE (1995). Pyrimethamine and proguanil resistance-conferring mutations in Plasmodium falciparum dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. Am. J. Trop. Med. Hyg. 52(6):565-568.

Rychlik-Sych M, Skrêtkowicz J, Gawroñska-Szklarz B, Górnik W, Sysa-Jêdrzejowska A, Skrêtkowicz-Szarmach K (2006). Acetylation genotype and phenotype in patients with systemic lupus erythematosus. Pharmacol. Rep. 58(1):22-29.

Sim E, Lack N, Wang CJ, Long H, Westwood I, Fullam E, Kawamura A (2008). Arylamine N-acetyltransferases: structural and functional implications of polymorphisms. Toxicology 254(3):170-183.

Vatsis KP, Weber WW, Bell DA, Dupret JM, Evans DA, Grant DM, Hein DW, Lin HJ, Meyer UA, Relling MV, Sim E (1995). Nomenclature for N-acetyltransferases. Pharmacogenet Genomics 5(1):1-17.

Walraven JM, Zang Y, Trent JO, Hein DW (2008). Structure/Function Evaluations of Single Nucleotide Polymorphisms in Human N-Acetyltransferase 2. Curr. Drug Metabol. 9(6):471-486.

WHO (2002). The importance of pharmacovigilance: safety monitoring of medicinal products. In. Geneva.

Wilkinson GR (2005). Drug metabolism and variability among patients in drug response. New Eng. J. Med. 352(21):2211-2221.

Yokogawa K, Nakaharu T, Ishizaki J, Ozaki E, Takeda Y, Mabuchi H, Matsushita R, Kimura K, Nakashima E, Ichimura F, Miyamoto KI (2001). Kinetic phenotypic diagnosis of N-acetylation polymorphism in patients based on ratio of urinary metabolites of salicylazosulfapyridine. Int. J. Pharm. 229(1-2):183-191.

academicJournals

Vol. 15(25), pp. 1292-1298, 22 June, 2016
DOI: 10.5897/AJB2015.15194
Article Number: 059F2CD59041
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Micropropagation of *Handroanthus heptaphyllus* (Vell.) Mattos from seedling explants

Evelyn Duarte, Pedro Sansberro and Claudia Luna*

Applied Biotechnology and Functional Genomics Laboratory, Northeast Institute of Botany (CONICET), Faculty of Agricultural Science, Northeastern University, Cabral St. 2131, CC: 209, W3402BKG, Corrientes, Argentina.

Received 28 December 2015, Accepted 23 May, 2016.

***Handroanthus heptaphyllus* (Vell.) Mattos (Bignoniaceae), a tropical forest tree, is a source of wood suitable for the manufacture of fine furniture and chemical compounds with medicinal and insecticidal properties. Poor seed viability of this species limits the conventional propagation practice. *In vitro* germination of seeds was achieved in semi-solid Murashige and Skoog's (MS) medium. A disinfection protocol was optimized for germination and growth of seedlings *in vitro*. Proliferation of shoots from cotyledonary and stem nodal segments was achieved in semi-solid MS medium supplemented with 22.2 µM 6-benzyladenine. Temporary immersion culture promoted elongation and rooting of shoots and reduced the shoot tip necrosis compared with culture on semisolid medium (6.7±2.7 and 41.7±7.0%, respectively; *P* = 0.001). Plantlets derived from temporary immersion showed better performance during the acclimatization phase. This is the first report on micropropagation of *H. heptaphyllus*.**

Key words: Seeds, uncoated seeds, cotyledon, stem nodes, axillary shoots, adventitious shoot, black lapacho.

INTRODUCTION

Handroanthus heptaphyllus (Vell.) Mattos (Bignoniaceae), synonym *Tabebuia heptaphylla* (Grose and Olmstead, 2007) also known as black lapacho is a heliophile meso hygrophytic species native to the tropical forests and riverine areas of Brazil and Argentina (Ramos et al., 2008; Olmstead et al., 2009; Mori et al., 2012). The characteristics of the wood, suitable for the manufacture of fine furniture, as well as the presence of certain secondary metabolites that impart insecticidal and medicinal properties, attracted the interest of companies for commercial exploitation of wild populations (Alonso, 2000; Martínez et al., 2012). Despite of the predatory exploration, use of the species on a commercial scale is very limited because their seeds exhibit reduced viability and do not tolerate extended storage periods (Mori et al., 2012). No genetic improvement programs have been set up to offer-improved seeds, either for commercial purposes or for the establishment of seed orchards.

In vitro propagation of plants is a biotechnological tool that helps preserve the genetic resources of trees and crops. However, the information on *in vitro* propagation of the *Handroanthus* genus is scarce. The studies of

*Corresponding author. E-mail: cluna@agr.unne.edu.ar.

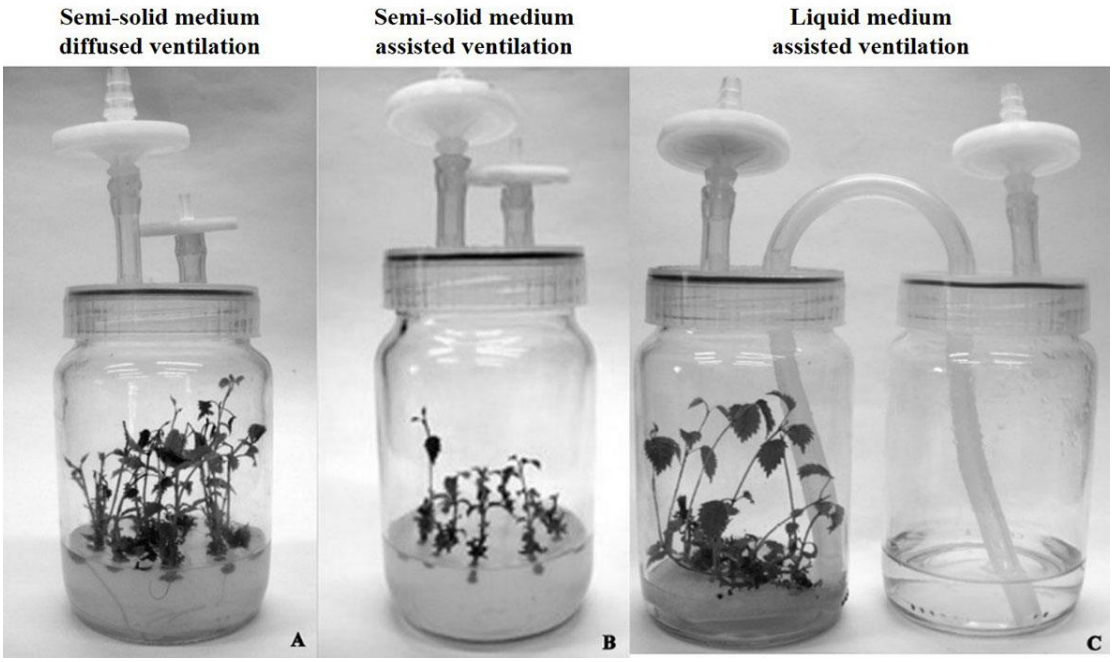

Figure 1. Methods of propagation tested for the elongation and *in vitro* rooting of *Handroanthus heptaphyllus* nodal segments. (A) Semisolid medium with diffused ventilation; (B) semisolid medium with assisted ventilation; (C) temporary immersion to liquid medium with assisted ventilation.

Handroanthus impetiginosus have been limited to morphology and physiological aspects during seed germination (Amaral da Silva et al., 2004) and the physiological abnormalities due to hyperhydricity of shoots cultured *in vitro* (Jausoro et al., 2010). More recently, Larraburu et al. (2012) reported a protocol for the micropropagation of *H. impetiginosus*.

The objective of this study was to establish seedlings *in vitro* as a source of explants for micropropagation of *H. heptaphyllus* through nodal segment cultures and produce plantlets for the enrichment of degraded forests or the establishment of new forest plantations.

MATERIALS AND METHODS

Plant

Pods with mature seeds of *H. heptaphyllus* were harvested from a 45-year-old tree grown in the wild reserve of the National University of Misiones (26°54'–59'S y 54°12'–18'W) and stored at 4°C until culture. The integument was removed and the uncoated seeds were surface disinfested with 70% ethanol for 1 min, transferred to 9 g·L^{-1} NaOCl aqueous solution for 15 min, and rinsed three times with sterile distilled water. Afterward, the seeds were cultured in 15 ml glass tubes (1 seed/tube) containing 4 ml of semi-solid Murashige and Skoog's (1962) medium with 30 g·L^{-1} sucrose (MS). The pH of the media was adjusted to 5.8 prior to addition of the gelling agent (Agar Sigma A-1296, 6.5 g·L^{-1}). The tubes were covered with aluminum foil and autoclaved at 143.2 kPa for 20 min. The cultures were incubated in a growth room at approximately 27±2°C under 14-h photoperiod (116 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), wavelength: 400 to 700 nm, from cool-white fluorescent lamps).

In vitro shoot multiplication and rooting

Cotyledon (CS) and stem nodal segment (SS) (1.5 to 2 cm long) with two axillary buds were collected from 15-day-old *in vitro* seedlings and singly cultured in 15 ml glass tubes containing 4 ml of semi-solid (6.5 g·L^{-1} agar) MS medium with 30 g·L^{-1} sucrose and 4.4 to 22.2 µM 6-benzyladenine (BA), with or without 1 µM indole-3-butyric acid (IBA). The numbers of responsive explants, sprouted buds per explant, and shoots with more than 5 mm in length were recorded after 15 days of culture.

To promote the elongation of the axillary shoots, CS and SS explants grown in MS supplemented with 22.2 µM BA were subcultured for 30 days to a 300 ml glass flask (10 shoots per flask) containing 100 ml of either semisolid (6.5 g·L^{-1} agar) or liquid half-strength MS (MS/2) medium with 15 g·L^{-1} sucrose and 0 to 4.5 µM gibberellin A_3 (GA_3). Flasks were closed with polypropylene caps containing two Millipore® filters that provided passive (Figure 1A) or assisted ventilation (Figure 1B). For the liquid media, the shoots were cultured in a temporary immersion (TI) system wherein the explants were in contact with the medium for 1 min every 4 h (Figure 1 C). To provide assisted ventilation to the shoots growing in semisolid medium, flasks were connected to the TI pneumatic system and the same frequency and duration of immersion was used for aeration. An ear pressure of 30 kPa was used for TI and assisted ventilation.

After 30 days of incubation, the elongated shoots grown in MS/2 medium without plant growth regulators were transferred to the rooting semi-solid or liquid induction medium consisting of MS/2

Table 1. Effect of IBA and BA on the shoot bud sprouting and growth from cotyledonal and stem nodal segments after 15 days of culture on semi-solid MS medium.

PGRs (µM)		Explants with sprouted-buds (%)		Number of shoots per responsive explant		Mean length of shoots (mm)	
IBA	BA	CS	SS	CS	SS	CS	SS
-	-	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	3.3 ± 0.1^{bc}	2. 0± 0.2^{d}	16.8 ± 2.1^{b}	8.8 ± 1.5^{b}
-	4.4	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	3.1 ± 0.4^{c}	3.1 ± 0.3^{c}	25.2 ± 0.4^{a}	13.6 ± 1.3^{a}
-	8.8	96.7 ± 5.8^{a}	96.7 ± 5.8^{a}	4.5 ± 0.5^{ab}	3.1 ± 0.4^{c}	16.5 ± 6.1^{bc}	8.7 ± 2.3^{b}
-	13.2	93.3 ± 5.8^{a}	93.3 ± 5.8^{a}	4.5 ± 0.8^{ab}	3.3 ± 0.2^{bc}	11.4 ± 2.5^{bcd}	5.1 ± 0.4^{c}
-	17.6	96.7 ± 5.8^{a}	100.0 ± 0.0^{a}	4.8 ± 0.0^{a}	3.8 ± 0.3^{abc}	9.6 ± 1.1^{d}	6.7 ± 0.2^{bc}
-	22.0	96.7 ± 5.8^{a}	96.7 ± 5.8^{a}	5.6 ± 0.6^{a}	4.2 ± 0.5^{a}	10.8 ± 0.9^{bcd}	7.4 ± 1.1^{bc}
1	4.4	96.7 ± 5.8^{a}	90.0 ± 10.0^{a}	4.7 ± 0.3^{a}	3.5 ± 0.3^{abc}	13.6 ± 1.4^{bcd}	9.3 ± 0.7^{b}
1	8.8	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	4.9 ± 0.6^{a}	4.1 ± 0.1^{ab}	10.5 ± 0.9^{bcd}	8.4 ± 0.4^{bc}
1	13.2	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	5.7 ± 0.4^{a}	4.0 ± 0.4^{ab}	10.0 ± 0.2^{cd}	8.5 ± 1.5^{bc}
1	17.6	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	5.2 ± 0.4^{a}	3.5 ± 0.1^{abc}	8.8 ± 0.8^{d}	7.1 ± 0.9^{bc}
1	22.0	96.7±5.8 ^{a}	96. 7 ± 5.8^{a}	5.3 ± 0.4^{a}	4.1 ± 0.3^{ab}	10.9 ± 0.3^{bcd}	7.6 ± 0.3^{bc}

Values are mean ± standard error of the mean (SEM) of three independent experiments (n= 30). Means in each column followed by different letters are different according to Tukey's Multiple Comparison Test (P≤0.05). CS and SS, cotyledonal and stem segment explants, respectively.

with 15 g·L^{-1} sucrose and 0, 25, 50, or 75 µM IBA for 3 days. They were then subcultured to the root expression medium of the same mineral composition, but without IBA for 15 days. Cultures were incubated under similar environmental conditions as described earlier.

Finally, the plantlets were transferred to 200 ml pots containing perlite plus controlled-release micro-fertilizer (Osmocote® 18-5-9, 0.6 mg·cm^{-3} substrate). They were grown for 4 weeks in a growth chamber providing day/night air temperature of 25 to 27/20 to 22°C, substrate temperature of 22 to 25°C with a 14-h photoperiod (420 µmol·m^{-2}·s^{-1} PPFD, wavelength: 400 to 700 nm, from high pressure mercury lamps). The hardened plants were transplanted to 3.2 L pots filled with pine bark, which provides proper drainage and aeration, plus controlled-release micro-fertilizer to promote and grown under greenhouse conditions for 12 weeks.

Experimental design and statistical analysis

All the experiments were arranged in a completely randomised design. Each treatment consisted of 30 explants and each experiment was repeated three times. For acclimatization, 15 plantlets per treatment were studied. The results presented are the means of the replications with the standard error of the media (±standard error of mean [SEM]). Data were subjected to analysis of variance (ANOVA; GraphPad Software, San Diego, CA) following Tukey's Multiple Comparison Test. To assess statistical significance, a probability level of 0.05 was chosen.

RESULTS AND DISCUSSION

Shoot multiplication and plantlet formation was achieved from nodal segments of aseptically germinated seedlings of *H. heptaphyllus* by combining semi-solid medium; to encourage breaking of apical dominance during the proliferation stage, and automatic temporary immersion in liquid medium; to promote further growth and rooting of the resulting shoots. Preliminary experiments indicated that more than 90% of cotyledonary and stem nodal segments cultured in semi-solid MS medium sprouted after 15 days of culture (Table 1). BA at 4.4 to 22.2 µM in the culture medium promoted the formation of new shoots from axillary shoots. However, the higher concentration negatively affected shoots elongation. Also, the addition of 1 µM IBA to the MS medium limited the growth of shoots due to callus proliferation at the bases of the explants (Figure 2A to B). An optimal growth of shoots with good appearance and vigor was observed when the explants were cultured on MS sucrose 30 g·L^{-1} plus BA 22.2 µM in which 96.7±5.8% of the CS and SS nodal buds sprouted and produced 5.6±0.6 and 4.2±0.5 shoots per explant having 10.8±0.9 and 7.4±1.1 mm in length, respectively (Table 1).

Neither the addition to the culture medium of GA_3 nor the assisted ventilation promoted shoot elongation (Table 2). Gibberellins are a class of phytohormones with demonstrated effects on a number of physiological processes (Zawaski and Busov, 2014). These include breaking dormancy of vegetative buds and promotion of internode elongation of woody angiosperms (Junttila, 1991). Among the 125 gibberellins identified in plants, fungi and bacteria, only three GA_1, GA_3 and GA_4, have been reported by studies with single gene dwarf mutants as directly effective in promoting shoot elongation (Nakayama et al., 1991). Our former results obtained from nodal segments of *Ilex paraguariensis*, a subtropical woody species, indicated that GA_1 and GA_4 were more effective in promoting shoot length when assayed at a wide range of concentrations while GA_3 did not stimulate

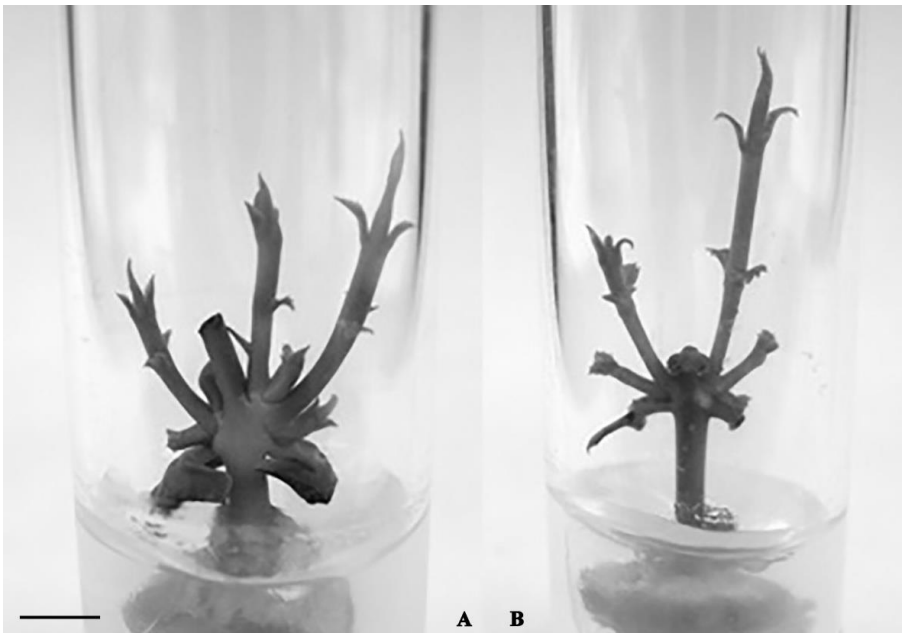

Figure 2. Rupture of apical dominance and proliferation of multiples shoots from cotyledon (A) and stem nodal segment (B) cultured on semi-solid MS medium plus IBA 1 µM and BA 22.2 µM. Bar indicate 5mm.

Table 2. Effect of different culture system and GA_3 on the elongation of shoots cultured on MS half-strength medium.

Culture system	GA_3 (µM)	Shoots with more than 5 mm in length (%)		Mean shoot length (mm)		Shoot tip necrosis (%)	
		CS	SS	CS	SS	CS	SS
Semi solid medium and diffuse ventilation	0.0	57.5±2.0^{a}	40.7±4.8^{a}	15.3±0.8^{bcd}	14.5±0.9^{bcd}	13.1±1.5^{bc}	13.7±0.8^{b}
	1.5	47.0±6.3^{a}	50.1±11.7^{a}	11.5±0.8^{bcd}	11.5±0.9^{de}	10.7±2.4^{bc}	14.9±2.6^{b}
	3.0	50.6±1.4^{a}	51.8±3.8^{a}	12.8±1.0^{bcd}	12.3±1.8^{cde}	10.3±0.4^{bc}	13.7±0.5^{b}
	4.5	45.2±4.7^{a}	44.8±7.9^{a}	12.2±2.6^{bcd}	11.9±1.1^{cde}	9.6±2.6^{b}	12.9±1.8^{b}
Semi solid medium and assisted ventilation	0.0	53.7±4.9^{a}	44.5±9.9^{a}	16.9±3.3^{bcd}	16.6±1.4^{bc}	14.4±2.2^{c}	20.5±3.7^{c}
	1.5	45.2±3.9^{a}	44.1±6.8^{a}	13.2±1.0^{bcd}	11.0±1.1^{de}	11.0±1.5^{bc}	15.1±1.2^{b}
	3.0	46.8±4.4^{a}	44.7±1.6^{a}	10.2±2.1^{cd}	9.5±1.2^{e}	11.6±1.1^{bc}	12.4±1.9^{b}
	4.5	49.2±5.5^{a}	43.5±5.2^{a}	9.3±2.0^{d}	12.0±1.0^{cde}	11.1±0.9^{bc}	15.1±0.7^{b}
Liquid medium and assisted ventilation (TI)	0.0	54.6±6.3^{a}	41.3±6.1^{a}	25.8±5.8^{a}	22.3±2.6^{a}	1.8±0.3^{a}	1.3±0.3^{a}
	1.5	52.3±5.8^{a}	40.6±1.4^{a}	18.8±4.8^{abc}	14.7±0.9^{bcd}	1.04±0.1^{a}	1.4±0.6 ^{a}
	3.0	51.5±6.5^{a}	45.3±5.1^{a}	19.5±2.9^{ab}	17.9±2.7^{ab}	1.3±0.5^{a}	1.3±1.1^{a}
	4.5	56.5±1.8^{a}	47.1±8.9^{a}	16.5±3.3^{bcd}	15.3±3.0^{bcd}	1.4±0.4^{a}	1.0±0.1^{a}

Values are mean ± standard error of the mean (SEM) of three independent experiments (n= 30). Means in each column followed by different letters are different according to Tukey's Multiple Comparison Test ($P \leq 0.05$). CS and SS indicate cotyledonary and stem nodal segment, respectively. TI: Temporary immersion.

internode elongation (Sansberro et al., 2001).

Additionally, the mean shoot length was greater and shoot tip necrosis was minimal when both explants type were cultured in a TI system. In fact, the mean shoot length varied between 9.3±2.0 to 25.8±5.8 mm while the shoot tip necrosis ranged from 1.0±0.1 to 20.5± 3.7%.

Table 3. Effect of different culture system and IBA on the induction of adventitious rooting from elongated shoots cultured on MS half-strength medium.

Culture system	IBA (µM)	Rooting (%)	Mean number of roots	Mean root length (mm)	Shoot/root dry weight ratio	Shoot tip necrosis (%)
Semi solid medium and diffuse ventilation	0	30.0 ± 20.0^{cd}	1.2 ± 0.3^{ab}	22.6 ± 2.3^{abcd}	4.9 ± 1.8^{bc}	33.3 ± 15.3^{bcd}
	25	73.3 ± 11.5^{abc}	2.2 ± 0.2^{ab}	46.1 ± 7.1^{a}	1.2 ± 0.5^{ab}	26.7 ± 11.6^{bcd}
	50	90.0 ± 17. 3^{ab}	2.2 ± 0.1^{ab}	38.0 ± 7.3^{abcd}	1.4 ± 0.5^{ab}	56.7 ± 5.8^{d}
	75	70.0 ± 30.0^{abc}	2.5 ± 0.2^{a}	44.9 ± 11.6^{ab}	1.2 ± 0.2^{ab}	50.0 ± 10.0^{d}
Semi solid medium and assisted ventilation	0	0.0 ± 0.0^{d}	-	-	-	43.3 ± 5.8^{cd}
	25	30.0 ± 30.0^{cd}	1.7 ± 1.0^{ab}	24.1 ± 20.9^{abcd}	0.6 ± 0.5^{a}	46.7 ± 15.3^{d}
	50	43.3 ± 20.8^{abcd}	1.5 ± 0.1^{ab}	41.9 ± 18.0^{abc}	0.7 ± 0.4^{a}	36.7 ± 20.8^{bcd}
	75	26.7 ± 15.3^{cd}	2.3 ± 0.9^{a}	30.7 ± 5.2^{abcd}	0.6 ± 0.2^{a}	46.7 ± 11.6^{d}
Liquid medium and assisted ventilation (TI)	0	73.3 ± 5.8^{abc}	2.0 ± 0.5^{ab}	16.1 ± 2.5^{abcd}	8.9 ± 1.1^{d}	13.3 ± 11.6^{abc}
	25	36.7 ± 32.1^{bcd}	1.2 ± 0.7^{ab}	11.0 ± 10.1^{d}	3.5 ± 3.2^{abc}	6.7 ± 5.8^{ab}
	50	63.3 ± 11.5^{abc}	1.3 ± 0.0^{ab}	13.2 ± 3.7^{cd}	6.3 ± 0.9^{cd}	6.7 ± 5.8^{ab}
	75	96.7 ± 5.8^{a}	2.4 ± 0.2^{a}	15.4 ± 4.3^{bcd}	4.0 ± 1.4^{bc}	0.0 ± 0.0^{da}

Values are mean ± standard error of the mean (SEM) of three independent experiments (n= 10). Means in each column followed by different letters are different according to Tukey's Multiple Comparison Test ($P \leq 0.05$). CS and SS indicate cotyledonary and stem nodal segment, respectively. TI: Temporary immersion.

BA in the culture medium promoted the formation of new sprouts from axillary buds without significantly affecting the growth and development of shoots. However, the incidence of the shoot tip necrosis was observed during the *in vitro* shoot multiplication and rooting stages. After 2 weeks of culturing, necrotic apices were observed in shoots growing in semisolid medium provided with either diffused or forced ventilation. This physiological disorder is commonly seen in *in vitro* cultures affecting a wide range of herbaceous and woody plants (Bairu et al., 2009a) and it is caused by a complex set of factors including plant growth regulators (Bairu et al., 2009b), calcium and boron deficiency (Hepler, 2005; Martin et al., 2007), medium type and salt strength (Jain et al., 2009; Takura and Kanwara, 2011), gelling agent (Singha et al., 1990), aeration (Bairu et al., 2009a), and pH (Pasqua et al., 2002). Our results indicated that the use of liquid medium through a TI system could overcome the shoot tip necrosis of *H. heptaphyllus* and promote growth and rooting of shoots. Because the use of assisted ventilation of the flask with semisolid medium did not overcome this physiological disorder, the advantage of TI for producing healthy shoots and plantlets may be related to better availability of nutrients in the absence of hyperhydricity. The rooting process was affected by the physical conditions of the culture medium (Table 3). The result of this study showed 73.3 ± 5.8% of SS explants cultured in TI rooted in a plant growth regulators (PGR)-free half-strength MS medium. Addition of IBA only slightly promoted the morphogenetic process probably due to the juvenility stage of the explants. Although, the use of TI stimulated growth of the aerial part at the expense of root growth by increasing the shoot/root ratio, the growth and development of the plantlets during the acclimatization phase were the highest under TI (Figure 3A to E). This could be attributed to better morphological and anatomical features of the leaves which enhance the water balance during the acclimatization phase (Yang and Yeh, 2008). The rooting process occurs without callus formation. The *in vitro* rooting of juvenile elongated shoots of *A. heptaphyllus* may be stimulated without plant growth regulators, but the results of our experiment showed that the pre-treatment of the elongated shoots with 75 µM IBA for 3 days and subculturing to fresh half-strength medium increased this morphogenic process, whereby 96.7± 5.8% of shoots regenerated 2.4 roots per shoot with a shoot/root ratio close to 4 and with no symptoms of necrosis.

To our knowledge, this is the first report on micro-propagation of *H. heptaphyllus*. The establishment and multiplication of shoot from nodal segments cultured in semi-solid medium and the elongation and rooting of shoots in a TI system are an effective combination for propagating these threatened forest trees.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was supported by grants from ANPCyT (PICTO 2011-0213, PICT 2014-1246) and SGCyT (PI-

Figure 3. In vitro rooting, plantlets formation and acclimatization of nodal segments cultured on semisolid medium with diffused ventilation (A, B), semisolid medium with assisted ventilation (C, D), and temporary immersion to liquid medium with assisted ventilation (E,F).

A005/11, PI- 001/14). The authors extend their deep appreciation to anonymous reviewers for their critical comments. E. Duarte received a CONICET scholarship. P. Sansberro and C. Luna are members of the Research Council of Argentina (CONICET).

REFERENCES

Alonso J (2000). El lapacho. Revista de Fitoterapia 1(2):107-117.

Amaral da Silva EA, Davide AC, Faria JM, de Melo DL, de Abreu GB (2004). Germination studies on *Tabebuia impetiginosa* Mart . Cerne Lavras 10:1-9.

Bairu MW, Jain N, Stirk WA, Dolezal K, Van Staden J (2009b). Solving the problem of shoot-tip necrosis in *Harpagophytum procumbens* by changing the cytokinin types, calcium and boron concentrations in the medium. South Afr. J. Bot. 75:122-127.

Bairu MW, Stirk WA, Van Staden J (2009a). Factors contributing to *in vitro* shoot-tip necrosis and their physiological interactions. Plant Cell Tissue Org. Cult. 98:239-248.

Grose SO, Olmstead RG (2007).Taxonomic Revisions in the polyphyletic genus *Tabebuia* s. l. (Bignoniaceae). Syst. Bot. 32:660-670.

Hepler PK (2005). Calcium: a central regulator of plant growth and development. Plant Cell 17:2142-2155.

Jain N, Bairu MW, Stirk WA, Dolezal K, Van Staden J (2009).The effect of medium, carbon source and explant on regeneration and control of shoot-tip necrosis in *Harpagophytum procumbens*. South Afr. J. Bot. 75:117-121.

Jausoro V, Llorente BE, Apostolo NM (2010). Structural differences between hyperhydric and normal *in vitro* shoots of *Handroanthus impetiginosus* (Mart. ex DC) Mattos (Bignoniaceae). Plant Cell, Tissue Organ Cult. 101:183-191.

Junttila O (1991). Gibberellins and the regulation of shoot elongation in woody plants. In: Takahashi N, Phinney BO and MacMillan J (eds), Gibberellins. Springer-Verlag, New York, pp. 199-210.

Larraburu EE, Apostolo NM, Llorente BE (2012). *In vitro* Propagation of Pink Lapacho: response surface methodology and factorial analysis for optimisation of medium components. Int. J. For. Res. P 9.

Martin KP, Zhang CL, Slater A, Madasser YJ (2007). Control of shoot necrosis and plant death during micropropagation of banana and plantains (Musa spp.). Plant Cell, Tissue. Organ Cult. 88:51-59.

Martínez M, Mancuello C, Brítez F, Pereira C, Arrúa J, Franco G, Conteiro M, Iañez V, González F, Benítez B, López T, Pérez S, Ferreira F (2012). Caracterización química y actividades biológicas de lapachol aislado de *Handroanthusheptaphyllus* (Vell.) Mattos. Steviana 4:47-64.

Mori NT, Teixeira de Moraes ML, Morita CM, Mori ES (2012).Genetic diversity between and within populations of *Handroanthus heptaphyllus* (Vell.) Mattos using microsatellite markers. Cerne, Lavras. 8:9-15.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plantarum 15:473-497.

Nakayama M, Yamane H, Murofushi N, Takahashi N, Mander L, Seto H (1991). Gibberellin biosynthetic pathway and the physiologically active gibberellin in the shoot of *Cucumis sativus* L. J. Plant Growth Regul. 10:115-121.

Olmstead RG, Zjhra ML, Lohmann LG, Grose SO, Eckert AJ (2009). A molecular phylogeny and classification of Bignoniaceae. Am. J. Bot. 96:1731-1743.

Pasqua G, Manes F, Monacelli B, Natale L, Anselmi S (2002). Effects of the culture medium pH and ion uptake in *in vitro* vegetative organogenesis in thin cell layers of tobacco. Plant Sci. 162:947-955.

Ramos V, Durigan G, Franco G, Siqueira M (2008). Árvores da floresta estacional semidecidual: guia de identificação de espécies. São Paulo: EDUSP; Biota/FAPESP, P 320.

Sansberro P, Mroginski L, Bottini R (2001). *In vitro* morphogenetic responses of *Ilex paraguariensis* nodal segments treated with different gibberellins and Prohexadione-Ca. Plant Growth Regul. 34:209-214.

Singha S, Townsend EC, Oberly GH (1990). Relationship between calcium and agar on vitrification and shoot-tip necrosis of quince (*Cydoniaoblonga*Mill.) shoots *in vitro*. Plant Cell, Tis. Org. Cult. 23:135-142.

Takura A, Kanwara JS (2011). Effect of phase of medium, growth regulators and nutrient supplementations on in vitro shoot-tip necrosis in pear. New Zeal. J. Crop Hort. Sci. 39:131-140.

Yang S, Yeh D (2008). *In vitro* leaf anatomy, *ex vitro* photosynthetic behaviors and growth of *Calathea orbifolia* (Linden) Kennedy plants obtained from semi-solid medium and temporary immersion systems. Plant Cell, Tis. Org. Cult. 93:201-207.

Zawaski C, Busov V (2014). Roles of gibberellin catabolism and signaling in growth and physiological response to drought and short-day photoperiods in *Populus* trees. PLOS ONE 9: e86217.

academicJournals

Vol. 15(25), pp. 1299-1305, 22 June, 2016
DOI: 10.5897/AJB2016.15398
Article Number: 5AF897B59043
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

A endophytic fungus, *Ramichloridium cerophilum*, promotes growth of a non-mycorrhizal plant, Chinese cabbage

Ling Xie[1,2], Erika Usui[1] and Kazuhiko Narisawa[1]*

[1]College of Agriculture, Ibaraki University, 3-21-1 Chuo, Ami, Ibaraki, Japan.
[2]Microbiology Research Institute, Guangxi Academy of Agriculture Science, Nanning, Guangxi, China.

Received 13 April, 2016; Accepted 26 May, 2016

A fungal endophyte, *Ramichloridium cerophilum*, was identified as a Class 2 endophytes species obtained from the leaf of common sowthistle (*Sonchus oleraceus* L). This fungus was found to grow endophylically in the roots of Chinese cabbage seedlings. Light microscopy of cross-sections of colonized Chinese cabbage roots showed that the fungus penetrates through the outer epidermal cells, grows further into the inner cortex, and colonizes the cortical tissue. Isolates of *R. cerophilum* 28L-9 have shown the ability to increase biomass of a nonmycorrhizal plant, Chinese cabbage. This finding is the first report of *R. cerophilum* could help to improve nonmycorrhizal plant.

Key words: *Ramichloridium cerophilum*, endophytic fungus, Chinese cabbage, plant growth promoting.

INTRODUCTION

The mycorrhizal symbiosis between plants and fungi is common and most plant species are dependent on this symbiosis for improving the productivity and sustainability, including current forest and agricultural systems (Toro and Delaux, 2016). The fungal symbiont generally aids the growth of the plant partner by improving nutrient uptake (Toro and Delaux, 2016), while reducing fertilizer use and pollution in the environment, which is important with relevance to agriculture and ecology. Chinese cabbage is an important and widely cultivated vegetable in East Asian countries, especially Japan, Korea and China, because of its rapid growth and is an economically important cash crop as well as an efficient food crop (Haque et al., 2016; Kang et al., 2010). However, members of the Cruciferae including this major commercial crop, Chinese cabbage, are known as non-mycorrhizal plants and other types of fungal symbionts were rarely found in these plants (Gilliam, 2016; Nouri and Reinhardt, 2015).

Endophytic fungi are defined as the endosymbionts that live within the plant intercellular and intracellular spaces for atleast part of their life cycle without causing apparent harm to their host (Rodriguez et al., 2009). Unlike mycorrhizal fungi that colonize plant roots and grow into the rhizosphere, endophytes reside entirely within plant tissues and may grow within roots, stems and/or leaves

*Corresponding author. E-mail: kan-moc@mx.ibaraki.ac.jp.

(Carroll, 1988; Stone et al., 2004). In nonclavicipitaceous endophytes (NC-endophytes), Class 2 endophytes may grow in both above- and below-ground tissues; by contrast, Class 3 and 4 endophytes (dark septate endophytes, DSE) are restricted to above-ground tissues and roots, respectively (Rodriguez et al., 2009). Class 2 endophytes have a broad host range of both monocot and eudicot plants and are the largest group of fungal symbionts and are readily culturable on artificial media and are thought to colonize all plants in natural ecosystems (Petrini, 1996). Studies have shown that they could increase host shoot and/or root biomass, possibly as a result of the induction of plant hormones by the host or biosynthesis of plant hormones by the fungi (Tudzynski and Sharon, 2002). Class 2 endophytes were the focus of this study which is distinct from the other NC-endophytes because in general they colonize roots, stems and leaves.

Dark septate endophytes (class 4 endophytes) *Heteroconium chaetospira*, *Phialocephala fortinii*, *Meliniomyces variabilis* and *Veronaeopsis simplex* which had been isolated from evergreen rainforests in Japan; and recent studies showed that beneficial traits toward Chinese cabbage, including nitrogen provision, growth promotion, and disease suppression could be associated with these isolates (Usuki and Narisawa, 2007; Khastini et al., 2012, 2014). Based on these findings, it was hypothesized that class 2 endophytes population in this area may be also diverse and there might possibly be more non-described species. In order to prove this hypothesis, key class 2 endophytes species were isolated from tissues of different naturally grown plant species. Here, the features of the selected key class 2 endophytes species that were effective in supporting the growth of a non-mycorrhizal plant, Chinese cabbage were described.

MATERIALS AND METHODS

Sample collection and fungal isolation

Leaves, stems and roots of 12 different plant species (Table 1) were collected from November to December 2007 within the campus and Field Science Centre of Ibaraki University in Ami, Ibaraki, Japan. Samples were washed with running tap water and cut into small pieces (leaf pieces size approximately 5.0 × 5.0 mm; stem and root pieces approximately 5.0 mm long). Fifteen pieces of respective tissues of each plant species were chosen randomly for fungal isolation. Leaf and stem pieces were immersed in 70% ethanol for 15 to 60 s and then in sodium hypochlorite (1% of household bleach) for 1 to 3 min. The root segments were rinsed three times in a 0.005% solution of Tween 20 and then three times with distilled water. Segments were air dried overnight and then plated on corn meal agar [corn meal (infusion form, Difco) 25 g L^{-1}; Bacto agar (Difco) 15 g L^{-1}; Ampicillin 50 µg L^{-1}; Streptomycin 50 µg L^{-1}]. After incubation for 2 weeks, pure cultures were obtained by transferring single hyphae to cornmeal malt yeast agar medium [CMMY; Malt extract (BD) 10 g L^{-1}, Yeast extract (BD) 2 g L^{-1}, Cornmeal infusion 8.5 g L^{-1}; Bacto agar 7.5 g L^{-1}]. Fungal isolates were identified based on morphology of sporulating structures.

Table 1. Pant samples and fungi isolates.

Plant sample	Number of isolates
Sonchus oleraceus L.	18
Sonchus spp.	17
Camellia japonica.	25
Eurya japonica Thunb.	16
Orchidaceae spp.	30
Rhododendron mucronatum G.Don.	31
Rhododendron obtusum planch.	36
Eurya japonica thumb.	25
Citrus reticulate.	14
Ilicium anisatum Linn.	31
Cornus officinalis.	30
Prunus jamasakura.	15
Total	288

Morphological observation and identification

To provide ideal observation conditions, slide cultures were made consisting of small inoculated pieces of approximately 3 × 3 mm pablum agar [Mead Johnson mixed Publum (Canadian Post Corporation, Ontario, Canada), 25 g; Bacto agar, 5 g; MiliQ water, 250 ml] sandwiched between two 18 × 18 mm cover glasses (Matsunami Class Ind., Osaka, Japan), and placed in a 9-cm water agar plate for providing humidity. After 2 to 4 weeks, when the culture growth was easily visible, the pablum agar was carefully removed and cover glasses were mounted on a 76 × 26 mm micro slide glass using a PVLG (Polyvinyl alcohol, 16.6 g; Lactic acid (Wako Chemical Ind., Osaka, Japan), 100 ml; Glycerin (Wako Chemical Ind., Osaka, Japan), 10 ml; MiliQ water, 100 ml) mounting medium. Observations were then made and the length of conidiogenous cells and conidia was measured under light microscope (Olympus BX51, Tokyo, Japan) with UPlanFLN FLN100x/1.30 Oil.

DNA extraction, amplification, sequencing and analysis

The genomic DNA was extracted from mycelium grown on oatmeal medium using the Prepman Ultra Sample Preparation Reagent Protocol (Applied Biosystems, California, USA). Primer set NS1 and NS4 (White et al., 1990), were used to amplified a part of the 18S rDNA region via polymerase chain reaction (PCR). The PCR reaction mixture (50 µl) contained 5 µl of 10x rTaq DNA polymerase buffer (TaKaRa Bio), 2.5 µl of each primer, 4 µl of dNTP mixture, 37.875 µl of MiliQ water, 0.125 µl of rTaq DNA polymerase (TaKaRa Bio), and 0.5 µl of template DNA. The amplification was carried out using a Takara PCR Thermal Cycler Dice (Takara Bio INC. model TP 600, Shiga, Japan) under the following conditions: 94°C for 4 min, 35 cycles 35 s at 94°C, 52°C for 55 s and 72°C for 2 min, followed by a final 10 min extension at 72°C. The PCR products were sequenced using a model 3130x DNA sequencer (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM TM Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems). For DNA sequencing, the 10 µl reaction mixture (Big Dye Terminator var. 3) contained 1.5 µl of 5 × Sequencing buffer; 1 µl of primer (3.2 µM), 1 µl of pre-mixture (kit), 6.2 µl of sterilized MiliQ water and 0.3 µl of template purified DNA. The sequencing conditions were: 96°C for 2 min, 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The determined sequences were compared with similar DNA sequences retrieved from the DDBJ/

EMBL/GenBank databases using the NCBI BLAST program.

Endophyte screening

In order to distinguish non-pathogenic fungi from pathogenic and other saprotrophic fungi, 13 isolates were selected based on their morphology as representative from different groups of fungi, and the priority was given to slow-growing isolates that usually start developing after at least 7 to 10 days of incubation. The endophyte screening on selected isolates was carried out after growing the fungus in 6-cm Petri dishes filled with oatmeal medium [Oatmeal, 10 g L^{-1}; Bacto agar, 18 g L^{-1}] enriched with nutrients [$MgSO_4 \cdot 7H_2O$ (Wako Chemical Ind., Osaka, Japan), 1 g L^{-1}; KH_2PO_4 (Wako Chemical Ind., Osaka, Japan), 1.5 g L^{-1}; $NaNO_3$ (Wako Chemical Ind., Osaka, Japan), 1 g L^{-1}], 4 replicate plates per fungal isolate. After the plates were largely covered by fungal colony, disinfected 2-day-old seedlings of Chinese cabbage c.v. W4107 (Watanabe Seed, Miyagi, Japan) were transplanted (three per plate) onto the growing fungal colony and placed into a sterile plastic pot and incubated for two weeks at 23°C with 18 h:6 h (L:D) and 180 mol m^{-2} s^{-2} light intensity. Negative impact of the fungal isolates was evaluated using a 0 to 3 scale (0: no visible symptom; 1: lightly yellowing leaves; 2: lightly yellowing leaves and smaller plants; 3: or death) (Mahmoud and Narisawa, 2013). Assessed plants were harvested and oven-dried at 40°C for 48 h. The dry weight of treated plants on per plate was taken and compared with the control plants. Four plates seedlings (twelve seedlings) were measured per isolate.

Statistical analysis

The mean dry biomass of each treatment was calculated and analyzed with one-way analysis of variance (ANOVA) and Duncan's multiple range test of SAS systems for Windows (version 8.02, SAS Institute Inc., Cary, NC, USA) was used to analyze the data.

Fungal re-isolation and colonization observation

Non-pathogenic isolates were evaluated for the ability to colonize Chinese cabbage. Root, stem and leaf segments, up to 1 cm in length taken from the same set of plants were surface sterilized using the protocol described in the fungal isolation. Roots were observed after washing, cross sectioning, and staining with 0.005% cotton blue in 50% acetic acid and examined under an Olympus BX51 microscope with UPlanFI40/0.75 and UPlanFI100/1.30 objectives to assess colonization levels.

RESULTS

Sample collection and fungal isolation

Two hundred eighty eight fungal isolates were successfully obtained from 540 leaf, stem and root segments cutting from 12 plant species. The frequencies of fungal isolation from these different tissues were 70.0, 77.6 and 61.0%, respectively. The dominant isolated fungi were species of *Pestalotiopsis* (72.8%), *Colletotrichum* (12.1%) and *Phomopsis* (8.9%). They are mostly isolated within 4 to 7days of placing root segments on the medium. *Ramichloridium cerophilum* was isolated from the leaf of common sowthistle (*Sonchus oleraceus* L.), it is a class 2 endophytes and able to symbiosis with plant and promote its growth.

Endophyte screening

To eliminate saprotrophic and/or pathogenic fungal isolates, 13 fungal isolates showing diverse morphology were used for endophyte screening. Compared to non-treated control plants, three of the isolates (approximately 2.7%) were not pathogenic to the Chinese cabbage seedlings. The plant biomass of 10L-4, 18R-10 and 28L-9 treatments showed no significant difference compared to the control (Figure 1) and no typical disease symptoms on Chinese cabbage growth. The isolates 10L-4 and 28L-9 were obtained from leaves, while 18R-10 from roots. Among three isolates, 28L-9 stimulated the seedling growth, with average biomass (64.5±8.2 mg) increasing 16.8% compared to control (55.2±4.2 mg) (Figures 1 and 2). Most of the tested isolates (over 97%) were pathogenic to Chinese cabbage with a negative impact index ranging from 1 to 3.

Morphological characteristics

Isolate 28L-9 was isolated from the leaf of common sowthistle (*Sonchus oleraceus* L.), its colonies were dark olivaceous grey with sharp margins on CMMY medium. They grew relatively slowly on the medium and reached around 30 mm after 2 weeks at 23°C (Figure 3). Hyphae were pale olivaceous-brown, smooth or verruculose, 2.0 to 3.4 (2.6 mean) µm wide. Conidiophores sometimes branched, arising from creeping aerial hyphae, smooth or verruculose, hardly tapering towards the apex, 2.1 to 3.3 (2.6) µm wide, 17 to 70 (44) µm long, mostly with 4 septa but ones with up to 10 septa have been observed (Figure 4). Conidiogenous cells are integrated, terminal, proliferating sympodially, rachis is short, straight and crowded, prominent, pigmented with non-thickened scars, minute, approximately 0.5 µm in diameter Conidia solitary, fusiform to clavate, thin-walled, smooth, 0 to 1-septate, subhyaline, 3.2 to 12.8 (7) × 2.0 to 4.2 (2.8) µm.

Molecular analysis

Based on the result of the molecular analysis, the isolate was affiliated with *R. cerophilum* EU041798.2 with 98% of similarity in 18S rDNA sequences. The sequence was deposited in GenBank at NCBI with accession number FJ514548. Isolate 28L-9 was identified as *R. cerophilum* based on the comparison of morphological characteristics (Arzanlou et al., 2007) and on the molecular analysis of the 18S rDNA sequences.

Fungal re-isolation and colonization observation

The re-isolation rate of 28L-9 from inoculated root tissues

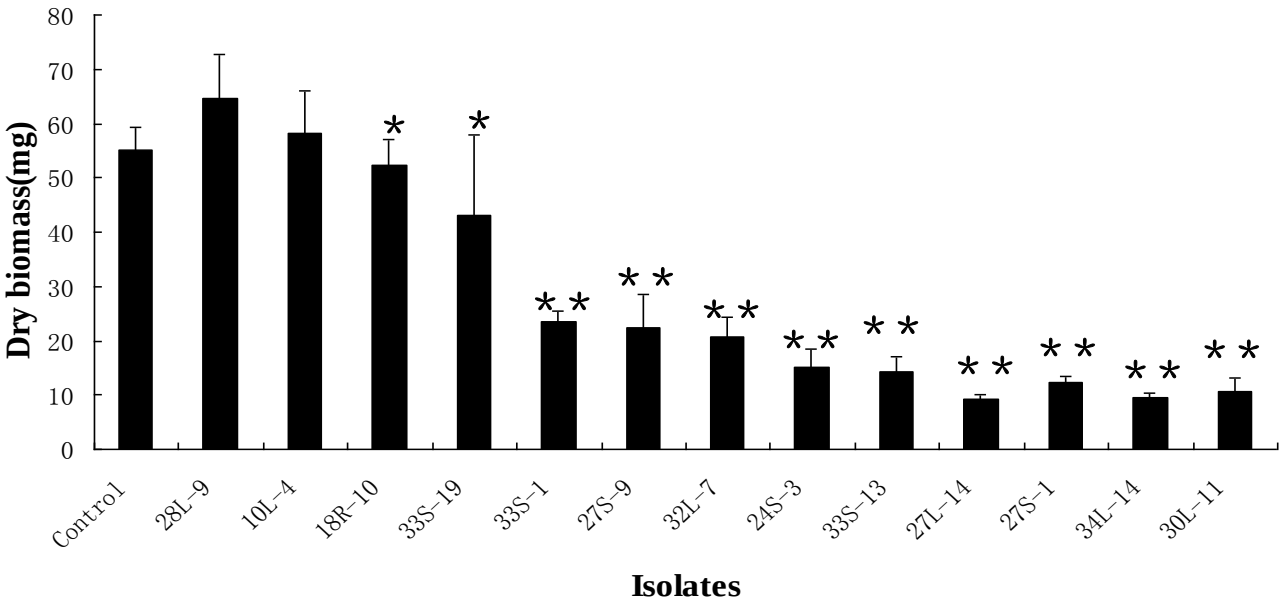

Figure 1. Dry biomass of Chinese cabbage inoculated with different fungal isolates. The filled columns represent the groups of selected fungi. Date are the means mean+SE; n=4. Asterisks present significant differences between each treatment and the control (**P<0.01, *P<0.05) following by Duncan's multiple range test.

Figure 2. The effect of 28L-9 on the growth of Chinese cabbage seedlings in Petri dish two weeks after transplanting. A, Non-treated control. B, 28L-9 treatment.

was consistently 100%. This result indicated that *R. cerophilum* was able to colonize root of Chinese cabbage without causing damage to the host plant. Roots inoculated with isolate 28L-9 showed pigmented hyphae of the isolate that grew mostly intercellularly, but also intracellularly in epidermal and inner cortical cells. However, the fungal hyphae were not observed in the vascular bundle. No visible sign of host reaction was seen in response to the colonization by the isolate, and the fungus external generally grew out of the root into the medium (Figure 5).

DISCUSSION

Only one isolate of *R. cerophilum* species was recovered during this study, representing an isolation rate of 0.11% among the total number of fungal isolates. It was yet

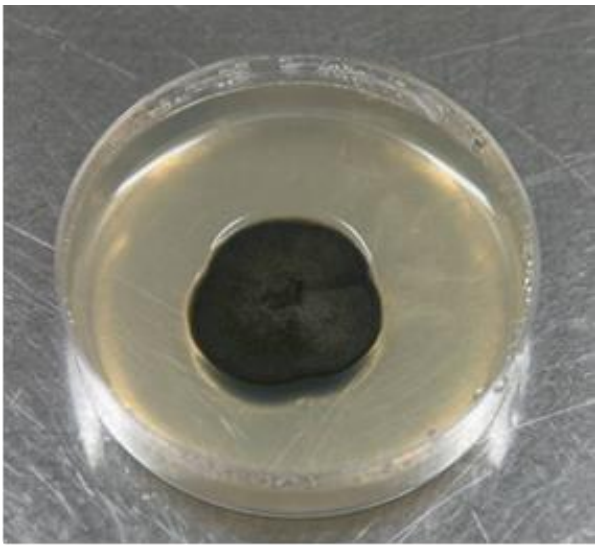

Figure 3. Colony of 28L-9 on CMMY medium after culturing for 2 weeks.

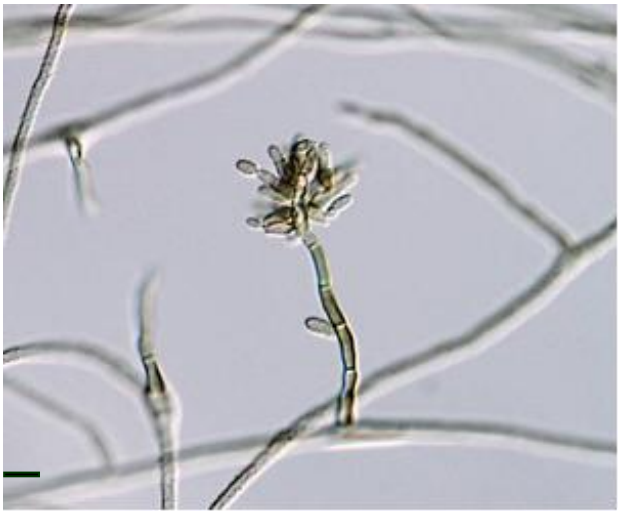

Figure 4. Conidial formation of 28L-9 on CMMY medium after 1 month of culturing. Bar=10 µm.

impossible to estimate its abundance, but this finding indicates that there are undescribed endophyte species that can be found in evergreen rainforests areas in Japan. Improved isolation media and protocols may enhance the recovery rate of this fungus, but the overall results of the study were comparable to those of previous studies in Japan using the same baiting method that discovered the endophyte *H. chaetospira* at 4.0% (Mahmoud and Narisawa, 2013). Fast-growing endophyte such as *P. fortinii* and *Leptodontidium orchidicola* with colony diameter reaching above 80 mm after 30 days on PDA (Currah et al., 1987) might be easier to recover as the probability of their obscuration by fast-growing fungi would be lower.

Endophytes are known to be able to colonize host tissues intracellularly and extracellularly without causing substantially negative impact (Diene et al., 2010). Depending on the fungal species involved, different responses have been observed with the host tissues. *Hetroconium chaetospira*, for example, when applied to Chinese cabbage colonized both epidermis and cortical layers, but was not observed in the vascular cylinder (Morita et al., 2003). Whereas *P. fortinii* can penetrate the vascular cylinder in addition to epidermal and cortical cells, consequently inducing undesirable effects on host plants under certain environmental conditions (Yu et al., 2001). The root colonization by *R. cerophilum* can be inter- and intra-cellular, and is limited to the epidermal and cortical layers, causing therefore no visible symptom or disruption to the growth of host plant. The potential environmental impact on the behavior of this endophyte is unknown but based on its inability to grow into the vascular tissue and on the lack of symptom in colonized host tissues; it is believed that this fungus has a low risk to be a plant pathogen. In the current study under controlled-environment conditions, however, *R. cerophilum* did not form any typical mycorrhizal structures in host plant, but hyphae of the fungus greatly grew along the surface of the root and colonized the epidermal layer. Although the abundance and ecological behavior of *R. cerophilum* under natural conditions in different habitats has not been studied. Our preliminary data indicate that the ability of this endophytic nature to produce varying amounts of surface hyphae on the root tissues of the plant seems essential to their ability to promote host plant growth.

Conclusion

This finding indicates that there are undescribed endophyte species that can be found in evergreen rainforests areas in Japan. This study found for the first time that *R. cerophilum* is a class 2 endophytes and is able to promote growth of a non-mycorrhizal plant, Chinese cabbage.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research (B) (No. 20380176) from the Japan Society for the Promotion of Science (to K.N.) and part of this work was also supported by the projects from China National Natural Science Foundation (No. 31460016). The authors are grateful to Emeritus Professor S. Tokumasu,

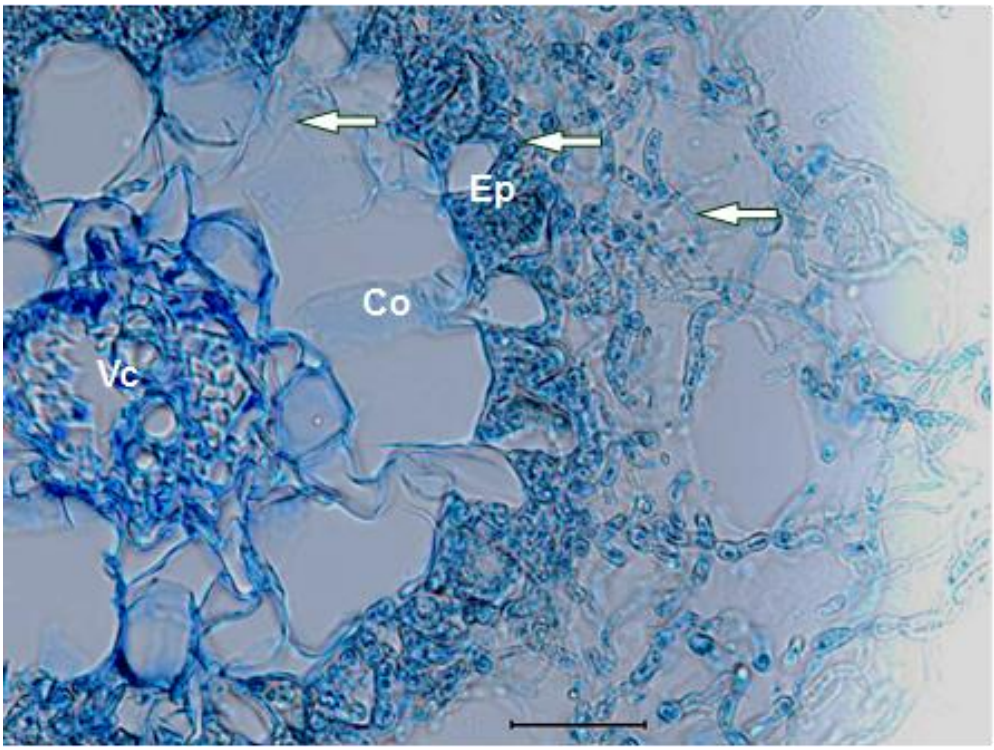

Figure 5. The cross-section of a 28L-9-treated Chinese cabbage root stained with 0.005% cotton blue 2 weeks after inoculation. Hyphae can be seen on the root surface (arrow), within epidermal and cortical cells (arrow). Ep: Epidermal; Co: cortical; Vc: vascular cylinder. Bar=20 µm.

University of Tsukuba, Japan for providing valuable suggestions for the fungal identification. They are also grateful to Dr. G. Peng, Agriculture and Agri-Food Canada, Saskatoon Research Centre, Canada, for critically reviewing the manuscript.

REFERENCES

Arzanlou M, Groenewald JZ, Gams W, Braun U, Shin HD, Crous PW (2007). Phylogenetic and morphotaxonomic revision of *Ramichloridium* and allied genera. Stud. Mycol. 58:57-93.

Carroll G (1988). Fungal endophytes in stems and leaves from latent pathogen to mutualistic symbiont. Ecology 69:2-9.

Currah RS, Smreciu EA, Seymour PND (1987). A national plant conservation program for Canadian botanic gardens. In. Bramwell D, Hamann O, Heywood V, and Synge H (Eds.) Botanic Gardens and the World Conservation Strategy. Academic Press, London, pp. 295-299.

Diene O, Takahashi T, Yonekura A, Nitta Y, Narisawa K (2010). A New Fungal Endophyte, *Helminthosporium velutinum*, Promoting Growth of a Bioalcohol Plant, Sweet Sorghum. Microbes Environ. 25:216-219.

Gilliam FS (2016). A novel mechanism to explain success of invasive herbaceous species at the expense of nativesin eastern hardwood forests. New Phytol. 209:451-453.

Haque MA, Yun HD, Cho KM (2016). Diversity of indigenous endophytic bacteria associated with the roots of Chinese cabbage (*Brassica campestris* L.) cultivars and their antagonism towards pathogens. J. Microbiol. 54(5):353-363.

Kang SM, Kim JT, Hamayun M, Hwang IC, Khan AL, Kim YH, Lee JH, Lee IJ (2010). Influence of prohexadione-calcium on growth and gibberellins content of Chinese cabbage grown in alpine region of South Korea. Sci. Hortic. 125:88-92.

Khastini RO, Ogawara T, Sato Y, Narisawa K (2014). Control of Fusarium wilt in melon by the fungal endophyte, *Cadophora sp*. Euro. J. Plant Pathol. 139:339-348.

Khastini RO, Ohta H, Narisawa K (2012). The role of a dark septate endophytic fungus, *Veronaeopsis simplex* Y34, in Fusarium disease suppression in Chinese cabbage. J. Microbiol. 50(4):618-624.

Mahmoud RS, Narisawa K (2013). A New Fungal Endophyte, *Scolecobasidium humicola*, Promotes Tomato Growth under Organic Nitrogen Conditions. PLoS One 8(11):e78746.

Morita S, Azuma M, Aoba T, Satou H, Narisawa K, Hashiba T (2003). Induced systemic resistance of Chinese cabbage to bacterial leaf spot and *Alternaria* leaf spot by the root endophytic fungus, *Heteroconium chaetospira*. J. Gen. Plant Pathol. 69:71-75.

Nouri E, Reinhardt D (2015). Flowers and mycorrhizal roots-closer than we think. Trends Plant Sci. 20(6):344-350.

Petrini O (1996). Ecological and physiological aspects of host-specificity in endophytic fungi. In: Redlin SC, and Carris LM (Eds.) Endophytic Fungi in Grasses and Woody Plants. APS Press, St Paul, pp. 87-100.

Rodriguez RJ, White JF, Arnold AE, Redman RS (2009). Fungal endophytes: diversity and functional roles. New Phytol. 182:314-330.

Stone JK, Polishook JD, White JRJ (2004). Endophytic fungi. In. Mueller G, Bills GF, and Foster MS (Eds.) Biodiversity of fungi: inventory and monitoring methods. Elsevier, Burlington, MA, USA, pp. 241-270.

Toro KS, Delaux PM (2016). Mycorrhizal symbioses: today and tomorrow. New Phytol. 209:917-920.

Tudzynski B, Sharon A (2002). Biosynthesis, biological role and application of fungal phyto-hormones. In. Osiewacz HD (Eds.) The mycota X. Industrial applications. Springer-Verlag, Berlin, Germany, Pp. 183-212.

Usuki F, Narisawa K (2007). A mutualistic symbiosis between a dark septate endophytic fungus, *Heteroconium chaetospira*, and a nonmycorrhizal plant, Chinese cabbage. Mycologia 99(2):175-184.

White TJ, Bruns T, Lee S, Taylor J (1990). Emplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In. Innis MA, Gelfand DH, Sninsky JJ, and White JT (Eds.) PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, pp. 315-322.

Yu T, Nassuth A, Peterson RL (2001). Characterization of the interaction between the dark septate fungus *Phialocephala fortinii* and Asparagus officinalis roots. Can. J. Microbiol. 47(8):741-753.

academicJournals

Vol. 15(25), pp. 1306-1314, 22 June, 2016
DOI: 10.5897/AJB2015.15154
Article Number: 103271B59045
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic diversity in two populations of *Limicolaria aurora* (Jay, 1839) from two ecological zones in Nigeria

Michael Olufemi AWODIRAN* and Zaccheaus Omololu OGUNJOBI

Department of Zoology, Obafemi Awolowo University, Ile-Ife, Nigeria.

Received 7 December, 2015; Accepted 6 May, 2016

Limicolaria aurora **belongs to the group of land snails commonly called garden snails. This study seeks to use shell morphology and random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) to examine gross morphological differences in populations of *L. aurora* from New Bussa (guinea savannah) and Benin City (tropical rain forest) in Nigeria and possibly delimit the populations into sub species. A total of one hundred and ten specimens of *L. aurora* made up of fifty five individuals from each of the two ecological zones were collected randomly for the study. Data on shell parameters including: height of shell (SH), width of shell (SW), aperture height (AH), aperture width (AW), spire length (SL), and first whorl length (1WL) measured on each snail were subjected to one way analysis of variance (ANOVA). Principal component analysis (PCA) and canonical variates analysis (CVA) were performed on the data using PAST statistical software. DNA which was extracted from the muscular tissues of the foot of eight individuals from each location using cetyltrimethylammonium bromide (CTAB) method was subjected to RAPD-PCR. Amplification of the DNA was done using five primers (OPB-12, OPB-18, OPH-08, OPD-11 and OPS-13). Analyses showed significant differences (P<0.05) in *L. aurora* populations within and between the vegetation zones revealing great heterogeneity in the populations. Both PCA and CVA clusters did not separate the populations into distinct sub-populations. SH was the most variable morphological characteristic and consequently the most suitable for the separation of *L. aurora* specimens into distinct populations. All the primers used in the amplification of the DNA produced polymorphic bands. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster diagram revealed two major clades within the snail populations with about 74% similarity. The study showed that RAPD-PCR analysis is more suitable for delimiting populations of *L. aurora* than morphometrics and that the basis for gross morphological differences in these populations might not only be environmental but also genetic factors.**

Key words: Achatinidae, biodiversity, environmental factors, morphometrics, phylogenetics, shell, sub-populations, variation.

INTRODUCTION

The genus *Limicolaria* belongs to the group of land snails of the family Achatinidae. The genus consists of snails that are commonly referred to as garden snails. The species of this genus that have been reported in the West African sub region include *Limicolaria aurora* and *Limicolaria flammea*. The genus *Limicolaria,* along with other achatinids has been reported to be serious crop pests and when introduced to a new environment can unbalance local ecosystem. Many achatinids have attained pest status even within their native range when

the habitat is modified for human habitation and farming (Raut and Barker, 2002).

L. aurora however, is not only agriculturally disastrous, but can as well be advantageous; the snail meat can be a good source of protein in fish feeds (Madu et al., 2006). The shell could be ground and used as a source of calcium carbonate in the formulation of animal feed. Land snail shells serve as a calcium source for various organisms that feed on them, especially for eggshell formation, muscle contraction, and osmo regulation. The alkalinity of the crushed shell is also useful in reduction of soil acidity (Graveland and van der Wal, 1996; Hotopp, 2002).

Moreover, in land snails, shell forms (morphology) often provide relevant morphometric data used in taxonomy and phylogenetic inference as well as in population biology. Shell morphometry is a useful tool in mollusc taxonomy and ecology. It has been used to discriminate between species, to recognize intraspecific morphological variation and to associate shell variations with environmental conditions and geographical distribution (Chiu et al., 2002; Wullschleger and Jokela, 2002; Pfenninger et al., 2003).

The extent of genetic diversity in natural population results from an interplay between forces generating local genetic differentiation and forces generating genetic homogeneity. Hence, the level of population genetic variation can be influenced by such processes as founder events, genetic drift, mutation, recombination, migration (gene flow) and selection. These forces may also interact with other factors, such as life-history traits, breeding system, dispersal and other ecological and evolutionary processes to determine the patterns of genetic structuring that are observed in the field (Gow et al., 2004).

In South Western Nigeria, biodiversity information of some achatinid snails exist (Oke and Odiete, 1996; Oke and Alohan, 2006; Oke, 2007; Oke et al., 2007, 2008; Oke and Chokor, 2010; Oke, 2013). However, genetic diversity data is lacking on most of the land snails. This study therefore combines the morphological analysis of the shell with random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) data to understand the basis for gross morphological differences in *L. aurora* populations and their possible delimitation into sub species.

MATERIALS AND METHODS

A total of 110 specimens (55 specimens from each location) were randomly collected for the study. The specimens collected were identified on the field according to Crowley and Pain (1970).

Specimens of *L. aurora* were collected from New Bussa (guinea savannah) and Benin City (tropical rain forest). New Bussa is a city in Niger state, Nigeria. The city sits at longitude 4°31'E (4.5167°E) and latitude 9°53'N (9.8833°N), while it is located at 40 km south of the original Bussa town. The city is located 499 feet above sea level. The climate is sub-humid, classified as tropical savannah and the average annual precipitation is 1,109 mm.

Benin City is the capital city of Edo State, Nigeria, sits at longitude 5°37'39"E (5.6275°E) and latitude 6°20'06"N (6.3350°N). Benin City is 259 feet above sea level. Climate is tropical with average annual precipitation of 2,025 mm. Figure 1 shows the map of Nigeria with the study areas.

Morphometric studies

The shell of each snail was described by six parameters as shown in Figure 2. The parameters measured are: height of shell (SH), width of shell (SW), spire length (SL), aperture height (AH), aperture width (AW), and 1st whorl length (Awodiran et al., 2012).

Measurements of each morphometric character were transformed to shell width (SW) to remove size-effect by growth allometry using Reist (1985) as described in Gunawickrama (2007) methods. Width corrected data were then analyzed by multivariate statistical method. Principal component analysis (PCA) and canonical variates analysis (CVA) were performed on the data using the software, PAST (Hammer et al., 2001). To find out the morphometric factors that can discriminate among the two populations, PCA was used in which factor loadings based on eigen values were used to determine the morphometric factors.

RAPD studies

DNA extraction

Total genomic DNA was extracted from foot tissue of the snails. Muscular foot sample was collected from the snails using a new razor blade. Different razor blade was used for each of the specimens. This was preserved in 80% ethanol and kept in the refrigerator till use. After which 0.1 g of the tissue sample was later collected from each of the preserved samples. DNA was extracted from these tissues using a CTAB protocol (Bucklin, 1992). DNA samples were then stored in a refrigerator. DNA concentration of all samples was measured on a spectrophotometer at 260 and 280 nm and the DNA purity was determined. The quality of DNA was detected by Agarose gel electrophoresis. Genomic DNA was used in PCR amplification using RAPD markers. A negative PCR controls was run to overcome one of the major limitation of RAPD marker which is the generation of artefact fragments and bands that appeared consistently in the negative controls were removed from the final analysis. Also, the dominance marker limitation was managed by estimating eight samples per locus for each primer coupled with the large number of polymorphism derived from the analysis. Code sequence and nucleotide lengths used in RAPD studies are shown in Table 1.

PCR reaction mix

The reaction mix was carried out in 20 µl final volume containing 50 to 60 ng genomic DNA, 0.1 µM of the primers, 2 mM $MgCl_2$, 125 µM of each deoxy Nucleotide Triphosphate (dNTP) and 1 unit of Taq DNA polymerase. The thermocycler profile has an initial denaturation temperature of 94°C for 3 min, followed by 45 cycles

*Corresponding author. E-mail: michfemi@yahoo.com. Tel: +234 806 208 8776.

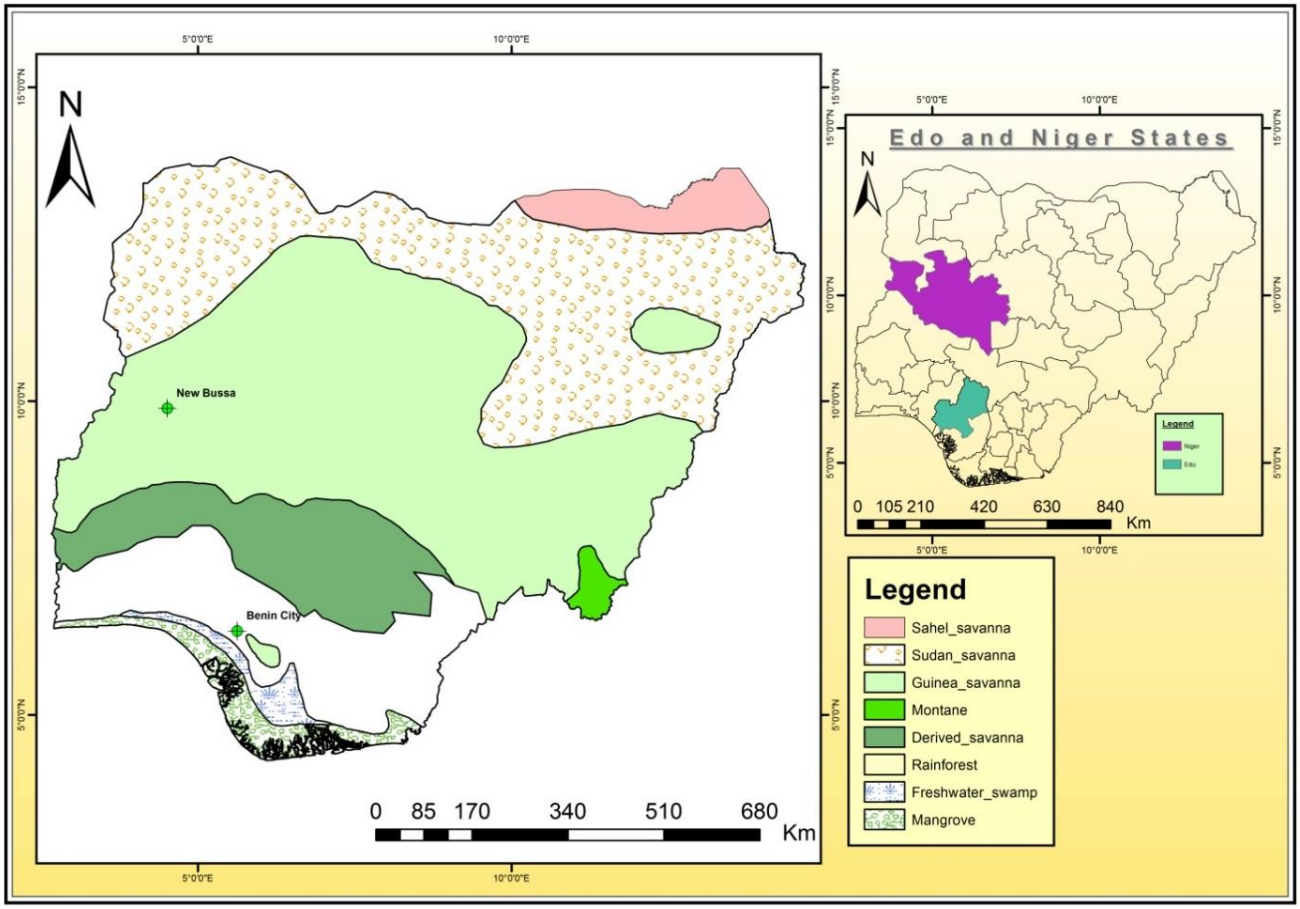

Figure 1. Map of Nigeria showing the study areas of Benin City (Edo State) and New Bussa (Niger State).

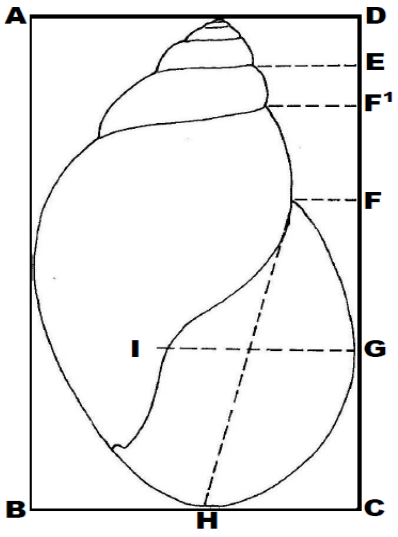

Figure 2. Measurements of shell morphology used in morphometric analysis (AB=Height of shell, SH; BC=Width of shell, SW; DF= Spire length, SL; FH= Aperture height, AH; GI=Aperture width, AW; and EF1=1st whorl length, 1WL).

of denaturation temperature of 94°C for 20 s, annealing temperature of 37°C for 40 s and primer extension temperature of 72°C for 40 s, followed by final extension temperature at 72°C for 5 min.

Gel electrophoresis

PCR amplicon electrophoresis was carried out by size fractionation on 1.4% Agarose gels. Agarose gels were prepared by dissolving and boiling 2.8 g agarose in 200 ml 0.5X Tris Boric Ethylenediamine Tetra-acetic Acid (TBE) buffer solution. The gels were allowed to cool to about 50°C and 10 µl of 5 mg/ml ethidium bromide was added, mixed together before pouring it into an electrophoresis chamber set with the combs inserted. After the gel has solidified, the PCR amplicon was loaded in the well created. Electrophoresis was done at 100 V for 2 h. The DNA was visualized and photographed on UV light source.

Band scoring and data analysis

Each gel was analyzed by scoring the present (1) or absent (0) polymorphic bands in individual lanes. The scoring can be done based on the banding profiles which is clear and transparent. The scores were then pooled for constructing a single data matrix. Purity of the DNA was carried out using spectrophotometer by measuring absorbance at 260 and 280 nm. The RAPD banding profiles were

Table 1. Primer sequences and the percentage polymorphism.

S/N	Primer name	Sequence	No. of polymorphic loci	No. of monomorphic loci	Total No. of Loci	Polymorphism (%)
1	OPB – 12	CCTTGACGCA	8	1	9	89
2	OPB – 18	CCACAGCAGT	10	1	11	91
3	OPH – 08	GAAACACCCC	8	1	9	89
4	OPD- 11	AGCGCCATTG	6	nil	6	100
5	OPS – 13	GTCGTTCCTG	9	nil	9	100
Total	-	-	41	3	44	

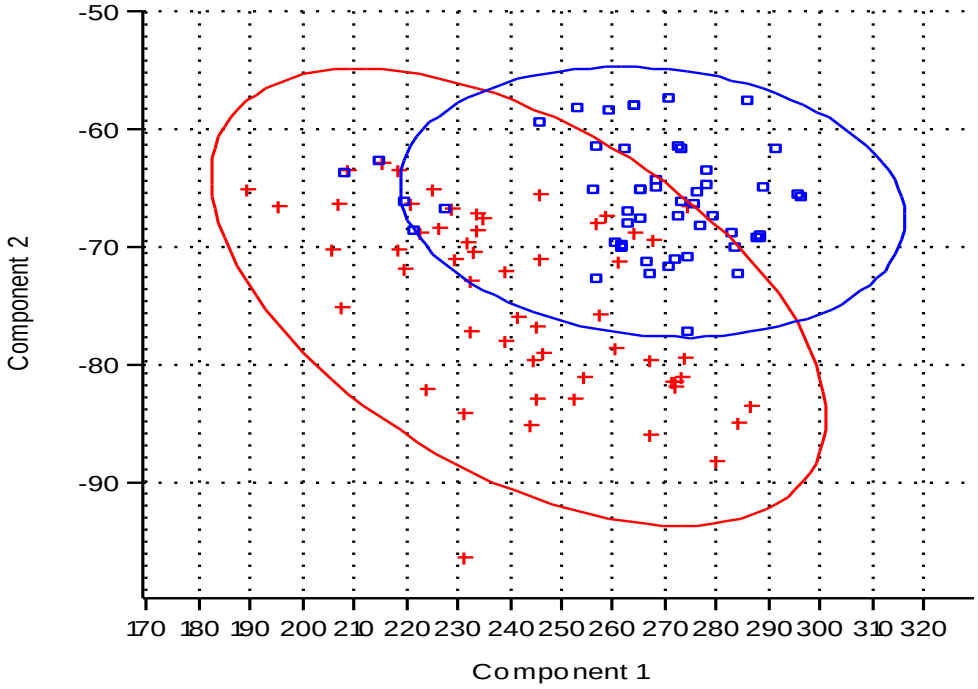

Figure 3. Principal component analysis of shell measurements of *Limicolaria aurora* from the two populations showing overlap of data between populations from New Bussa and Benin city (+ represents New Bussa, while □ represents Benin City).

visually scored for all the DNA samples and for each primer. Similarity coefficients were calculated across all the possible pair wise comparisons of snail samples among populations, using the formula:

$$Sxy = \frac{2nxy}{nx + ny}$$

where nxy is the number of common bands shown in both individuals x and y, and nx and ny are the total numbers of bands observed in individual x and y, respectively (Nei, 1978). As a means of providing a visual representation of genetic relationships, a dendrogram was constructed based on the similarity coefficient values (1 - Sxy) between pairs of snail samples. The NTSYS-PC software program was used to estimate genetic similarities with the Jaccard's coefficient (Rohlf, 2000) and a dendrogram was constructed using the Unweighted Pair Group Method of Arithmetic Averaging (UPGMA) employing the Sequential, Agglomerative, Hierarchical and Nested clustering module (SAHN).

RESULTS

Morphometric studies

Figure 3 is the PCA diagram of the morphometric measurements of *L. aurora* from the two locations studied. The clusters produced overlapped. Figure 4 shows the relationship between shell characteristics and the loadings on PCA. This shows that Height of Shell (E) is the characteristic responsible for most of the variation among

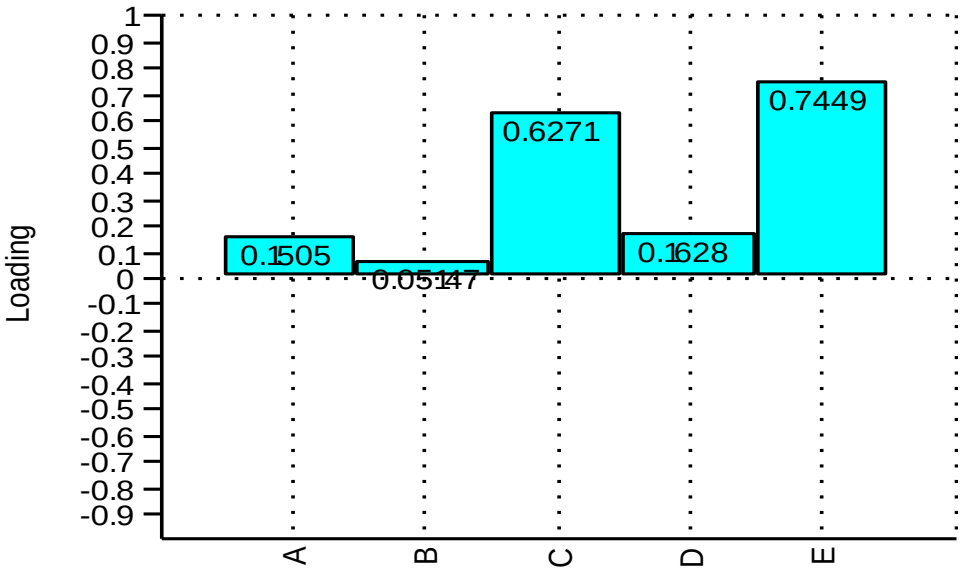

Figure 4. Snail shell characteristics and their loadings on PC1 of the principal component analysis showing height of shell as the character most responsible for variation among the populations studied. A: Aperture height (AH); B: aperture width (AW); C: spire length (SL); D: 1st whorl length (1WL); E: height of shell (SH).

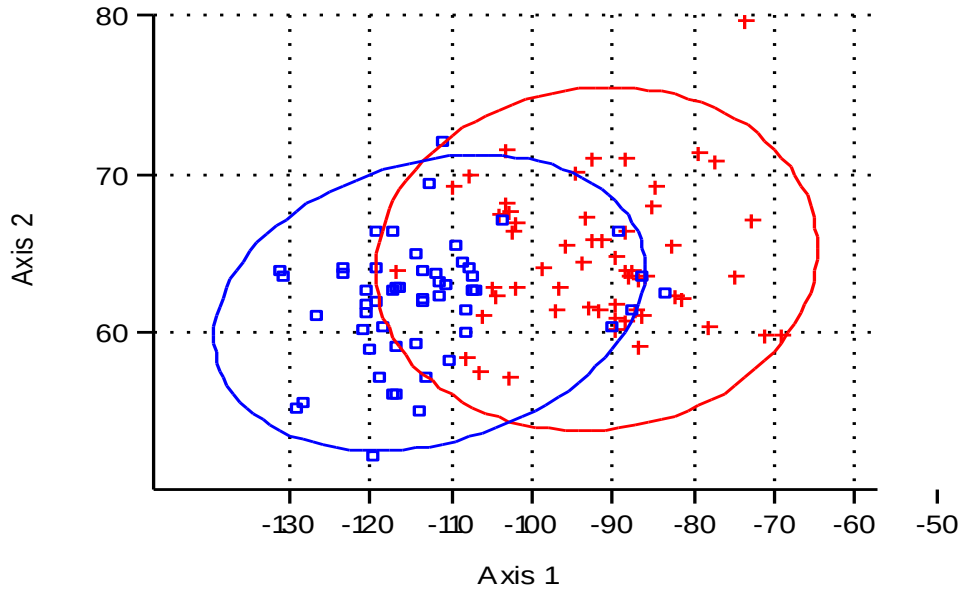

Figure 5. Canonical variates analyses of shell measurements of *L. aurorafrom* New Bussa and Benin City showing homogeneity of characters (+ represents New Bussa, while □ represents Benin City).

the populations of *L. aurora*, followed by spire length (C) while aperture width (B) is the characteristic which contributes least to the variation. CVAs were carried out to determine if there are significant differences among the populations of *L. aurora*. Figure 5 shows the CVAs of the shell of *L. aurora.* The CVA plots showed overlapping of clusters of specimens from the locations studied.

Data for these characteristics were also compared in box plot (Figure 6). The box plots represent summaries of measurements of the two highest loading characters (that is, SH and SL) and when subjected to ANOVA, these measurements were not significantly different ($P > 0.05$)

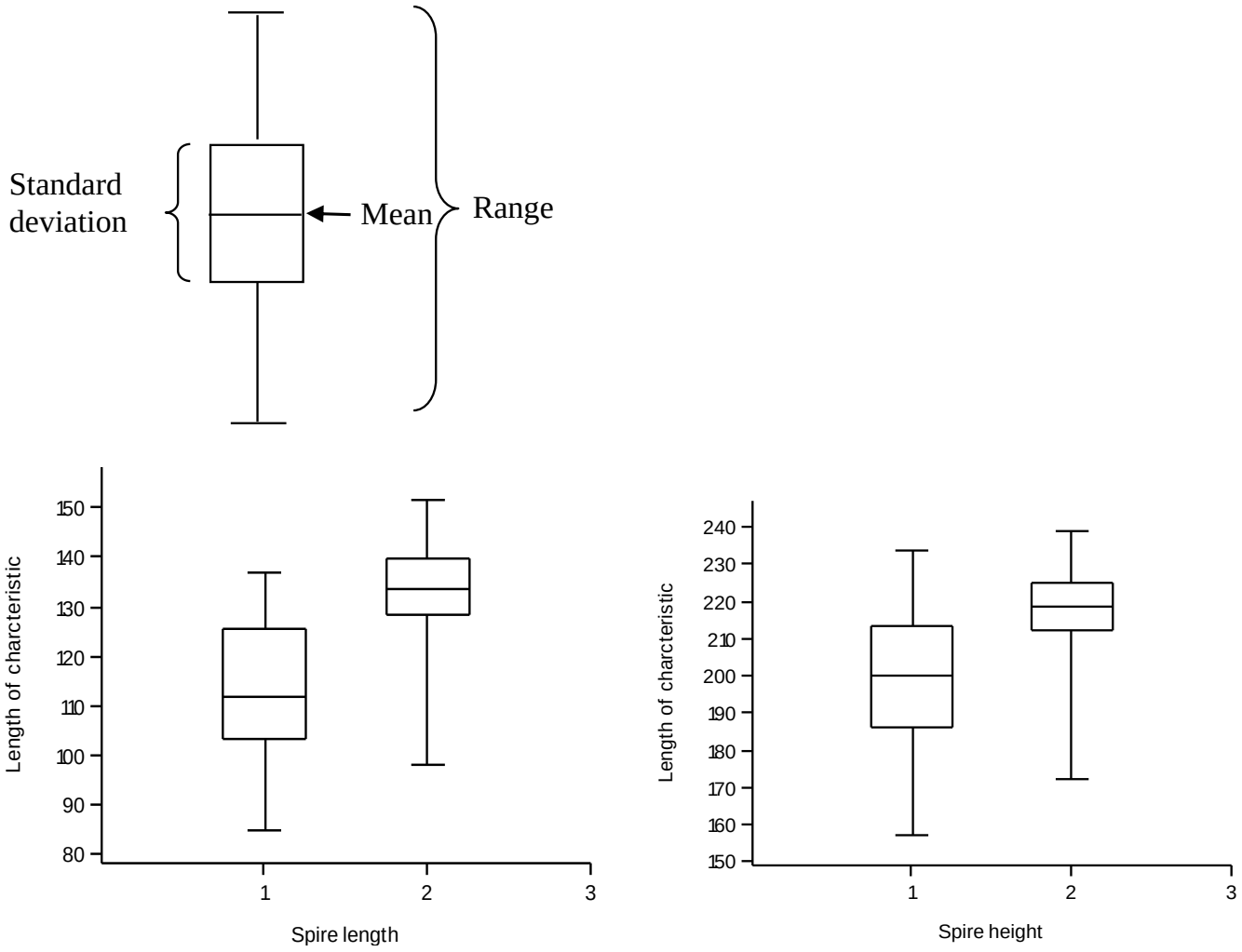

Figure 6. Box plots of morphometric characteristics *for Limicolaria aurora* from 1= New Bussa and 2=Benin City.

for both SH and SL.

RAPD result

The primers used for this study, their sequences as well as their percentage polymorphism are shown in Table 1. All the primers produced good RAPD amplifications with varying bands. Forty four loci were generated from the five primers, of these 41 (93%) were polymorphic, while three (7%) were monomorphic. The average number of loci per primer is nine.

The total number of RAPD bands produced by the primers for the two locations was 178. Samples of *L. aurora* from New Bussa has the highest band score of 103 (65% polymorphism), followed by Benin City population with the band score of 75 with 96% polymorphism (Tables 2 and 3). There was no band that was population specific. OPB-18 primer generated the highest number of bands among all the primers, while OPB-11 produced the least number of bands. The UPGMA cluster diagram revealed two major genotypic groups within the snail populations with about 74% similarity (Figure 7). The first clade consists of all the samples of *L .aurora* from New Bussa, while the second clade consists of all the samples from Benin City.

DISCUSSION

Morphometric studies

Morphological homogeneity was evident between the two populations of *L. aurora* as shown by overlapping of the clusters produced from both PCA and CVA. CVA did not separate the specimens into two distinct populations as shown by the overlapping of clusters (Figure 5). Among the shell characteristics measured, SH was the most variable and consequently the most suitable for the separation of the populations. Another significant

Table 2. Estimates of genetic variation.

Population	Number of polymorphic loci	Number of monomorphic loci	Total	Proportion of polymorphic loci (%)
New Bussa	17	9	26	65
Benin City	26	1	27	96
Combined	41	3	44	93

Table 3. Total band scored in each population.

Population	Total band score
New Bussa	103
Benin city	75
Total	178

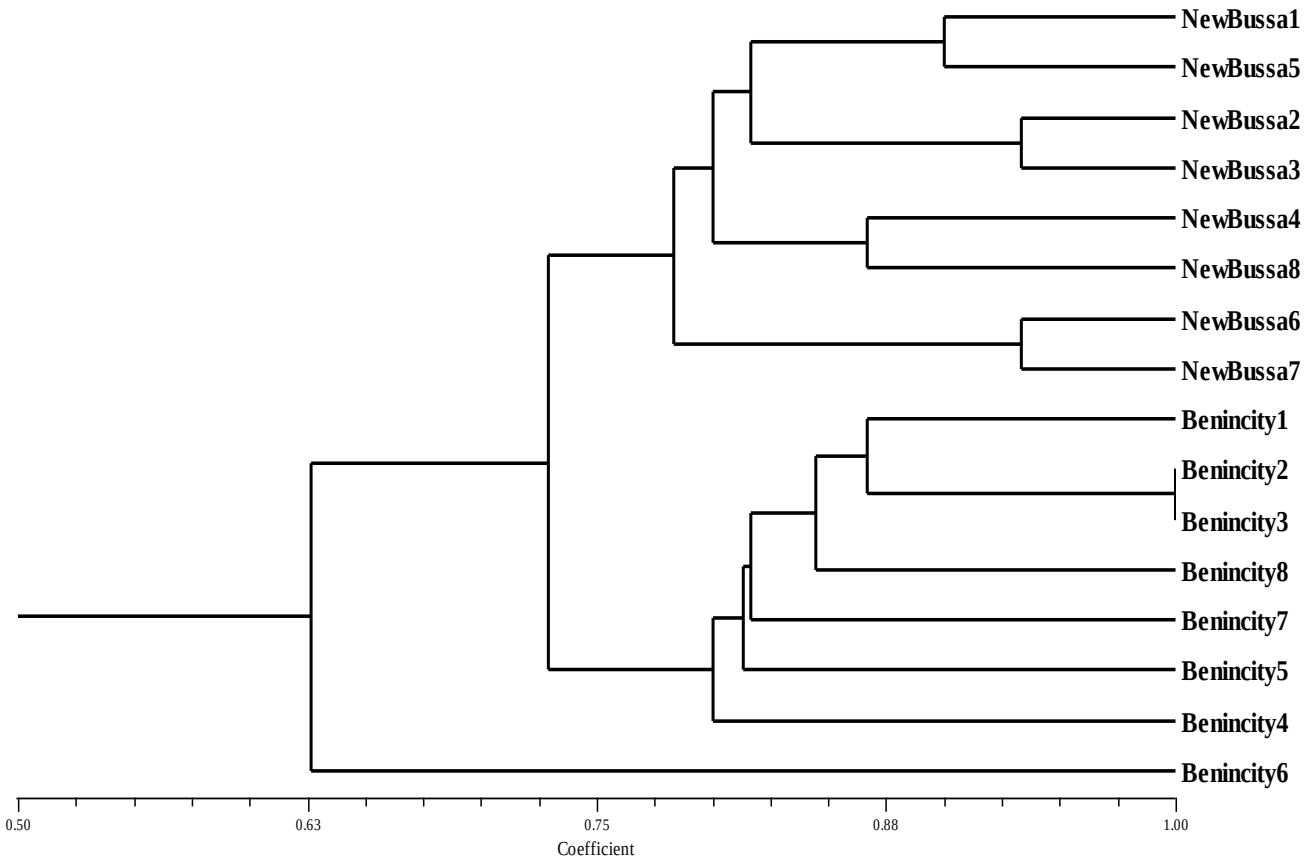

Figure 7. UPGMA dendrogram summarizing the data on differentiation between *Limicolaria aurora* populations according to RAPD analysis.

diagnostic characteristic was the SL.

Conchological variations observed on the shells of the *L. aurora* from different geographical locations may be due to many possible factors including genetic, biotic and abiotic factors. Since *L. aurora* exhibited morphological differences among populations, it is argued that the difference or variations in size can be attributed to environmental conditions as well as genetic factors. Dela et al. (2010) argued that variation in snail shells is not only genetic, but also affected by the growth rate and population density of the snails. It may also be possible that the diversity within populations observed could be

due to the numerous introduction and reintroduction of several gene pools of snails to the study areas by local snail marketers.

The variations in the snail's shell morphology could also indicate phenotypic plasticity or genetic differentiation. Plasticity influences the evolution and adaptive responses of organisms, because it can alter the relationship between the phenotype and the genotype (Trussell and Etter, 2001).

It is also possible that since the snails were collected from different geographical locations which have varied climatic and environmental factors, these may have direct effects on the snail shell shapes.

RAPD studies

Percentage polymorphism was high in the two populations studied. This was 96% in Benin City and 65% in New Bussa (Table 2). Analysis of the proportions of polymorphic loci and band sharing based similarity indices for within-location samples indicates that a relatively high level of genetic similarity exists in New Bussa population than in Benin City population, hence higher genetic diversity in Benin City population than New Bussa population. The higher within location genetic similarity and lower level of frequency of polymorphic loci and gene diversity estimates for New Bussa population could be an indication of comparatively closer relationship among individuals within the location.

Aestivation stage of the organism, humidity and temperature range in a habitat of the land snail are factors that were argued to have influenced variations in land snail (Vinic et al., I998; Albuquerque et al., 2009). Environmental stress such as drought could possibly have influenced genetic diversity in New Bussa with lower annual rainfall compared to Benin City. It may relate to reduced genetic variability in the population, genotype-specific survivorship, that is, individuals with particular genotypes which are tolerant to drought might survive while less tolerant genotypes might be wiped out, hence resulting in reduced genetic variability and diversity.

Moreover, breeding experiments have shown that a large part of the variation leading to the characterization of some morphotypes is genetically determined (e.g. the 'giant' form *Helix aspersa maxima* or the 'conical' form *Helix aspersaconoidea*), but within a given form, the variation in the trait itself is sometimes subject to a strong environmentally induced component (Madec and Guiller, 1993; Madec et al., 1998).

Genetic drift and natural selection are the two primary evolutionary mechanisms that cause population differentiation (Hufford and Mazer, 2003). Natural selection by ecological factors will result in development of ecological adaptation or ecotypes. It remains to be determined whether the observed population differentiation in this study resulted from any natural selection.

The UPGMA cluster diagram revealed two major clades. The first cluster comprises of New Bussa specimens, while the second cluster comprises mainly specimens from Benin city. UPGMA cluster separation of the specimens into two distinct groups reveals that the two populations are genetically distinct from each other. Estimate of genetic distance between the populations is 0.9245.

In conclusion, DNA based analysis unlike morphometrics employed in this study differentiates the populations of *L. aurora* under study into two sub populations which may imply that the basis of gross morphological differences in these populations might not be due to environmental factors only but also genetic. The RAPD analysis differentiates the populations better, revealing two distinct groups. According to Thorpe and Sol-Cave (1994), the average genetic distance for conspecific populations is 0.05 (range: 0.02 to 0.07), the high genetic distance (0.9245) between the populations in this study revealed that the two populations of *L. aurora* are not categorized in conspecific value, hence a subspecies of *L. aurora* is suspected. Further studies involving the use of DNA sequence analysis is needed to maximize the efficiency of this study.

Conflicts of Interest

The authors declare no conflict of interest.

REFERENCES

Albuquerque FS, Peso-Aguiar MC, Assunção-Albuquerque MJT, Gálvez L (2009). Do climate variables and human density affect Achatinafulica (Bowditch) (Gastropoda: Pulmonata) shell length, total weight and condition factor? Braz. J. Biol. 69(3):879-885.

Awodiran MO, Awopetu JI, Odekanyin OO, Ogunbosoye O (2013). An electrophoretic study of protein diversity in five species of land snails (Achatinidae) from Nigeria. Int. J. Biol. Chem. Sci. 7(1):86-95.

Bucklin A (1992). Use of formalin-preserved samples for molecular analysis. Newsl. Crustacean Mol. Tech. 2:3.

Chiu Y, Chen H, Lee S, Chen CA (2002): Morphometric analysis of shell and operculum variations in the Viviparid snail, *Cipangopaludina chinensis* (Mollusca: Gastropoda), in Taiwan. Zool. Stud. 41(3):321-331.

Crowley TE, Pain T (1970).A monographic revision of the African Land Snails of the Genus *Limicolaria.* Sci. Zool. 177:1-61

Dela RVG, Torres MAJ, Demayo CG. (2010). Geometric Morphometric Tools in the Analysis of Shell Shape of Twelve Local Populations of the Invasive Snail *Achatina fulica* Bowdich from the Philippines. International Conference on Environmental Engineering and Applications 2010: 91-95.

Gow J, Noble, LR, Rollinson D, Mimpfoundi R, Jones CS (2004). Breeding system and demography shape population genetic structure across ecological and climatic zones in the African freshwater snail, *Bulinus forskalii* (Gastropoda, Pulmonata), host for *schistosomes*. Mol. Ecol. 13:3561-3573.

Graveland J, van der Wal R (1996). Decline in snail abundance due to soil acidification causes eggshell defects in forest passerines. Oecologia 105:351-360.

Gunawickrama KBS (2007). Morphological heterogeneity in some estuarine populations of the catfish *Arius jella* (Ariidae) in Sri Lanka. Ceylon J. Sci. (Biol. Sci.) 36(2):101-108.

Hammer O, Harper DAT, Ryan PD (2001). PAST: Paleontological Statistics Software Package for Education and Data Analysis.

Paleontol. Electronica 4(1):50-56.
Hotopp KP (2002). Land snails and soil calcium in central Appalachian Mountain forest. Southeast. Nat. 1:27-44.
Hufford KM, Mazer SJ (2003): Plant ecotypes: Genetic differentiation in the age of ecological restoration. Trends Ecol. Evol. 18:147-155.
Madec L, Giller A (1993). Observations on distal genitalia and mating activity in three conchologically distinct forms of the land snail *Helix aspersa*. J. Molluscan Stud. 59:455-460.
Madec L, Guiller A, Coutellec-Vreto MA, Desbuquois C (1998). Size-fecundity relationships in the land snail *Helixaspersa*: preliminary results on a form outside the norm. Invertebr. Reprod. Dev. 34:83-90.
Madu CT, Sogbesan OA, Ugwumba AA (2006) Nutritive potentials and utilization of garden snail (*Limicolaria aurora*) meat meal in the diet of *Clarias gariepinus* fingerlings. Afr. J. Biotechnol. 5(20):1999-2003.
Nei M (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583-590.
Oke OC (2007). Land snail diversity in a patch of cocoa plantation in Erin-Ijesha hills, Osun State, Nigeria. African Scientist 8 (2): 61-68.
Oke OC (2013). Terrestrial mollusc species richness and diversity in Omo Forest Reserve, Ogun State, Nigeria. Afr. Invertebr. 54(1):93-104.
Oke OC, Alohan FI (2006).The land snail diversity in a square kilometre of tropical rainforest in Okomu National Park, Edo State, Nigeria. Afr. Sci. 7(3):135-142.
Oke OC, Chokor JU (2010). Land snail species richness in Idanre hills, Ondo State, Nigeria. Afr. J. Ecol. 48:1004-1008.
Oke OC, Odiete WO (1996).New species of land molluscs from south western Nigeria. I. A new species attributed to *Ptychotrema* (Mollusca, Gastropoda). J. Afr. Zool. 110:61-64.
Oke OC, Alohan FI, Edosom wan EU (2007). Land snail diversity and abundance in a patch of secondary tropical rainforest in Ekpoma, Edo State, Nigeria. Afr. Sci. 8(2):55-60.
Oke OC, Alohan FI, Uzibor MO, Chokor JU (2008).Land snail diversity and species richness in an oil palm agroforest in Egbeta, Edo State, Nigeria. Biosci. Res. Commun. 20(5):249-256.
Pfenninger M, staubach S, Albrecht C, Streit B, Schwenk K (2003). Ecological and morphological differentiation among cryptic evolutionary lineages in freshwater limpets of thenominal form-group *Ancylus fluviatilis*. Mol. Ecol. 12:2731-2745.
Raut SK, Barker GM (2002). *Achatinafulica* and other *Achatinidae* as pests in tropical agriculture; In: Molluscs as crop pests. CABI Publishing, New Zealand, Pp. 55-114.
Reist JD (1985). An empirical evaluation of several univariate methods that adjust for size variation in morphometric data. Can. J. Zool. 63: 1429-1439.
Rohlf J (2000). NTSYS pc Version 2.0.Exeter software, Setauket, New York.
Thorpe JP, Sol-cave AM (1994).The use of allozyme electrophoresis in invertebrate systematics. Zool. Scr. 23:3-18.
Trussell GC, Etter RJ (2001).Integrating genetic and environmental forces that shape the evolution of geographic variation in a marine snail. Genetica 112–113:321-337.
Vinic GK, Unnithan VK, Sugunan VV (1998). Farming of the Giant African Snail, *Achatina fulica*. India: Central Inland Capture Fisheries Research Institute. 56:1-24.
Wullschleger EB, Jokela J (2002). Morphological plasticity and divergence in life-history traits between two closely related freshwater snails; *Lymnea ovata* and *Lymnae aperegra*. J. Molluscan Stud. 68:1-5.

academicJournals

Vol. 15(25), pp. 1315 -1319, 22 June, 2016
DOI: 10.5897/AJB2015.15093
Article Number: 64D601559047
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic polymorphism of bone morphogenetic protein receptor 1B (BMPR-1B) gene and its association with litter size in Indonesian fat-tailed sheep

Maskur M.*, Tapaul R., and Kasip L.

Animal Breeding and Genetics Laboratory, Faculty of Animal Science, University of Mataram, Majapahit St. 62. Lombok Nusa Tenggara Barat, Indonesia, 83125.

Received 9 November, 2015; Accepted 23 May, 2016

The Indonesian fat-tailed sheep (IFTS) is a local sheep that has been long time raised and well adapted to the extreme environments of Lombok Island. The present study was conducted to determine the polymorphism of bone morphogenetic protein receptor 1B (BMPR-1B) gene and its association with litter size in the IFTS breed by employing forced polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods in the IFTS. Polymorphism of BMPR-1B genes on IFTS population were identified by the forced PCR-RFLP methods. The result of this study showed that mutation of BMPR-1B gene resulted in two allele of wild type (+) and mutan allele (B) with frequencies of 0.807 and 0.193, respectively and three genotype of BB (110 bp/110 bp), B+ (110 bp/140 bp), and ++ (140 bp/140 bp) with almost unequal frequencies distribution of 0.060, 0.268, and 0.672. Genetic diversity of BMPR-1B genes caused different litter sizes on individual IFTS. A highly significant difference (P<0.01) was observed in the average of litter size in different genotypes. The highest average of litter size was found in the genotype of BB, that is, 1.685 lambs.

Key words: Bone morphogenetic protein receptor 1B (BMPR-1B), gene, mutation, polymorphism, litter size.

INTRODUCTION

Prolificacy in sheep is regulated by three major genes: bone morphogenetic protein receptor 1B (BMPR-1B), bone morphogenetic protein 15 (BMP-15), and growth differentiation factor 9 (GDF9). BMPs are members of the transforming growth factor β (TGF-β) superfamily. BMP is multifunctional protein which controls growth, differentiation and apoptosis in many types of cells and plays indispensable roles during embryogenesis and the fertility in mammals (Davis, 2005).

BMPR-1B (fecundity booroola/*Fec*B) gene is an autosomal gene located on sheep chromosome 6 which is syntenic with human chromosome 4 and is the first major gene for prolificacy identified in sheep (Wilson et al., 2001). The BMP receptor is expressed by oocytes and granulosa cells from the primary stage to the late antral stage of follicle development and binds to BMP15. Mutation in the BMPR-1B gene is a single nucleotide non-conservative substitution that has an additive effect

*Corresponding author. E-mail: maskur07@yahoo.co.id.

on ovulation rate (Davis, 2006; Pramod et al., 2013). A single copy of the gene in heterozygous ewes produced about 1.5 extra eggs and gave birth to about 1.0 extra lamb per ewe lambing. Homozygous ewes carrying two copy of the gene produced about 3.0 extra eggs resulting in about 1.5 extra lambs per ewe lambing (Davis, 2004).

The *Fec*B mutation in sheep is due to a single nucleotide substitution (Arginine to Glutamine) at 746th position of open reading frame (ORF) in exon 6 that induces a nonsynonymous substitution of glutamine with an arginine corresponding to 249th position (Q249R) of the mature peptide (Mulsant et al., 2001). This mutation causes loss of reaction capabilities to BMP-4 that plays a central role in determining the formation of the primordial germ cell (PGC) in the ovary (Mulsant et al., 2001; Wilson et al., 2001). The demage on the BMP system during follicle development led to increase average ovulation (Fabre et al., 2006) and was reported in Australian Merino sheep, Indian Garole, Kendrapara, and Bonpala sheep (Kumar et al., 2008; Roy et al., 2011).

Genetic mechanism caused by mutations in BMPR-1B and BMP-15 genes, which has a relationship with the average number of ovulation in sheep, is still not widely known. The tendency for sheep producing twins (twining) or three (triplets) lambs are the same, although there are differences in the level of gene regulation. The Indonesian fat-tailed sheep (IFTS) is a local prolific and high economic value sheep in Indonesia. It is essential to do research on the genetics and reproduction in IFTS to identify genes with major effect on prolificacy which will be useful for increasing and accelerating the rate of genetic improvement on litter size. The aim of this study was to identify polymorphism in BMPR-1B gene and its possible association with litter size in IFTS.

MATERIALS AND METHODS

The University of Mataram, Faculty of Medicine, Ethical Committee for Medical Research, Mataram, Indonesia approved all animal procedures for this experiment (Register No.35/UN18.8/ETIK/2015).

Phenotyping (measurement of the litter size)

Two hundred and fifty IFTS (aged 1.5 to 3 years) reared under extensive communal conditions were used in this study. All animals were ear tagged. The number of lambs born for every birth of each ewe was recorded. The average litter size for each ewe of the first three parities was recorded and used for further analysis.

Sample collection and DNA isolation

Blood samples for DNA analysis were collected from the jugular vein of each animal. Blood was collected on K_2EDTA 0.5 M and stored at -25°C for few weeks or at -75°C up to several months. Genomic DNA was extracted from whole blood by the phenol-chloroform method, then dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), and kept at -20°C (Sambrook et al., 1989).

Detection of the *Fec*B mutations

A primer pair was designed to detect single nucleotide polymorphisms in exon 6 of the BMPR-1B gene in prolific IFTS by PCR-RFLP as described by Wilson et al. (2001). Primers amplified a 140-bp band. After digestion with Ava II (G|GWCC), the BB animals had a 110-bp band, the B+ animals had 140- and 110-bp bands, and the ++ animals had a 140-bp band. The primer sequences were as follows: Forward, 5′-GTCGCTATGGGGAAGTTTGGATG-3′ and Reverse, 5′-CAAGATGTTTTCATGCCTCATCAACACGGTC-3′.

Amplification was carried out in a volume of 25 µl. The PCR reaction contained 100 ng DNA, 0.5 µM of each primer (10 pmol/µl), 1x PCR buffer (10 mM Tris-HCl pH 9.0), 1.5 mM $MgCl_2$ and 50 mM KCl, 5% deionized formamide, 200 µM dNTPs, and 0.025 U Taq DNA polymerase (Pharmacia). Amplification was performed for 35 cycles using DNA thermal Cycler (Perkin Elmer Cetus Corp.). The first cycle was at 95°C for 5 min followed by 35 subsequent cycles of 94°C × 45 s, 60°C × 45 s, then 72°C × 60 s, and the last cycle at 72°C for 5 min. The PCR products of 5 µl were digested separately with 10 U of *Ava* II (Fermentas) at 37°C over night in a 15 µl reaction mixture. The DNA fragments of BMPR-1B were separated by electrophoresis on 2.5% agarose gels. The gels were visualized with ethidium bromide staining and analyzed using an AlphaImager EP Documentation and Analysis Systems (Alpha Innotech Corporation, USA).

Statistical analysis

Genotype and allele frequency within and among genetic groups were determined by the method of Goodman adapted by Maskur et al. (2014). Association analysis was performed using general linear model (GLM) and the least square means of the genotypes were compared by *t*-test (as implemented in the SAS programme). The linear model used was as follows:

$$Y=A+LS+Gi+eij$$

where Y is phenotypic value of litter size, A is population mean, LS is lambing season effect, Gi is fixed effect of the BMPR-1B genotype, and eij is random error.

RESULTS AND DISCUSSION

Detection of polymorphism in BMPR-1B (*Fec*B) gene in IFTS

The PCR product is about 140 bp, located in exon 6 of the BMPR-1B gene. Mutations at the gene can be identified using restriction enzyme *Ava* II (G|GACC). The digestion using *Ava* II (G|GACC) produces two alleles, namely the wild-type allele (+) is 140 bp (uncut) and the mutant allele (*B*) is 110 bp/30 bp (cut). The wild-type allele (+) was not sensitive to *Ava* II, while the mutant allele (*B*) was cut by *Ava* II producing two DNA fragments 110 and 30 bp. The analysis of the *FecB* locus implies that mutation occured to make it sensitive to *Ava* II enzyme which recognized the sequence (G|GACC) as the site of cutting.

Mutations at the BMPR-1B gene locus are transition mutations that change adenine bases into guanine (A/G transition) at base 746 of the coding region of the BMPR-

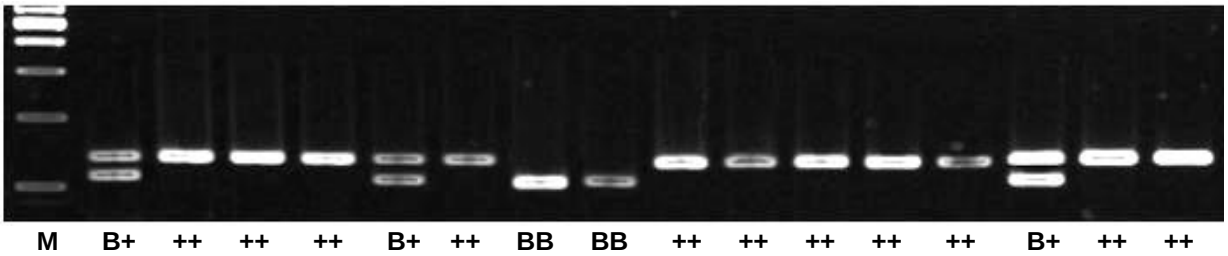

Figure 1. The *FecB* mutation of *BMPR-1B* gene was digested by *AvaII*. M= 100 bp DNA marker. "wild-type"(+) 140 bp and mutant allele (B) 110 and 30 bp.

Table 1. Allelic and genotypic frequency of BMPR1B gene in Indonesia fat tailed sheep.

Gene	N	Frequency					χ^2 (HWE)
		Genotype			Allele		
BMPR-1B	250	BB	B+	++	B	+	5.114**
		0.060	0.268	0.672	0.193	0.807	

H-WE: Hardy-Weinberg equilibrium. The number of individuals for the BB, B+ and ++ genotypes are given in Table 3. **P<0.01.

1B gene. This point mutation results in a change in the amino acid sequence of BMPR-1B in which a glutamine (CAG) in the wild-type to an arginine (CGG) in the mutant (CAG→CGG, Q249R). The alteration of amino acid sequence causes functional change in the intracellular kinase domain of mature protein and was reported to affect ovulation rate in Australian Merino sheep, Indian Garole, Kendrapara and Bonpala sheep (Kumar et al., 2008; Roy et al., 2011), British Milk sheep, Chinese Small Tail Han and Hu sheep (Chu et al., 2007).

The *FecB* mutation of the *BMPR-1B* gene has been studied in various sheep breeds. The results indicated that IFTS carried the same *FecB* mutation as found in Small Tailed Han ewes (Chu et al., 2007), Greek Chios and Florina sheep (Michailidis et al., 2008) and Booroola Merino ewes (Mulsant et al., 2001; Souza et al., 2001; Wilson et al., 2001). The single nucleotide polymorphisms (SNPs) identified in exon 8 (GenBank accession number GQ863578) of Mehraban sheep was found to be associated with reproduction traits (Abdoli et al., 2013).

Allelic and genotypic frequency of BMPR1B gene in IFTS

Allelic and genotypic identification of exon 6 BMPR-1B gene using the forced PCR-RFLP technique produces two alleles, namely, the wild-type allele (+) and the mutant allele (B) with three genotypes BB, B+ and ++ (Figure 1). There are contrasting allelic frequencies distributions between B and + allele in BMPR-1B gene of IFTS. The distribution of allelic frequency of the + allele was higher than B allele, respectively of 0.807 and 0.193, while the frequency distribution of the genotypes BB, B+ and ++ were respectively 0.060, 0.268 ,and 0.672 (Table 1).

Tabel 2. Genetic index of Indonesian fat tail sheep population.

Gene	H_o	H_e	SE	PIC
BMPR-1B	0.268	0.313	0.0190	0.264

H_o: Heterozygosity observation; H_e: heterozygosity expectation; SE: standard error; PIC: polymorphic information content.

Chi-square (χ2) test showed that the genotype distributions of exon 6 BMPR-1B gene was not at Hardy-Weinberg equilibrium (H-WE) in IFTS. The genotype frequencies at polymorphic loci of exon 6 BMPR-1B gene showed a highly significant difference (P<0.01). This contrasts with the same *Fec*B mutation in the Small Tailed Han ewes in China, as reported by Chu et al. (2007) where the allelic frequencies distributions between the B and + allele differed, the + allele (0.27) being lower than that of the B allele (0.73), while the frequencies of genotypes BB, B+, and ++ were 0.52, 0.42, and 0.06, respectively.

Genetic index has an important meaning to get the description about the genetic variability (Marson et al., 2005). Data in Table 2 show the results of genetic index measurements in IFTS population. These data indicate a genotypic imbalance in the population where genotype heterozygote frequencies are higher than the Hardy-Weinberg expectation agreement. This could be due to intensive selection, resulting in a tendency towards the

Table 3. Average litter size in Indonesian fat tail sheep based on the genotype of the BMPR-1B gene.

Genotype	N	Litter size
BB	15	1.685 ± 0.165*
B+	67	1.455 ± 0.254*
++	168	1.145 ± 0.108**

*P<0.05; **P<0.01.

accumulation of certain genotypes (Tambasco et al., 2003) and the possibility of inbreeding (Machado et al., 2003). Polymorphism information content (PIC) value is commonly used in genetics as a measure of polymorphism for a marker locus used in linkage analysis. Based on the PIC value of 0.264, it can be stated that the genetic diversity of BMPR-1B gene within IFTS population is at the medium level. This statement is based on PIC levels of polymorphism as determined by Botstein et al. (1980) in which levels of ≤0.25 are classified as low, 0.25≤PIC≤0.5 are classified as medium and PIC≥0.5 are classified as high polymorphism.

Association of BMPR-1B gene polymorphism with litter size in IFTS

The association between genotype of the BMPR-1B gene and average litter size in IFTS is shown in Table 3. The effect of genotype of BMPR-1B gene was found to be significant for litter size on IFTS. BMPR-1B is a receptors for majority of the TGF-β superfamily members. One explanation could be that the action of BMPR-1B gene on the target gene through formation of the receptor complexes, causes the phosphorylation of intracellular signaling molecules called Smads, which then translocate to the nucleus and regulate transcription of target genes (Moore et al., 2002).

The effect of *Fec*B mutation on litter size in the IFTS is lower than other breeds that had been reported by any researcher. Expression of BMPR-1B gene can varied between different breed of sheep and different environmental conditions or the interaction between breed of sheep and environment will give different expression (Fogerty, 2008). The difference could be due to the background genotype, environmental factors (feeding and management) such as the relatively low nutritional value of the tropical forages available to these ewes or combination of these factors. IFTS were reared under the extensive farming system in the region with dry weather along 8 to 9 months per year. This condition causes low nutritional value of forage that is available to support the production and reproduction of IFTS. Several studies showed the different expression of BMPR-1B gene in different environmental conditions. Guan et al. (2007) reported that the expression of BMPR-1B gene on Garole and Hu sheep varied between different location with different environmental conditions. The litter size of Garole sheep is 2.27 in humid rice paddies of the Sundarbans region, but lower mean litter size is 1.74 in the semiarid environment of the Deccan plateau of Maharashtra and 1.68 in Rajhastan. The avarage litter size of Hu sheep in conservation area in China is 2.12, higher than in Shanghai and Suzhou is 1.78 and 1.90 respectively. The Small Tailed Han ewes with genotypes *Fec*B^{B}/*Fec*B^{B} (BB); *Fec*B^{B}/*Fec*B^{+} (B+) and *Fec*B^{+}/*Fec*B^{+} (++) has litter size 2.65±0.10, 2.36±0.12, and 1.25±0.17, respectively (Chu et al., 2007). In the Awassi sheep, litter size of ++, B+, and BB ewes was 1.28, 1.90, and 1.92, respectively (Gootwine et al., 2008). Litter sizes of the Javanese sheep with genotypes *Fec*B^{B}/*Fec*B^{B} (BB), *Fec*B^{B}/*Fec*B^{+} (B+), and *Fec*B^{+}/*Fec*B^{+} (++) for *FecB* have been measured at 2.59, 1.95, and 1.24, respectively (Inounu, 1996).

The data in Table 3 indicates a significant increase in litter size of IFTS that carry FecB mutations in both heterozygous and homozygous condition. This implies that the sheep with FecB mutations has granulosa cells and are more sensitive to follicle stimulating hormorne (FSH); thus, the cells divides more actively leading to ovarian follicles becoming adults and mature (Davis 2008; Fabre et al*.,* 2006). The prolific nature occurs based on the concept that increased ovulation rate lead to an increasing number of ovulated oocytes and if fertilization occurs and embryo viability can be maintained by the parent, it will be followed by the birth of a child more than one (Wilson et al., 2001).

Conclusion

Polymorphism of *Fec*B gene was identified in IFTS by forced PCR-RFLP method. Restriction enzyme digestion with *Ava*II for *Fec*B gene resulted in three genotypes of BB, B+ and ++ with a frequency of 0.060, 0.268 and 0.672, respectively and two alleles of wild type (+) and mutan (B) with almost unequal frequency distribution of 0.807 and 0.193. Genotypic polymorphism of BMPR-1B has a significant influence on litter size in IFTS. Higher litter size was observed in BB genotype as compared to B+ and ++ gentopes of *Fec*B gene.

Conflict of Interests

The authors have not declared any conflict of interests.

Abbreviations

IFTS, Indonesian fat-tailed sheep; **PCR-RFLP,** polymerase chain reaction-restriction fragment length polymorphism; ***FecB*, Fecundity Booroola; BMPR-1B,** bone morphogenetic protein receptor type-1B; **BMP-15,**

bone morphogenetic protein 15; **GDF9,** growth differentiation factor; **TGF-β,** transforming growth factor β; **FSH,** follicle stimulating hormorne.

REFERENCES

Abdoli R, Zamani P, Deljou A, Rezvan H (2013). Association of BMPR1B and GDF9 genes polymorphisms and secondary protein structure changes with reproduction traits in Mehraban ewes. Gene 524(2):296-303.

Botstein D, White RL, Skolnick M, Davis RW (1980). Construction of a genetic linkage map in human using restriction fragmenth length polymorphisms. Am. J. Hum. Genet. 32:314-331.

Chu MX, Liu ZH, Jiao CL, He YQ, Fang L, Ye SC, Chen GH, Wang JY (2007). Mutations in BMPR-IB and BMP-15 genes are associated with litter size in Small Tailed Han sheep (*Ovis aries*). J. Anim. Sci. 85:598-603

Davis GH (2004). Fecundity genes in sheep. Anim. Reprod. Sci. 82-83:247-253.

Davis GH (2005). Major genes affecting ovulation rate in sheep. Genet. Sel. Evol. 37(Suppl. 1):S11-S2.

Davis GH (2008). The Booroola gene: origin, distribution, use and management of the *FecB* mutation. Use of the FecB (Booroola) gene in sheep-breeding programs. Proc. of the Helen Newton Turner Memorial International Workshop held in Pune. Maharashtra. India. 1:22-29.

Davis GH, Balakrishnan L, Ross IK, Wilson T, Galloway SM, Lumsden BM, Hanrahan JP, Mullen M, Mao XZ, Wang GL, Zhao ZS, Zeng YQ, Robinson JJ, Mavrogenis AP, Papachristoforou C, Peter C, Baumung R, Cardyn P, Boujenane I, Cockett NE, Ey-thorsdottir E, Arranz JJ, Notter D (2006). Investigation of the Booroola (FecB) and Inverdale (FecXI) mutations in 21 prolific breeds and strains of sheep sampled in 13 countries. Anim. Reprod. Sci. 92:87-96.

Fabre S, Pierre A, Mulsant P, Bodin L, Di Pasquale E, Persani L, Monget P, Monniaux D (2006). Regulation of ovulation rate in mammals: contribution of sheep genetic models. Reprod. Biol. Endocrinol. 4:20.

Fogerty (2008). Environmental modulation of FecB expression. Use of the FecB (Booroola) gene in sheep-breeding programs. Proc. of the Helen Newton Turner Memorial International Workshop held in Pune. Maharashtra. India. 1:66-75.

Gootwine E, Reicher S, Rozov A (2008). Prolificacy and lamb survival at birth in Awassi and Assaf sheep carrying the FecB (Booroola) mutation. Anim. Reprod. Sci. 108(3-4):402-411.

Guan F, Liu SR, Shi GQ, Yang LG (2007). Polymorphism of *FecB* gene in nine sheep breeds or strains and its effects on litter size, lamb growth and development. Anim. Reprod. Sci. 99:44-52.

Inounu I (1996). Keragaan produksi ternak domba prolifik [disertasi]. Bogor: Program Pascasarjana. Institut Pertanian Bogor.

Kumar S, Mishra AK, Kolte AP, Dash SK, Karim S (2008). Screening for Booroola (FecB) and Galway (FecXG) mutations in Indian sheep. Small Rumin. Res. 80(1-3):57-61.

Machado MA, Schuster I, Martinez ML, Campos AL (2003). Genetic diversity of four cattle breeds using microsatellite markers. Rev. Bras. Zootec. 32:92-98.

Marson EP, Ferraz JB, Meirelles FV, Balieiro JC, Eler JP, Figueiredo LG, Mourao GB (2005). Genetic characterization of European-Zebu composite bovine using RFLP markers. Genet. Mol. Res. 4(3):496-505.

Maskur, Rodiah, Chairussyuhur Arman (2014). Association of a novel single nucleotide polymorphism in growth hormone receptor gene with production traits in Bali cattle. Ital. J. Anim Sci. 13:3461

Michailidis G, Melpomeni A, Pappa V (2008). Reproductive performance and investigation of BMPR-IB and BMP-15 gene mutations in Greek Chios and Florina sheep breeds. Arch. Zootech. 11(1):24-31.

Moore RK, Otsuka F, Shimasaki S (2003). Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. J. Biol. Chem. 278:304-310.

Mulsant P, Lecerf F, Fabre S, Schibler L, Monget P, Lanneluc I, Pisselet C, Riquet J, Monniaux D, Callebaut I, Cribiu E, Thimonier J, Teyssier J, Bodin L, Cognie Y, Chitour N, Elsen JM (2001). Mutation in the bone morphogenetic protein receptor-IB is associated with increased ovulation rate in Booroola ewes. Proc. Natl. Acad. Sci. U.S.A. 98: 5104-5109.

Pramod KR, Sharma SK, Kumar R, Rajan A (2013). Genetics of ovulation rate in farm animals. Vet. World 6(11):833-838.

Roy J, Polley S, De S, Mukherjee A, Batabyal S, Pan S, Brahma B, Datta TK, Goswami SL (2011). Polymorphism of fecundity genes (FecB, FecX, and FecG) in the Indian Bonpala sheep. Anim. Biotechnol. 22(3):151-162.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning : A laboratory Manual. 2nd edition. Cold Spring Harbor Laboratory Press, USA.

Souza CJH, Mac Dougal C, Campbell BK, McNeilly AS, Baird DT (2001). The Booroola (*FecB*) phenotype is associated with a mutation in the bone morphogenetic receptor type 1B (BMPR-1B) gene. J. Endocrinol. 169:1-6.

Tambasco DD, Paz CCP, Tambasco-Studart, Pereira AP, Alencar MM, Freitas AR, Coutinho LL, Packer IU, Regitano LCA (2003). Candidate genes for growth traits in beef cattle crosses *Bos taurus* X *Bos indicus.* J. Anim. Breed. Genet. 120:51-56.

Wilson T, Wu XY, Juengel JL, Ross IK, Lumsden, JM, Lord EA, Dodds KG, Walling GA, McEvan JC, O'Connel AR, McNatty, KP, Montgomery GW (2001). Highly prolific Booroola sheep have a mutation in the intracellular kinase domain of bone morphogenetic protein IB receptor (ALK-6) that is expressed in both oocytes and granulosa cells. Biol. Reprod. 64:1225-1235.

academicJournals

Vol. 15(25), pp. 1320-1329, 22 June, 2016
DOI: 10.5897/AJB2015.15183
Article Number: 74FFC3659049
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Does the transgenic Cry1Ac toxin adversely affect the population dynamics of floral-visiting insects in soybean crop?

Oliveira, F. and Fernandes, M. G.*

Programa de Pós Graduação em Entomologia e Conservação da Biodiversidade, Dourados, Mato Grosso do Sul, Brazil.

Received 18 December 2015, Accepted 29 April, 2016

One of the strategies of Integrated Pest Management (IPM)-soybean is the use of genetically modified plants. However, there are concerns about the unknown effects associated with this technology on non-target organisms. The objective of this study was to determine the population dynamics of species of floral-visiting insects in *Bacillus thuringiensis* (Bt) and non-Bt soybean. In the experiment, two soybean treatments were assessed, one of soy DM 6563 Intacta and the other BMX Potência RR. Floral-visiting insects were collected in the flowering period every 15 min/h for 12 h/day, every 3 to 4 days. 549 floral-visiting insects (Class Insecta) were collected, comprising 8 orders, 30 families, and 92 species. Of these, 279 were present in the cultivar DM 6563 Intacta and 270 in the cultivar BMX Potência. The most abundant species were *Apis mellifera* (35.15%), *Musca* species 1 (10.01%), and *Lagria villosa* (5.28%). Species composition was similar for the two cultivars. The highest number of species visited the flowers of the cultivars between 8:00 and 11:00 am, while the lowest intensity of visitation occurred between 12:00 and 15:00 pm. After diversity was determined, it was observed that cultivar BMX Potência (non-Bt) had a higher diversity than cultivar DM 6563 Intacta (Bt).

Key words: Pollinators, species diversity, transgenic.

INTRODUCTION

Soybean culture has been affected by many agricultural pests, including arthropods, resulting in decreased crop yields and reduced seed and grain quality (Roggia, 2010). Frequent pest infestations have resulted in increased use of synthetic chemical insecticides in all producing regions in Brazil. Currently, the harmful effects of insecticides on pollination by entomophilous agents, an important production factor of this agricultural crop, are of concern (Freitas and Pinheiro, 2012; Malaspina and Silva-Zacarin, 2006; Spadotto et al., 2004). Besides increasing the number of fruits or pods, effective pollination can also contribute to increased oil in the seeds, number of seeds per pod, seed weight, shortened crop cycles, uniform seed ripening, and reducing crop losses (Free, 1993;

*Corresponding author. Email: MarcosFernandes@ufgd.edu.br

Freitas, 1997; Nogueira-Couto, 1998). To reduce risks to human health, environmental contamination, and possible harmful effects on pollinating insects, including bees, the restricted use of insecticides in soybean culture has been encouraged (Anvisa, 2015). To minimize consequences from insect pests in soybean culture and regulate the use of pesticides, the Integrated Pest Management of soy (IPM-Soy) was implemented, integrating the use of various strategies and control tactics (Corrêa-Ferreira et al., 2010), one of which is the use of genetically modified plants (transgenic) resistant to pests. Currently, available transgenic plants resistant to caterpillars contain genes of the bacterium *Bacillus thuringiensis* (Bt) that encode lethal toxins for certain insect groups. Bt plants have the potential to minimize losses caused by insect pests, especially the order Lepidoptera, as well as reduce insecticide use (Yu et al., 2011). Bt insecticidal proteins kill insects by a process comprising the following steps: crystal solubilization processing, conversion to an active protoxin form, high-affinity binding to the midgut receptors, irreversible insertion of the toxin into the membrane, and the formation of infection pores (Bravo et al., 2005; de Maagd et al., 2001).

Despite numerous advantages of using genetically modified insect-resistant plants, there are still concerns about the unknown effects associated with this technology (Kouser and Qaim, 2011; Nunes, 2010). Several studies show that the effect this technology may have on non-target organisms is still unclear (Faria et al., 2007; Liu et al., 2005; Naranjo, 2005; Silva, 2013; Whitehouse et al., 2007). Thus, the large-scale use of genetically modified crops for resistance to pests can pose a risk to biodiversity as a result of possible effects on non-target organisms (Dutra et al., 2012), which underscores the importance of conducting studies that assess such interactions.

Non-target organisms are defined as species that may be exposed to Bt proteins for a long time, but which are not direct targets of transgenic technology (Andow and Hilbeck, 2004). Among these organisms, one of the most important groups are the pollinating insects that may be both directly exposed to the toxins of these plants, through consumption of the plant, nectar, or pollen (Nunes, 2010), as indirectly through the food chain when arthropods feed on herbivores or honeydew from insects that have fed off transgenic plants (Groot and Dicke, 2002; Faria et al., 2006).

There is evidence that the expression of Bt toxins in transgenic plants can affect (directly or indirectly) the populations of non-target species (Birch, 1997; Monnerat and Bravo, 2000) by reducing the populations of pollinating insects and floral visitors, including beetles, butterflies, and a number of beneficial arthropods, which can lead to reduced biological pest control (Hong et al., 2008). In Bt maize that expresses the Cry1Ab toxin, as does the soybean used in the present study, a negative effect on the non-target species *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and its larval parasitoid *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) was determined (Vojtech et al., 2005). Similar negative effects were also observed on the natural enemy *Pirata subpiraticus* (Araneae: Lycosidae) in cultures of transgenic rice expressing the same protein Cry1Ab (Chen et al., 2009). Other studies also show that the survival of bees feeding on pollen of Bt cotton expressing this toxin was negatively affected (Liu et al., 2000). Therefore, the objective of the current study was to determine the characteristics of the population dynamics of species of floral-visiting insects in Bt and non-Bt soybean *Glycine max* (L.) Merrill cultivars.

MATERIALS AND METHODS

Experimental area

The experiment was conducted at the experimental farm of the Federal University of Grande Dourados, Dourados-MS, Brazil (22°14′ 20.51″south, 54° 59′M 58.4″ west; altitude 394 m). The studied areas comprised two fields of 1.0 ha each. One field was sown with the cultivar DM 6563 Intact (MON 87701 × MON 89788) and the other with the cultivar BMX Potência RR (M 8360 RR). Both cultivars, which have a similar phenological cycle, were sowed on 5 November, 2014.

Survey of floral visitors

The collection of floral visitors took place throughout the flowering period (26 December, 2014 to 11 January, 2015) of the cultivars. The sampling method was adapted from that of Pires et al. (2006a). The collection of floral visitors was done to determine the abundance and diversity of these insects in the Bt and non-Bt soy, as well as visitation characteristics and species composition for each period in both cultivars.

Four evaluations were carried out every three or four days and in each assessment. Active insect collection was carried out every 15 min for 12 h in each cultivar. Insects were collected using an insect net or directly in a deadly vial. All insects found resting on or taking off from flowers were captured. After the 15 min of collection, the dead insects were placed in an envelope, stating date/time, collector, and treatment and then stored in a styrofoam box to be taken to the Entomology Laboratory of the Federal University of Grande Dourados (UFGD) for later sorting.

Identification of floral visitors

The insects were identified to the lowest taxonomic level possible using specific keys for the groups and confirmed with experts in the field.

Statistical analysis

To analyze the diversity of the two communities studied, the Shannon-Wiener function was calculated (Shannon and Weaver, 1949), as well as Simpson' index (Pinto-Coelho, 2002). To obtain representative gradients of the floral visitors community structure, based on the species composition at each time and day of collection, the normality of the data of all individuals of each species

in each day and time of collection, Kolmogorov-Smirnov, and Shapiro-Wilk tests were used. Then, comparative analysis was performed between the different schedules and treatments using the Kruskal-Wallis test.

RESULTS

Both cultivars flowered for approximately 15 days. During flowering, 549 species of floral-visiting insects (Class Insecta) were collected, comprising 8 orders, 30 families, and 92 species. Of this total, 279 individuals (50.81%) were present in the Bt cultivar, whereas 270 (49.18%) were collected from flowers of non-Bt cultivar (Table 1). No significant differences were noted in the abundance of insect species in relation to the cultivars, as similar numbers of individuals were observed in both cultivars. The Hymenoptera, to which the largest number of collected specimens belonged, showed the highest abundance among all of the orders collected, both in the Bt cultivar where 122 individuals were sampled (43.72% of the total found in this cultivar), as well as in the non-Bt cultivar that presented 121 individuals of this order (44.81%). Among the specimens from this order collected, representatives of the Apidae family were the most abundant, representing 40.86 and 38.14%, respectively, of all insects found in the Bt and non-Bt cultivars, totaling 9 species in both cultivars, and *Apis mellifera* was the most abundant.

Diptera had the highest diversity, with 15 species in the non-Bt cultivar and 17 species observed in the Bt cultivar, with Hymenoptera having 22 species in the Bt cultivar and 16 in the non-Bt cultivar. Coleoptera had 10 species in the Bt cultivar and 13 species in the non-Bt cultivar and Lepidoptera had 9 in the Bt cultivar and 11 species in the non-Bt cultivar. Hemiptera had six species in both cultivars, while Neuroptera, Mantodea, and Dermaptera had only one species each. Although, the distribution of species was very similar between the two evaluated cultivars, the results of the Shannon-Wiener and Simpson tests demonstrated that the Bt cultivar had greater diversity (H = 1.30280; D = 0.86471) than the non-Bt cultivar (H = 1.22871; D = 0.84557).

The greatest number of species was observed in the third evaluation on January 6 with a total of 34 species in the BMX Potência and 35 in the DM 6563 IPRO (Figure 1). The first (December 30) and the fourth (9 of January) evaluations had nearly equal number of species, while at the second evaluation (02 January) was noticed the lowest value of the species found during the period of sampling.

The greatest number of floral visitor species in the soybean cultivars occurred between 8:00 and 11:15 am. The least species visitation to the soybean flowers occurred between 12:00 and 15:15 pm (Figure 2). Among the 103 species collected in the two studied soybean cultivars, *A. mellifera* L. had the highest number of individuals (102 individuals in the cultivar Intacta and 91 in the cultivar BMX Potência), representing 36.55 and 33.70%, respectively, of the total insects found in both crop cultivars (Figure 3). The total number of individuals of *A. mellifera* observed during the period of the evaluated day (from 07:00 am to 18:15 pm) (Figure 4) did not indicate normality by the Kolmogorov-Smirnov and Shapiro-Wilk tests, that evaluated block (evaluation days) and treatment (evaluated times). Therefore, the nonparametric Kruskal-Wallis test (α = 5%) was performed. The variation in the abundance of *A. mellifera* in the different classes of times was determined and no significant differences were noted between the classes of times in both cultivars.

DISCUSSION

Because there was no significant difference in the abundance and richness of floral-visiting insects between the two treatments of *G. max*, this group of insects is therefore not directly affected by the Bt toxin expressed in the evaluated cultivar (Intacta). Higgins (2009) examined non-target arthropods at the community level in corn in the United States for three years, and did not observe a significant difference in the abundance of the community, when fields of Bt and non-Bt corn were compared.

In the present study, the Shannon-Wiener diversity (H') index showed similar values in both evaluated cultivars. These close results between Bt and non-Bt cultivars represent equality in richness and abundance in relation to species among the evaluated cultivars. However, lower species richness was initially expected for the Bt cultivar, owing to a higher degree of environmental disturbance caused by the Cry1Ac toxin, since less altered environments tend to have greater species richness (Odum, 1988; Freitas and Pinheiro, 2012). Nonetheless, in non-Bt cultivars or tolerant insects, the generally required use of insecticides can also provide a disturbance of the environment.

Considering the Hymenoptera collected, representatives of the Apidae family were the most abundant. *A. mellifera* was the most abundant. Similar results were also observed in Mato Grosso do Sul, Brazil, regarding the diversity of floral-visiting insects in cotton crops (Dutra et al., 2012), as well as in other regions of Brazil, such as Bahia, Goiás, Mato Grosso, São Paulo, and in Distrito Federal (Pires et al., 2006b).

The high abundance of *A. mellifera* can be attributed to this species visiting flowers in exchange for offered floral resources (Machado, 2006; Dutra et al., 2012); the peak of visitors during the period between 08:00 and 11:15 am is possibly due to higher production and resources offered to insects by the soybean plants to attract pollinators to flowers. This species is an important pollinator, very common, and has the highest number of interactions with plants (Mouga et al., 2012). The presence of these bees is fundamental for soybean

Table 1. Species of floral-visiting on Intacta DM 6563 IPRO and BMX Potência cultivars. Diversity index Shannon-Wiener (H), Simpson (D) and number of individuals. 2014 December and 2015 January, Dourados/MS.

Táxon	Bt		Non Bt	
	Nº	P*logP	Nº	P*logP
Hymenoptera				
Halictinae				
Augochloropsis spp. 1	1	-0.00876	2	-0.01578
Augochloropsis spp. 2	2	-0.01537	2	-0.01578
Augochloropsis spp. 3	0	0	1	-0.00901
Augochloropsis spp. 4	1	-0.00876	0	0
Dialictus spp. 1	1	-0.00876	0	0
Dialictus spp. 2	1	-0.00876	0	0
Dialictus spp. 3	3	-0.02116	0	0
Halictus spp. 1 (Latreille, 1804)	0	0	1	-0.00901
Halictus spp. 2 (Latreille, 1804)	2	-0.01537	1	-0.00901
Ichneumonidae				
Ichneumonidae Gênero A spp. 1	0	0	2	-0.01578
Ichneumonidae Gênero B spp. 1	0	0	1	-0.00901
Ichneumonidae Gênero C spp. 1	0	0	1	-0.00901
Ichneumonidae Gênero C spp. 2	0	0	1	-0.00901
Braconidae				
Braconidae Gênero A spp. 1	0	0	1	-0.00901
Braconidae Gênero B spp. 1	0	0	1	-0.00901
Sphecidae				
Ammophila spp. 1 (Kirby, 1798)	0	0	1	-0.00901
Sphex dorsalis (Lepeletier, 1845)	1	-0.00876	2	-0.01578
Apidae				
Xylocopa brasilianorum (Linnaeus, 1767)	1	-0.00876	4	-0.0271
Eulaema nigrita (Lepeletier, 1841)	0	0	1	-0.00901
Trigona spinipes (Fabricius, 1793)	2	-0.01537	2	-0.01578
Apis mellifera (Linnaeus, 1758)	102	-0.15976	91	-0.15919
Pompilidae				
Aplochares spp. (Banks, 1944)	1	-0.00876	1	-0.00901
Agenioideus spp. (Ashmead, 1902)	1	-0.00876	0	0
Chalcochares spp. 1 (Banks, 1917)	1	-0.00876	0	0
Chalcochares spp. 2 (Banks, 1917)	0	0	2	-0.01578
Protonectarina sylveirae (de Saussure, 1854)	0	0	1	-0.00901
Vespidae				
Polistes spp. 1	0	0	1	-0.00901
Polistes spp. 2	1	-0.00876	0	0
Polybia paulista (Ihering, 1896)	1	-0.00876	0	0
Pachodynerus guadalupensis (De Saussure, 1853)	0	0	1	-0.00901
Total of individuals from Order Hymenoptera	122	-	121	-
Number of species from Order Hymenoptera	16	-	22	-
Hemiptera				
Pentatomidae				
Nezara viridula (Linneaus, 1758)	2	-0.01537	1	-0.00901
Piezodorus guildinii (Westwood, 1837)	1	-0.00876	2	-0.01578

Table 1 contd.

Euchistus heros (Fabricius, 1794)	1	-0.00876	1	-0.00901
Pyrrhocoridae				
Dysdercus maurus (Distant, 1901)	2	-0.01537	4	-0.0271
Dysdercus spp. 1 (Boisduval, 1835)	2	-0.01537	3	-0.02171
Dysdercus spp. 2 (Boisduval, 1835)	1	-0.00876	3	-0.02171
Total of individuals from Order Hemiptera	9	-	14	-
Number of species from Order Hemiptera	6	-	6	-
Coleoptera				
Coccinelidae				
Scymnus spp. (Kugelann, 1794)	0	0	1	-0.00901
Cicloneda sanguínea (Linnaeus, 1763)	1	-0.00876	0	0
Eriopis conexa (Germar, 1824)	0	0	1	-0.00901
Carabidae				
Odontochila spp. 1 (Agassiz, 1847)	1	-0.00876	0	0
Odontochila spp. 2 (Agassiz, 1847)	0	0	1	-0.00901
Tenebrionidae				
Lagria villosa (Fabricius ,1783)	16	-0.07119	13	-0.06343
Chrysomelidae				
Diabrotica speciosa (Germar, 1824)	9	-0.0481	5	-0.03208
Colaspis joliveti (Bechyne, 1955)	1	-0.00876	0	0
Colaspis sp. (Fabricius, 1801)	7	-0.04015	6	-0.03674
Cerotoma arcuatus Oliver, 1791	0	0	1	-0.00901
Scarabaeidae				
Macrodactylus spp. (Dejean, 1821)	1	-0.00876	3	-0.02171
Euphoria lúrida (Fabricius, 1775)	1	-0.00876	0	0
Curculionidae				
Anthonomus spp. (Germar, 1817)	1	-0.00876	0	0
Sternechus mrázi (Voss, 1934)	3	-0.02116	0	0
Staphylinidae				
Staphylinidae spp. 1 (Latreille, 1802)	2	-0.01537	0	0
Staphylinidae spp. 2 (Latreille, 1802)	0	0	3	-0.02171
Elateridae				
Elaterinae spp. 1	1	-0.00876	0	0
Elaterinae spp. 2	1	-0.00876	0	0
Conoderus malleatus (Germar, 1824)	0	0	1	-0.00901
Total of individuals from Order Coleoptera	45	-	34	-
Number of species from Order Coleoptera	13	-	10	-
Lepidoptera				
Nymphalidae				
Dione juno (Cramer, 1779)	6	-0.03585	6	-0.03674
Dione spp. 1 (Hübner, 1819)	1	-0.00876	0	0

Table 1 contd.

Dione spp. 2 (Hübner, 1819)	0	0	1	-0.00901
Doxocopa agathina (Cramer, 1777)	2	-0.01537	2	-0.01578
Hamadryas februa (Hübner, 1821)	4	-0.02643	4	-0.0271
Dryas iulia (Fabricius, 1775)	0	0	2	-0.01578
Diaethria clymena (Cramer, 1775)	1	-0.00876	0	0
Hypothyris euclea (Godart, 1819)	1	-0.00876	1	-0.00901
Dynamine postverta (Cramer, 1771)	2	-0.01537	1	-0.00901
Theclinae				
Strymon spp. (Hübner, 1818)	0	0	1	-0.00901
Strymon spp. (Hübner, 1818)	1	-0.00876	0	0
Hesperiidae				
Pyrgus orcus (Stoll, 1780)	1	-0.00876	0	0
Noctuidae				
Anticarsia gemmatalis (Hübner, 1818)	7	0.04015	19	-0.08111
Heliconiinae				
Euptoieta hegesia (Cramer, 1779)	1	-0.00876	0	0
Total of individuals from Order Lepidoptera	27	-	37	-
Number of species from Order Lepidoptera	11	-	9	-
Diptera				
Syrphidae				
Allograpta oblíqua (Say, 1823)	6	-0.03585	5	-0.03208
Allograpta exótica (Wiedemann, 1830)	6	-0.03585	7	-0.04113
Ornidia obesa (Lepeletier & Serville, 1828)	1	-0.00876	1	-0.00901
Toxomorus politus (Say, 1823)	0	0	1	-0.00901
Tachinidae				
Tachinidae spp. 1	5	-0.0313	2	-0.01578
Tachinidae spp. 2	1	-0.00876	0	0
Tachinidae spp. 3	2	-0.01537	0	0
Tachinidae spp. 4	1	-0.00876	0	0
Tachinidae spp. 5	8	-0.04422	2	-0.01578
Tachinidae spp. 6	1	-0.00876	4	-0.0271
Tachinidae spp. 7	1	-0.00876	0	0
Tachinidae spp. 8	2	-0.01537	0	0
Tachinidae spp. 9	0	0	1	-0.00901
Tachinidae spp. 10	0	0	1	-0.00901
Tachinidae spp. 11	1	-0.00876	0	0
Tachinidae spp. 12	3	-0.02116	2	-0.01578
Muscidae				
Musca spp. 1	29	-0.10219	26	-0.09787
Musca spp. 2	0	0	1	-0.00901
Musca spp. 3	3	-0.02116	2	-0.01578
Calliphoridae				
Calliphoridae spp. 1	1	-0.00876	1	-0.00901

Table 1 contd.

Tabanidae				
Tabanidae spp. 1	2	-0.01537	4	-0.0271
Total of individuals from Order Diptera	73	-	60	-
Number of species from Order Diptera	17	-	15	-
Mantodea				
Mantidae				
Mantidae	0	0	1	-0.00901
Total of individuals from Order Mantodea	0	-	1	-
Number of species from Order Mantodea	0	-	1	-
Neuroptera				
Chrysopidae				
Chrysopidae	3	-0.02116	0	0
Total of individuals from Order Neuroptera	3	-	0	-
Number of species from Order Neuroptera	1	-	0	-
Dermaptera				
Forficulidae				
Doru luteipes (Scudder, 1876)	0	0	2	-0.01578
Total of individuals from Order Dermaptera	0	-	2	-
Number of species from Order Dermaptera	0	-	1	-
Total	279	-	270	-
H		1.22871		1.3028
D		0.84557		0.86471

P: Sample proportion having individuals from i species; Bt: number of individuals found in Bt cultivar; Non Bt: number of individuals found in non Bt cultivar.

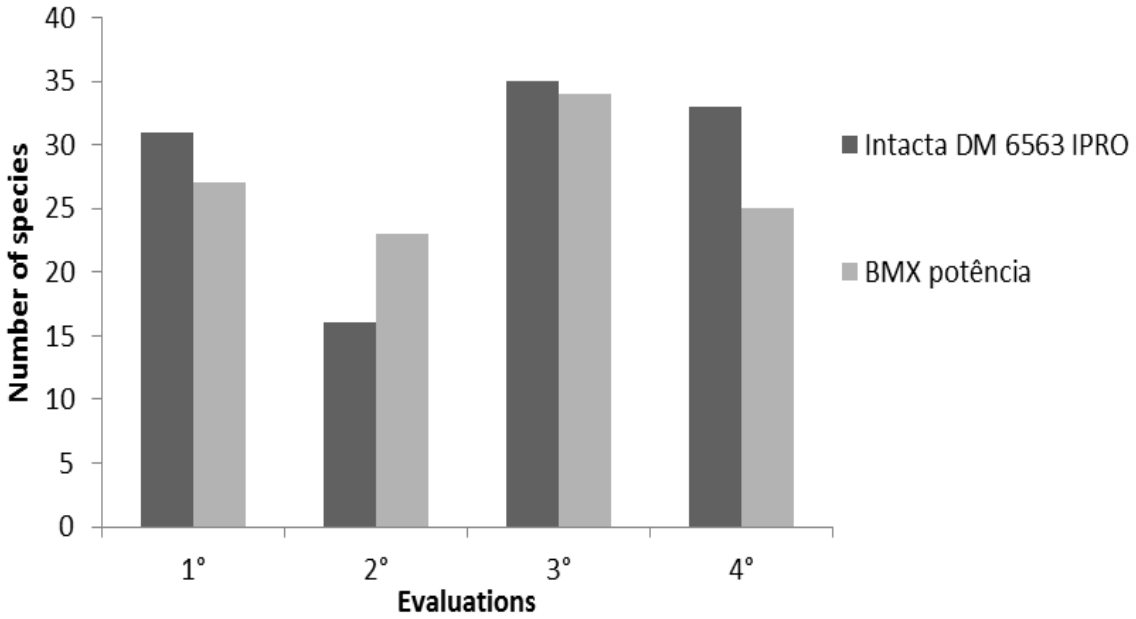

Figure 1. Richness of floral-visiting species on the soybean crop. Dourados, MS, from 2014 December to 2015 January.

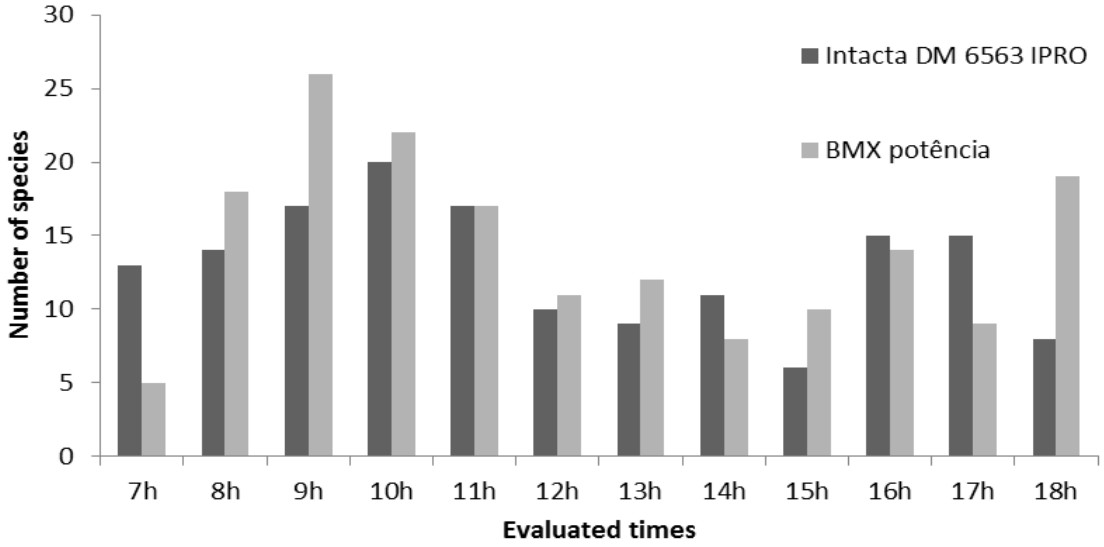

Figure 2. Richness of floral-visiting species (total number of species in four evaluations) on the soybean crop at the different times. Dourados, MS, from 2014 December to 2015 January.

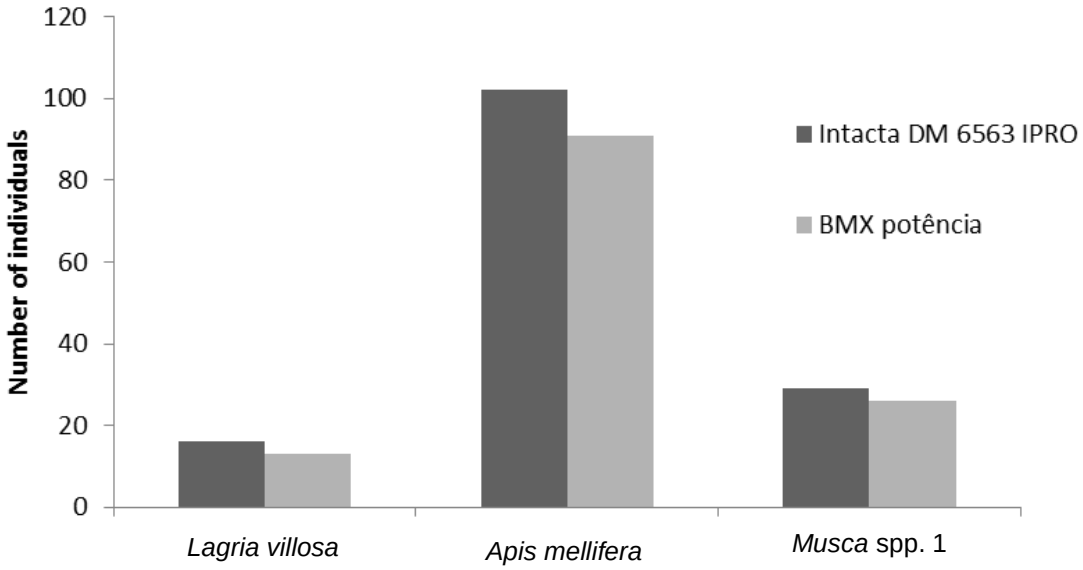

Figure 3. Abundance of most frequent species of floral-visiting insects on soybean crop (total number of species in four evaluations). Dourados, MS, from 2014 December to 2015 January.

production since it positively influences the production of grains and seed quality due to its pollination efficiency of 97.43% when compared with that of other insects (Chiari et al., 2008).

An important disturbance called Colony Collapse Disorder (CCD) has been the focus of many studies globally due to the disappearance of individuals that form bee colonies, as seen by the absence of dead bees in apiaries, the rapid decrease in the number of adult bees, and the lack of cleptoparasites (Cox-Foster and van Engelsdorp, 2009). One of the most important probable causes of this disorder is the effect of chemical insecticides on or near the colonies. Therefore, in efforts to protect bee species, mainly *A. mellifera*, from CCD, the data obtained in this study regarding the time of least visitation to the flowers, can determine the most sustainable insecticide applications, when necessary.

Based on the results of the current study, it can be concluded that the Cry1Ac toxin from Bt did not affect the population dynamics of the species of flower-visiting insects in soybean crops. Therefore, it is possible to add that this technology led to no significant differences in

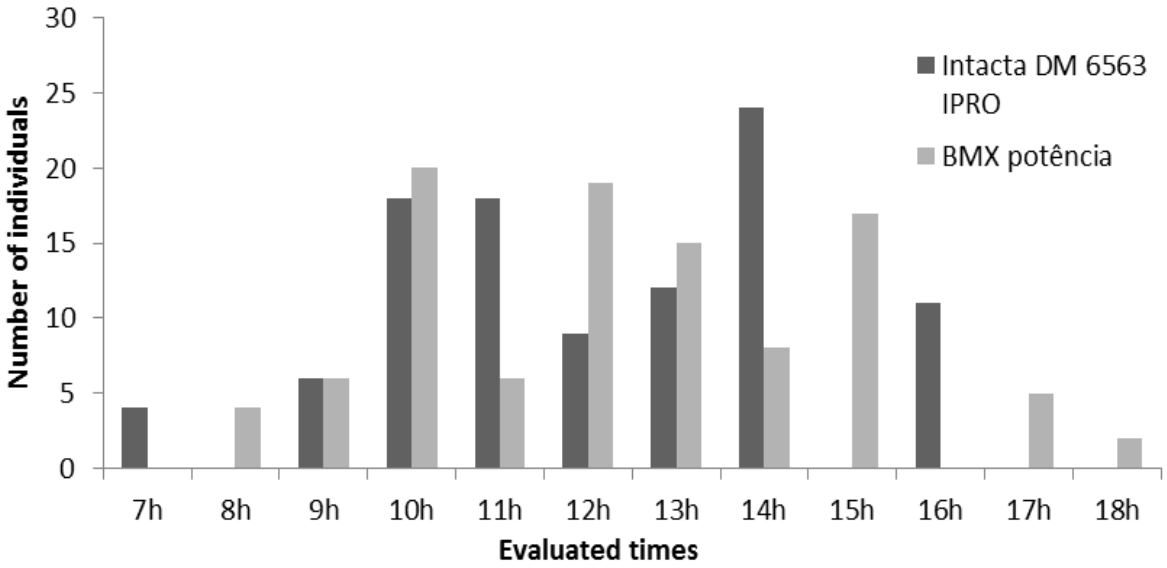

Figure 4. Abundance of *Apis mellifera* floral-visiting on soybean crop (total number of individuals in four evaluations). Dourados, MS, from 2014 December to 2015 January.

diversity, species composition, frequency, richness, or time of occurrence among the species of floral visitors to the culture.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors would like to thank FUNDECT/CAPES, chamada nº 44/2014-PAPOS-MS/Manoel A. Uchoa-Fernandes.

REFERENCES

Andow DA, Hilbeck A (2004). Science-based risk assessment for non-target effects of transgenic crops. BioScience. 54:637-649.

Agencia Nacional de Vigilância Sanitária (2015). ANVISA. WWW.anvisa.gov.br/divulga/noticias. Acesso em: 28 July 2015.

Birch RG (1997). Plant transformation: Problems and Strategies for Practical Application. Ann. Rev. Plant Physiol. Plant Mol. Biol. 48:297-326.

Bravo A, Gill SS, Soberon M (2005). *Bacillus thuringiensis* mechanisms and use. In: Gilbert LI, Iatrou K, Gill SS (eds.). Compr. Mol. Insect Sci. Elsevier. 6:175-206.

Chen M, Liu ZC, Frag Q, Hu C, Peng YF, Shelton AM (2009). Analysis of Cry1Ab toxin bioaccumulation in a food chain of Bt rice, an herbivore and a predator. Ecotoxicology 18:230-238.

Chiari WC, Toledo VAA, Hoffmann-Campo CB, Rúvolo-Takasusuki MCC, Toledo TCSOA, Lopes TS (2008). Polinização por *Apis mellifera* em soja transgênica [*Glycine max* (L.)] Roundup Ready ™ cv. BRS 245 RR e convencional cv. BRS 133. Acta Sci. Agron. 30:267-271.

Corrêa-Ferreira BS, Alexandre TM, Pellizzaro EC, Moscardi F, Bueno AF (2010). Práticas de manejo de pragas utilizadas na soja e seu impacto sobre a cultura. Londrina:Embrapa Soja. Circ. Técnica.78:15.

Cox-Foster D, vanEngelsdorp D (2009). Solving the mystery of the disappearing bees. Scientific American, New York.

de Maagd RA, Bravo A, Crickmore N (2001). How *Bacillus thuringiensis* has envolved specific toxins to colonize the insect world. Trends in Genetics. 17:193-199.

Dutra CC, Meotti C, Fernandes MG, Raizer J (2012). Riqueza e composição de insetos visitantes florais em algodoeiro Bt e não Bt. Arq. Inst. Biol. 79:353-361.

Faria CA, Wackers FL, Pritchard J, Barrett DA, Turlings TCJ (2007). High susceptibility of Bt maize to aphids enhances the performance of parasitoids of lepidopteran pests. PLoS One. 7:1-11.

Faria MF, Lundgren JG, Fontes EMG, Fernandes OA, Schimidt F, Nguyen VT, Andow DA (2006). Assessing the effects of Bt Cotton on Generalist Arthropod Predator. In: Hilbeck, A., Andow, D.A., Fontes, E.M.G. (ed). Environmental risk assessment of genetically modified organisms methodologies for assessing Bt cotton in Brazil. CABI Publishing pp. 175-199.

Free JB (1993). Insect pollination of crops. London: Academic Press.

Freitas BM (1997). Changes with time in the germinability of cashew (Anacardium occidentale) pollen grains found on different body areas of its pollinator bees. Rev. Bras. Biol. São Carlos 57:289-294.

Freitas BM, Pinheiro JN (2012). Polinizadores e pesticidas: princípios e manejo para os agroecossistemas brasileiros. Brasília: MMA. P 112

Groot AT, Dicke M (2002). Insect-resistant transgenic plants in amultitrophic context. Plant J. 31:387-406.

Higgins LS, Babcock J, Neese P, Layton RJ, Moellenbeck D, Storer N (2009). Three-year field monitoring of Cry1F, Event DASØ15Ø7–1, maize hybrids for non-target arthropod effects. Environ. Entomol. 38:281-292.

Hong LTT, Tâm DQ, Tuân HA (2008). Potencial effects of transgenic Cotton on flower visitors in Vietnam. In: Andow, DA, Hillbeck A, Van Tuat AN (Ed.). Environmental Risk Assessment of Genetically Modified Organisms: challenges and opportunities with Bt cotton in Vietnam. Wallingford: Centre Agric. Biosci. Int. 9:432.

Kouser S, Qaim M. (2011). Impact of Bt cotton on pesticide poisoning in smallholder agriculture: A panel data analysis. Ecol. Econ. 70:2105-2113.

Liu XX, Sun CG, Zhang QW (2005). Effects of transgenic Cry1A+CpTI cotton and Cry1Ac toxin on the parasitoid, Campoletis chlorideae (Hymenoptera: Ichneumonidae). Insect. Sci. 12:101-107.

Liu W, Wang F, Zhang F, Meng Z, Wang F (2000). Evaluation on role of predators in Helicoverpa armigera control. Chinese Journal of Biological Control. 16:97-101.

Machado CS (2006). Aspectos de interesse da polinização entomófila de *Helianthus annus* L. no Recôncavo Baiano. Dissertação de mestrado. Universidade Federal da Bahia. Cruz das Almas, Bahia.

Malaspina O, Silva-Zacarin ECM (2006). Cell markers for ecotoxicological studies in target organs of bees. Braz. J. Morphol. Sci. 23:303-309.

Monnerat RG, Bravo A (2000). Proteínas bioinseticidas produzidas pela bactéria *Bacillus thuringiensis*: modo de ação e resistência. In: Melo IS, Azevedo JL (Ed.). Controle biológico 3. Jaguariúna: Embrapa Meio Ambiente.163-200.

Mouga DMDS, Noble CF, Bussmann DBG, Krug C (2012). Bees and plants in a transition area between atlantic rain forest and araucaria forest in southern Brazil. Revue d'Ecologie. 67:313-327.

Naranjo SE (2005). Long-Term Assessment of the Effects of Transgenic Bt Cotton on the Abundance of Nontarget Arthropod Natural Enemies. Environ. Entomol. 34:1193-1210.

Nogueira-Couto RH (1998). Manejo das colméias de abelhas africanizadas para polinização. In: XII Congresso Brasileiro de Apicultura. Salvador: Confederação Brasileira de Apicultura. 2:129-134.

Nunes DH (2010). Efeitos do algodoeiro geneticamente modificado Bollgard® em organismos não-alvo. Tese Doutorado em Ciências. Universidade de São Paulo, Piracicaba. 211f.

Odum EP (1988). Ecologia. Ed. Guanabara, Rio de Janeiro.

Pinto-Coelho RM (2002). Fundamentos em Ecologia. Porto Alegre, Ed:Artmed.

Pires C, Silveira, FA, Pereira FO, Paes JS, Sujii ER, Fontes E (2006a). Protocolo de amostragem de visitantes florais em algodoeiro (*Gossypium* spp.). Brasília: Embrapa Recursos Genéticos e Biotecnologia, Boletim de Pesquisa e desenvolvimento P 131.

Pires C, Silveira F, Cardoso CF, Oliveira GM, Pereita FFO, Souza VV, Nakasu EYT, Paes JSO, Teles É, Silvie P, Rodrigues S, Miranda J, Scomparini J, Bastos C, Oliveira GS, Oliveira JE, Santos JB, Barroso PAV, Sujii E, Fontes E (2006b). Visitantes florais em espécies cultivadas e não cultivadas de algodoeiro (Gossypium spp.), em diferentes regiões do Brasil. Brasília: Embrapa Recursos Genéticos e Biotecnologia. Boletim de pesquisa e desenvolvimento. 148:40.

Roggia S (2010). Caracterização de fatores determinantes dos aumentos populacionais de ácaros tetraniquídeos em soja. Tese, Doutorado em Entomologia, Universidade de São Paulo, Piracicaba.

Shannon CE, Weaver W (1949). The mathematical theory of communication. Urbana: University of Illinois Press.

Silva GV (2013). Efeito das plantas Bt de soja e milho sobre pragas não-alvo e seus inimigos naturais. Dissertação, Mestrado em Ciências Biológicas, Universidade Federal do Paraná, Curitiba.

Spadotto CA, Gomes MAF, Luchini LC, Andrea MM (2004). Monitoramento de riscoambiental de agrotóxicos: princípios e recomendações. Jaguariúna, SP: Embrapa Meio Ambiente. 29 p.

Vojtech E, Meissle M, Poppy GM (2005). Effects of Bt maize on the herbivore Spodoptera littoralis (Lepidoptera: Noctuidae) and the parasitoid Cotesia marginiventris (Hymenoptera: Bracondiae). Transgenic Res. 14:133-144.

Yu HL, Yun HL, Kong MW (2011). Risk assessment and ecological effects of transgenic *Bacillus thuringiensis* crops on non-target organisms. J. Integr. Plant Biol. 53:520-538.

Whitehouse MEA, Wilson L J, Constable GA (2007). Target and non-target effects on the invertebrate community of Vip cotton, a new insecticidal transgenic. Aust. J. Agric. Res. 58:383-385.

academicJournals

Vol. 15(25), pp. 1330-1338, 22 June, 2016
DOI: 10.5897/AJB2015.14950
Article Number: A52532059051
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Morpho-phenological diversity among natural populations of *Medicago polymorpha* of different Tunisian ecological areas

Mounawer Badri*, Najah Ben Cheikh, Asma Mahjoub and Chedly Abdelly

Laboratory of Extremophile Plants, Centre of Biotechnology of Borj Cedria, B.P. 901, Hammam-Lif 2050, Tunisia.

Received 27 August, 2015; Accepted 13 January, 2016

Medicago polymorpha **is a herbaceous legume that can be a useful pasture plant, in particular, in regions with a Mediterranean climate. The genetic variation in 120 lines of *M. polymorpha* sampled from five regions in Tunisia was characterized on the basis of 16 morpho-phenological characters. Results from analysis of variance (ANOVA) showed that differences among populations and lines existed for all traits, with population explaining the greatest variation for measured traits. The populations of Enfidha and Soliman were the earliest flowering, while those of El Kef, Bulla Regia and Mateur were the latest. El Kef and Mateur exhibited the highest aerial dry weight while the lowest value was found for Soliman. Moderate to lower levels of heritability (H^2) were registered for investigated traits. There was no significant association between pairwise population differentiation (Q_{ST}) and geographical distances. Studied lines were clustered into three groups with 59 for the first group, 34 for the second group, and 27 lines for the third group. The lines of the first two groups showed the largest length of stems while those of the second group had the highest number of leaves. The variation of quantitative traits among populations was influenced by the altitude, temperature and relative humidity. Overall, the high levels of within population variation and the lack of correlation between population differentiation and geographical distances suggest a potentially important rate of long-distance seed dispersal and confirm the role played by natural selection in the population structure of Tunisian populations of *M. polymorpha*.**

Key words: *Medicago polymorpha*, populations, quantitative traits, population differentiation, environmental parameters.

INTRODUCTION

The genus *Medicago,* with about 87 species of herbs and shrubs widespread from the Mediterranean to central Asia (Small and Jomphe, 1989; Small, 2010), includes the widely cultivated major forage crop *Medicago*

*Corresponding author. E-mail: mounawer_badri@yahoo.fr, mounawer.badri@cbbc.rnrt.tn. Tel: +216 79 325 848. Fax: +216 79 325 638.

sativa (alfalfa, lucerne) and the legume model species *Medicago truncatula*. In Tunisia, the genus *Medicago* is well represented and it is an extremely rich and diverse gene pool (Seklani et al., 1996). *Burr Medic (Medicago polymorpha*) is an annual herbaceous legume, diploid (2n = 14) and self-compatible species. *M. polymorpha* can be a useful pasture plant, in particular, in regions with a Mediterranean climate (Salhi Hannachi et al., 1998). It had aroused great interest due to high nutritious quality, highly palatability and N-fixing plan in neutral soil (Loi et al., 1993, 1995). *M. polymorpha* is a species of Mediterranean origin, but its species range is wide spread throughout the world. The wide diffusion and adaptability can be explained by its low sensitivity to photoperiod and vernalization (Aitken, 1981). Heyn (1963) identified three botanical varieties of this species: *brevispina* with spineless or tubercled pods; *polymorpha*, spined and *vulgaris* spined but with pods smaller than *polymorph*a. In Tunisia, *M. polymorpha* grows in a range of environments from humid to upper arid stages (Abdelkefi et al., 1996).

In general, plant growth and reproduction is dependent on the effects of genotype and the abiotic and biotic factors. Sufficient genetic diversity is very important for plants to survive in changing climate conditions. Traditionally, plants diversity is assessed by morphological descriptors. Morpho-phenological characterization of natural populations of *M. polymorpha* may provide valuable insights into the traits and underlying genetics needed for meeting challenges of the future environmental conditions. Morphological characters are generally quantitative, having a mono- or polygenic determinism. Understanding of morphological characters facilitates the identification of desirable traits and their genetic determinants (Tar'an et al., 2005; Arraouadi et al., 2011; Badri et al., 2011). Sixteen quantitative traits considered as descriptors for *M. truncatula*, *Medicago laciniata* and *Medicago ciliaris* populations (Badri et al., 2007, 2010; Arraouadi et al., 2009; Lazrek et al., 2009) were measured for the lines of *M. polymorpha*. Genetic diversity may appear spatially structured at different scales, such as among neighboring individuals, subpopulation, and population (Escudero et al., 2003).

Knowledge of spatial genetic structures provides a valuable tool for inferring the evolutionary forces such as selective pressures and drift (De Kort et al., 2012). Low gene flow due to spatial isolation of populations may even increase the degree of local differentiation (Hendry, 2002). Nevertheless, phenotypic plasticity rather than genetic differentiation may be an alternative way of matching genotypes to environment; indeed increasing environmental variation favors higher levels of plasticity (Hahn et al., 2012).

The current study aims to: (i) analyze the morpho-phenological diversity within and among natural populations of *M. polymorpha*, and (ii) assess the relationship between phenotypic variation among populations and the site-of-origin environmental parameters. In this study plant performance was studied under greenhouse conditions, where plants grew in common environments for multiple generations. Thus, population and within population effects should be due to genetics rather than due to maternal environmental effects.

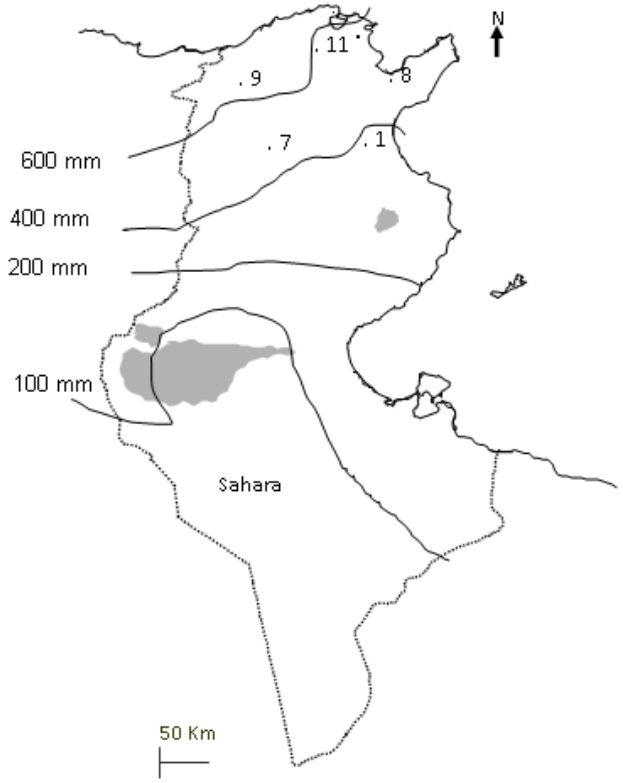

Figure 1. Map of Tunisia with the location of natural populations of *Medicago polymorpha* from which studied lines were collected. Enfidha (1), El Kef (7), Soliman (8), Bulla Regia (9), and Mateur (11).

MATERIALS AND METHODS

Plant material

Five populations of *M. polymorpha* collected from different eco-geographical regions (Enfidha, El Kef, Soliman, Bulla Regia, and Mateur) (Figure 1) in Tunisia were used. These populations were sampled in July, 2009 and represented three different bioclimatic stages, ranging from sub-humid to lower semi-arid environments (Table 1). At each location, 24 pods were collected randomly from each population. The neighboring samples were four meters apart to avoid sampling the same genotype more than once. Distances between populations were at least 43 km inbred lines. To minimize maternal environmental effects on trait expression, inbred lines of *M. polymorpha* were developed by single-seed descent at the F3 generation under greenhouse conditions.

Table 1. Environmental factors of the collection sites of Tunisian populations of *Medicago polymorpha.*

Population (code)	Number of studied lines	Longitude (E)	Latitude (N)	Bioclimatic stage	Altitude (m)	Texture	Electro-conductivity (mmho/cm)	Mean annual rainfall (mm)	Mean temperature (°C)	Mean relative Humidity (%)
Enfidha (TNP1)	24	10°220'	36°070'	Lower semi-arid	2	Clay	0.6	350	20.40	68.33
El Kef (TNP7)	24	35°831'	94°255'	Upper semi-arid	500	Sandy loam	0.8	450	17.60	61.00
Soliman (TNP8)	24	10°320'	36°410'	Upper semi-arid	2	Loamy sand	1.4	600	19.20	77.00
Bulla Regia (TNP9)	24	25°844'	37°429'	Sub-humid	200	Sandy loam	0.8	600	19.17	62.67
Mateur (TNP11)	24	09°400'	37°010'	Sub-humid	37	Loamy sand	3.2	600	18.9	60.8

Morpho-phenological characterization

The experiment was conducted under greenhouse conditions at the Centre of Biotechnology of Borj Cedria (CBBC) in spring, 2014. Twenty four lines per population were used. Seeds were scarified using sandpaper and were transferred in pots (diameter = 17 cm; deep = 13 cm) of two liters filled with soil of the CBBC and compost of sphagnum (2:1). Six replicates per line were used, giving a total of 720 plants, which were organized into a randomized complete three blocks design. Each plant was grown in an individual pot in greenhouse with a mean temperature of 25°C. Plants were grown in well-irrigated treatment (100% of field capacity). Three replicates of each genotype from each block were harvested at the formation of the first green pod and three at the end of plants' lifecycle. Sixteen quantitative traits were measured for the lines of M. *polymorpha*. Eleven of these were related to vegetative growth: days from emergence to first true leaf (D1L, days), days from emergence to sixth leaf (D6L, days), length of stems (LS, cm), length of roots (LR, cm), number of ramifications (NR), number of leaves (NL), aerial fresh weight (AFW, g), aerial dry weight (ADW, g), root dry weight (RDW, g), root dry weight and aerial dry weight ratio (RDW/ADW), aerial dry weight at second harvest (ADWh2, g). The remaining characters were related to flowering time and pods production: days from emergence to first flower (FLOR, days), number of pods (NPOD), weight of pods (WPOD, g), weight of 100 pods (W100P, g), and harvest index (HI). The aerial and root dry weights was estimated after drying in an oven at 70°C for 48 h.

Statistical analyses

To test for population and line nested within population effects on the 14 traits measured from greenhouse grown plants, ANOVAs were performed using general linear models (GLM) procedure (type III) in SPSS version 16 (2007 Rel 1600 SPSS Inc., Chicago, IL, USA) where population and lines were considered as fixed factors. Comparison of population means of measured traits was performed using the Duncan's multiple range test at 5%.

The estimation of variance among populations (Vp) and lines (Vg) was performed using the VARCOMP procedure in SPSS treating the population and lines as random factors, relying on Restricted Maximum Likelihood (REML) method. The residual variance between the replicates of the same genotype (=line) was considered as the environmental variance (Ve). Broad-sense heritability (H^2) of the traits was estimated as the ratio of the genetic variance on the sum of the genetic and environmental variances (Badri et al., 2007). Population differentiation (Q_{ST}) for quantitative traits was computed as reported for a predominantly autogamous species (Badri et al., 2015) as $Q_{ST}=Vp/(Vp+Vwp)$, where Vp is the variance among populations and Vwp the variance within populations. Pairwise Q_{ST} was only estimated for the traits showing significant variation among populations. Correlations between Q_{ST} and geographical distances were analyzed using the Mantel test (Mantel, 1967) in XLSTAT software v 7.5 (Addinsoft, USA). The matrix of geographical distance between populations was calculated by measuring the shortest distance between two points in the map, using geographical coordinates for each site.

Pearson correlations between measured traits were estimated using Correlate procedure in SPSS software. Clustering analysis of lines and populations was performed based on dissimilarity matrix using Euclidean distances estimated on the mean line and population values, respectively, with the Ward's method in XLSTAT software. A discriminant analysis (DA) was performed on means of measured traits for the groups of lines and populations. Cluster and discriminant analyses were performed on means of 7 and 12 traits showing significant differences for lines and populations, respectively.

Five environmental parameters of sampling sites of populations of *M. polymorpha* were examined: altitude (m), electro-conductivity (mmho/cm), mean annual rainfall (mm), temperature (°C) and relative humidity (%). Soil samples were collected from three points at each site and they were analyzed at the Laboratory of Soils at the Ministry of Agriculture, Tunisia. The temperature (T), the mean annual rainfall (R), and the relative humidity (H) were provided by the National Institute of Meteorology, Tunis, Tunisia. Pearson correlations between 12 traits, showing significant variation among populations, and environmental parameters were estimated using Correlate procedure in SPSS software. Significance level was set to 0.05 and adjusted for multiple comparisons by Bonferroni correction (Badri et al., 2007).

RESULTS

Morpho-phenological variation among populations

Results from the ANOVA showed that the variation of measured traits was explained by the effects of population and lines within population (Table 2). The largest effect was found for population. Of the 16 traits, 11 differed among populations and 7 differed among lines. The populations of El Kef, Bulla Regia and Mateur were the latest flowering while those of Enfidha

Table 2. Contribution of population line within population treatment population x treatment (P x T) and line x treatment (L x T) interaction effects on measured traits for populations of *Medicago polymorpha.*

		D1L	D6L	FLOR	LS	LR	NR	NL	AFW	ADW	RDW	RDW/ADW	ADWh2	NPOD	WPOD	W100P	HI
Population	F	6.07	1.44	23.02	1.82	2.02	3.11	4.05	3.46	17.80	3.57	0.71	21.12	5.05	3.29	2.96	1.62
	P	0.000	0.228	0.000	0.134	0.101	0.020	0.005	0.012	0.000	0.010	0.585	0.000	0.001	0.015	0.025	0.17
Line(Pop)	F	1.80	0.81	3.68	2.12	1.12	2.19	1.96	0.98	4.87	0.83	0.87	2.19	1.08	1.00	1.10	1.29
	P	0.005	0.829	0.000	0.001	0.305	0.000	0.002	0.536	0.000	0.793	0.738	0.000	0.371	0.507	0.345	0.130

Significant (P ≤ 0.05), non significant (P>0.05), F: coefficient of Snedecor-Fisher. Days from emergence to first true leaf (D1L days); days from emergence to sixth leaf (D6L days); days from emergence to first flower (FLOR days); length of stems (LS cm); length of roots (LR cm); number of ramifications (NR); number of leaves (NL); aerial fresh weight (AFW g); aerial dry weight (ADW g); root dry weight (RDW g); root dry weight and aerial dry weight ratio (RDW/ADW); aerial dry weight at second harvest (ADWh2 g); number of pods (NPOD); weight of pods (WPOD g); weight of 100 pods (W100P g); harvest index (HI).

Table 3. Mean values and coefficient of variation (CV), variance among populations (Vp), genetic variance (Vg), environmental variance (Ve), heritabilities (H^2) and population differentiation for quantitative traits (Q_{ST}) for measured traits for populations of *Medicago polymorpha.*

Parameter	TNP1	TNP7	TNP8	TNP9	TNP11	F	*P*	CV	Vp	Vg	Ve	H^2	Q_{ST}
D1L	9.75±1.73^{a}	7.99±7.48^{c}	9.31±1.95^{ab}	9.03±1.89^{bc}	9.06±1.52^{c}	6.07	0.000	2.18	0.23	0.06	13.77	0.00	0.02
D6L	26.13±4.54^{a}	25.50±3.01^{a}	25.34±3.39^{a}	25.56±3.00^{a}	25.08±3.68^{a}	1.44	0.228	0.74	0.00	0.00	12.74	0.00	0.00
FLOR	50.48±11.25^{b}	60.38±18.88^{a}	47.10±9.91^{b}	57.47±8.99^{a}	54.69±9.41^{a}	23.02	0.000	1.32	26.13	103.37	66.39	0.61	0.13
LS	32.06±8.56^{b}	38.61±13.01^{a}	31.41±17.70^{ab}	37.48±8.41^{a}	33.63±10.73^{ab}	1.82	0.134	2.22	7.71	37.83	116.93	0.24	0.05
LR	7.86±3.40^{ab}	9.10±3.76^{a}	7.26±3.05^{b}	10.06±14.10^{ab}	8.63±3.59^{ab}	2.02	0.101	5.19	0.18	14.44	37.29	0.28	0.00
NR	2.38±1.16^{ab}	3.81±8.75^{a}	2.03±1.04^{b}	2.66±1.29^{a}	2.33±1.10^{a}	3.11	0.020	8.90	0.19	0.74	15.32	0.05	0.01
NL	65.80±31.58^{bc}	70.60±34.40^{abc}	58.91±31.61^{c}	80.78±37.93^{a}	76.36±43.01^{ab}	4.05	0.005	3.19	49.33	277.66	1046.00	0.21	0.04
AFW	3.19±1.58^{ab}	3.97±2.59^{a}	2.30±1.21^{c}	3.81±1.90^{a}	3.12±1.73^{ab}	3.46	0.012	3.67	0.37	0.81	2.94	0.22	0.09
ADW	0.59±0.33^{bc}	1.10±0.78^{a}	0.42±0.26^{c}	0.73±0.35^{b}	0.79±1.09^{a}	17.8	0.000	5.71	0.06	0.10	0.34	0.23	0.12
RDW	0.04±0.04^{b}	0.15±0.37^{a}	0.93±5.74^{b}	0.04±0.03^{b}	0.06±0.07^{b}	3.57	0.010	66.53	0.01	0.02	5.90	0.00	0.00
RDW/ADW	0.07±0.07^{a}	0.13±0.33^{a}	1.60±9.37^{a}	0.08±0.09^{a}	0.09±0.09^{a}	0.71	0.585	74.42	0.03	0.02	16.67	0.00	0.00
ADWh2	1.05±0.87^{b}	1.63±1.17^{a}	0.87±0.44^{b}	1.11±0.58^{b}	1.05±1.95^{b}	21.12	0.000	46.23	0.00	0.00	264.01	0.00	0.00
NPOD	24.02±13.22^{a}	14.92±8.35^{b}	22.42±13.27^{ab}	24.77±14.99^{a}	24.47±13.40^{a}	5.05	0.001	3.49	14.48	6.17	168.38	0.04	0.08
WPOD	0.84±0.51^{a}	0.71±0.46^{ab}	0.71±0.46^{ab}	0.73±0.50^{ab}	0.67±0.39^{b}	3.29	0.015	28.50	0.00	0.04	25.15	0.00	0.00
W100P	3.70±1.59^{b}	4.53±1.90^{a}	3.42±2.56^{bc}	3.28±2.86^{bc}	2.99±1.18^{c}	2.96	0.025	67.46	0.00	0.00	15830.00	0.00	0.00
HI	0.98±0.64^{a}	0.79±0.94^{a}	1.92±0.68^{a}	0.75±0.63^{a}	0.79±0.59^{a}	1.62	0.17	23.22	0.21	0.87	16.92	0.05	0.01

Enfidha (TNP1), El Kef (TNP7), Soliman (TNP8), Bulla Regia (TNP9), and Mateur (TNP11). Days from emergence to first true leaf (D1L days); days from emergence to sixth leaf (D6L days); days from emergence to first flower (FLOR days); length of stems (LS cm); length of roots (LR cm); number of ramifications (NR); number of leaves (NL); aerial fresh weight (AFW g); aerial dry weight (ADW g); root dry weight (RDW g); root dry weight and aerial dry weight ratio (RDW/ADW); aerial dry weight at second harvest (ADWh2 g); number of pods (NPOD); weight of pods (WPOD g); weight of 100 pods (W100P g); harvest index (HI). Standard deviation (SD), means of each trait followed by the same letters are not significantly different between studied populations of *M. polymorpha.*

and Soliman were the earliest (Table 3). The highest aerial dry weight (ADW) was registered for El Kef population and Mateur while the lowest values were for Enfidha and Soliman. The largest weight of pods (WPOD) was observed for Enfidha, El Kef, Soliman and Bulla Regia while

Table 4. Correlations between pairwise Q_{ST} matrix and geographical distances (GD) and between measured traits for natural populations of *Medicago polymorpha*.

		D1L	D6L	FLOR	LS	LR	NR	NL	AFW	ADW	RDW	RDW/ADW	ADWh2	NPOD	WPOD	W100P	HI
GD	**r**	**-0.29**	**ND**	**0.85**	**ND**	**ND**	**0.76**	**0.64**	**0.69**	**0.48**	**-0.66**	**ND**	**0.92**	**0.16**	**ND**	**-0.22**	**0.88**
	P	**0.770**	**ND**	**0.325**	**ND**	**ND**	**0.357**	**0.473**	**0.415**	**0.604**	**0.459**	**ND**	**0.639**	**0.863**	**ND**	**0.844**	**0.323**
D1L		1.00															
D6L		0.44*	1.00														
FLOR		0.19*	0.17*	1.00													
LS		-0.07	0.06	0.48*	1.00												
LR		0.02	0.09	0.11	0.23*	1.00											
NR		-0.03	-0.04	0.27*	0.16*	0.03	1.00										
NL		-0.03	-0.10	0.39*	0.43*	0.11	0.05	1.00									
AFW		-0.14*	-0.01	0.43*	0.44*	0.08	0.27*	0.52*	1.00								
ADW		-0.05	0.00	0.56*	0.42*	0.11	0.24*	0.67*	0.45*	1.00							
RDW		-0.02	-0.01	-0.01	0.02	-0.02	0.00	0.07	0.02	-0.01	1.00						
RDW/ADW		-0.01	0.00	-0.01	0.02	-0.03	0.00	0.06	0.01	-0.02	1.00*	1.00					
ADWh2		-0.01	-0.06	0.01	-0.01	-0.04	0.01	0.05	-0.03	-0.02	-0.02	-0.04	1.00				
NPOD		0.05	0.00	-0.11	0.06	0.22*	0.12*	0.18*	0.11	0.09	0.03	0.03	0.09	1.00			
WPOD		0.03	0.22*	-0.01	-0.01	0.29*	0.03	0.05	0.05	-0.05	0.11	0.12	0.01	-0.01	1.00		
W100P		0.03	0.22*	0.01	-0.09	0.05	-0.16*	-0.16*	-0.14*	-0.15*	0.06	0.06	-0.08	-0.08	1.00*	1.00	
HI		0.05	0.11	-0.07	-0.24*	-0.04	-0.03	-0.26*	-0.31*	-0.35*	0.29*	0.44*	-0.13*	0.08	0.11	0.03	1.00

*Significant ($P \leq 0.05$), non significant ($P>0.05$). Non determinant (ND); Days from emergence to first true leaf (D1L days); days from emergence to sixth leaf (D6L days); days from emergence to first flower (FLOR days); length of stems (LS cm); length of roots (LR cm); number of ramifications (NR); number of leaves (NL); aerial fresh weight (AFW g); aerial dry weight (ADW g); root dry weight (RDW g); root dry weight and aerial dry weight ratio (RDW/ADW); aerial dry weight at second harvest (ADWh2 g); number of pods (NPOD); weight of pods (WPOD g); weight of 100 pods (W100P g); harvest index (HI).

the lowest value was for Mateur. Higher variation within populations (CV > 50%) was observed for root dry weight (RDW), the ratio RDW/ADW and weight of 100 pods (W100P), moderate levels (20% < CV < 50%) were registered for aerial dry weight at the second harvest (ADWh2), WPOD and HI and lower values (CV < 20%) were noted for the remaining characters.

Higher broad-sense heritability ($H^2 > 0.4$) was found for flowering time, moderate levels ($0.2 \leq H^2 \leq 0.4$) were noted for length of stems (LS), length of roots (LR), number of leaves (NL), aerial fresh weight (AFW), and ADW, and lower values ($H^2 < 0.2$) were for the remaining traits (Table 3). Moderate population differentiation (Q_{ST}) was registered for flowering time (FLOR) and ADW, and lower levels were for the remaining traits. There was no significant association ($P > 0.05$) between the pairwise Q_{ST} matrix and geographical distances as confirmed by a Mantel test (Table 4).

Among the 120 possible correlations between measured traits, 38 correlations were significant and 27 of them were positive (Table 4). Most of correlations between the parameters of aerial growth were positive. The flowering time was positively correlated with days from emergence to first true (D1L) and sixth (D6L) leaves and the LS, the number of ramifications (NR), NL, AFW, and ADW. The NPOD was positively correlated with LR, NR, and NL. The HI was negatively correlated with LS, NL, AFW, ADW, and ADWh2.

Cluster analysis

The hierarchical cluster analysis was used to examine the aggregation patterns of 120 lines of *M. polymorpha*. Studied lines were clustered into three groups (Figure 2). A first group contained 59

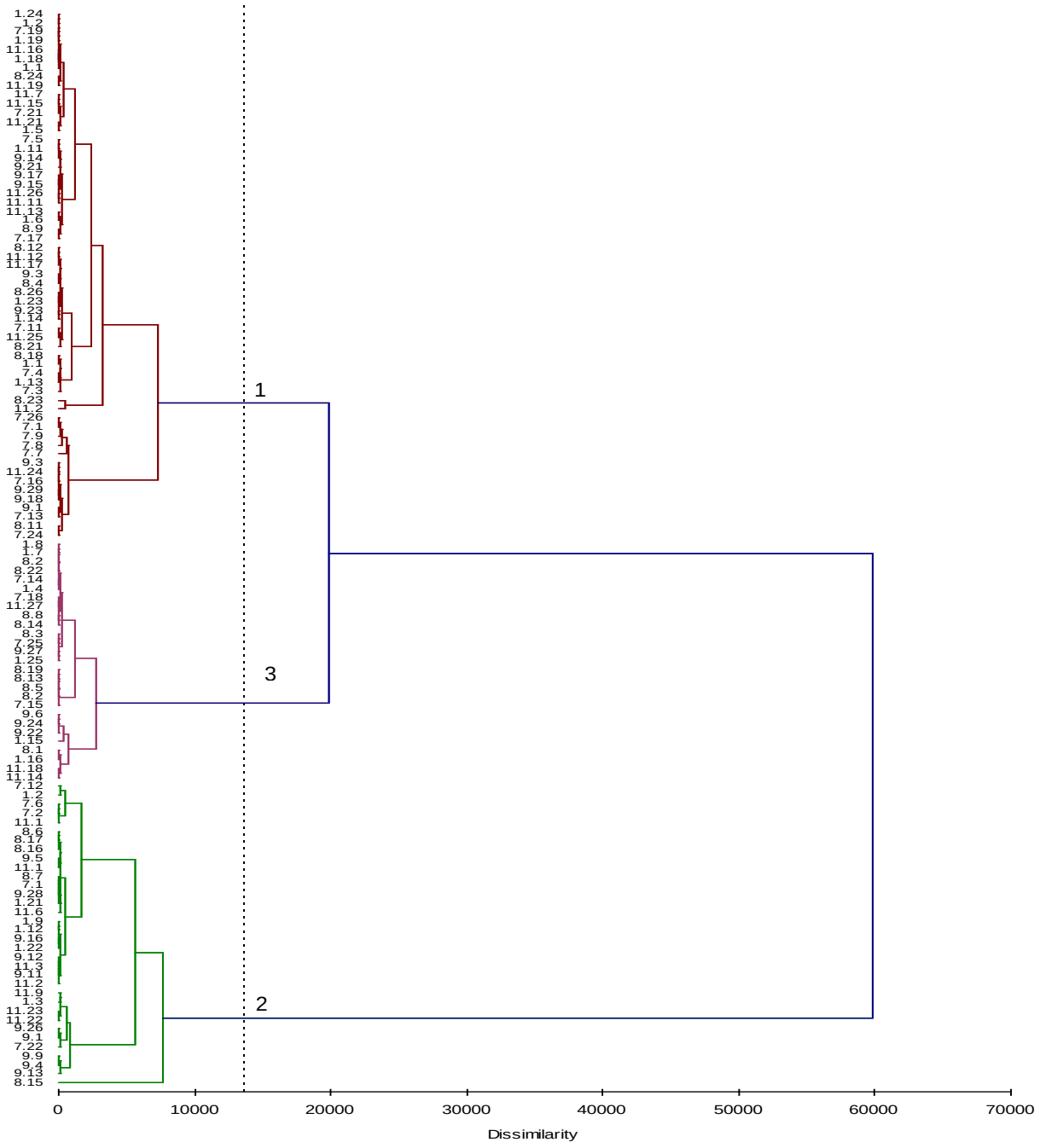

Figure 2. Dendrogram of the lines of *Medicago polymorpha* clustered based on Euclidean distances of dissimilarity matrix with the Ward's method. Enfidha (1), El Kef (7), Soliman (8), Bulla Regia (9), and Mateur (11).

lines including 12 lines from Enfidha, 13 lines from Mateur, 15 lines from El Kef, 9 from Soliman, and 10 lines from Bulla Regia. A second group is constituted by 34 lines with 6 lines from Enfidha, 8 from Mateur, 5 from El Kef, 5 from Soliman, 10 from Bulla Regia. A third group was composed by 6 lines from Enfidha, 3 from Mateur, 4 lines of El Kef, 10 of Soliman, and 4 lines of Bulla Regia. Lines of the first and second groups showed the highest values of LS while those of the third group had the lowest level (Table 5). On the other hand, the lines of the second group exhibited the highest NL while the lowest values were recorded for lines of the first and third groups. Studied populations were clustered into three groups (Figure 3). A first group was formed by Enfidha population, a second group included the population of El Kef, and a third group was constituted by

Table 5. Means of measured traits for classes of lines and populations of *Medicago polymorpha*.

Class\Variable	Lines						Populations					
	1	2	3	λ	F	p-value	1	2	3	λ	F	p-value
D1L	9.13	9.10	9.56	-	-	-	9.75[a]	8.96[b]	9.13[b]	0.941	3.678	0.028
FLOR	55.04	58.98	45.98	-	-	-	50.67	60.28	53.21	-	-	-
LS	36.47[a]	38.74[a]	26.29[b]	0.75	19.56	< 0.0001	-	-	-	-	-	-
NR	2.89	2.83	1.66	-	-	-	2.32	3.58	2.36	-	-	-
NL	65.53[b]	104.08[a]	35.88[c]	0.19	244.39	< 0.0001	64.53	70.99	71.12	0.991	0.547	0.580
AFW	-	-	-	-	-	-	3.11	4.15	3.09	-	-	-
ADW	0.73	1.01	0.33	-	-	-	0.59[b]	1.09[a]	0.64[b]	0.833	11.689	< 0.0001
RDW	-	-	-	-	-	-	0.04	0.15	0.33	-	-	-
ADWh2	1.15	3.96	0.79	-	-	-	0.95	1.68	2.23	-	-	-
NPOD	-	-	-	-	-	-	24.16[a]	15.23[b]	23.62[a]	0.855	9.895	0.000
WPOD	-	-	-	-	-	-	0.88	0.71	1.10	-	-	-
W100P	-	-	-	-	-	-	4.16	4.79	13.33	-	-	-

λ: Lambda of Wilks F: coefficient of Snedecor-Fisher. Days from emergence to first true leaf (D1L days); days from emergence to first flower (FLOR days); length of stems (LS cm); number of ramifications (NR); number of leaves (NL); aerial fresh weight (AFW g); aerial dry weight (ADW g); root dry weight (RDW g); aerial dry weight at second harvest (ADWh2 g); number of pods (NPOD); weight of pods (WPOD g); weight of 100 pods (W100P g). Means of each trait followed by the same letters are not significantly different between the three lines and populations' groups.

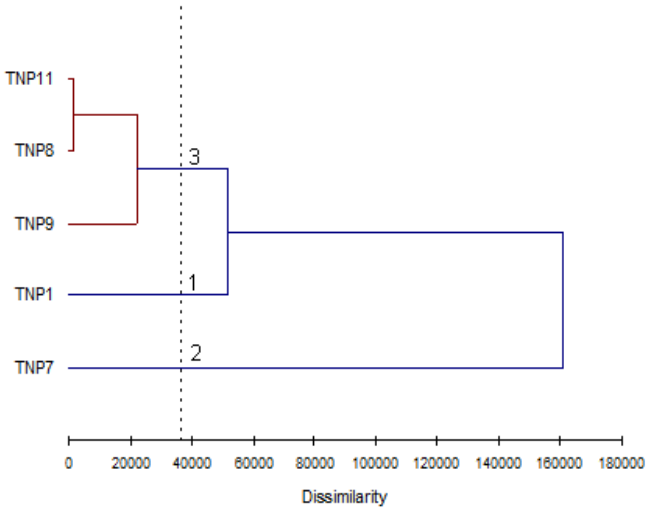

Figure 3. Dendrogram of populations of *Medicago polymorpha* clustered based on Euclidean distances of dissimilarity matrix with the Ward's method. Enfidha (TNP1), El Kef (TNP7), Soliman (TNP8), Bulla Regia (TNP9), and Mateur (TNP11).

Soliman, Bulla Regia and Mateur populations. The populations of first group showed the highest number of days from emergence to first true leaf (D1L) while the populations of second group showed the highest ADW (Table 5). Furthermore, the populations of first and third groups exhibited the highest NPOD.

Correlations between morphological traits and environmental parameters

Among the 55 correlations between the traits and environmental parameters, 9 correlations were significant and 6 of them were negative (Table 6). The altitude was positively correlated with FLOR, AFW and ADW while it was negatively correlated with NPOD. Furthermore, the temperature was negatively correlated with FLOR and ADW. The relative humidity was negatively associated with FLOR, AFW and RDW.

Table 6. Correlations between measured traits and environmental parameters for natural populations of *Medicago polymorpha*.

	Altitude	EC	Ann rain	T	RH
D1L	-0.14	-0.08	-0.10	0.17	0.16
FLOR	0.42*	-0.05	0.00	-0.36*	-0.40*
NR	0.26	-0.09	-0.06	-0.21	-0.16
NL	0.12	0.01	0.06	-0.08	-0.23
AFW	0.43*	-0.16	-0.10	-0.29	-0.39*
ADW	0.48*	-0.05	-0.10	-0.43*	-0.40*
RDW	-0.06	0.00	0.07	0.00	0.17
ADWh2	-0.04	0.01	0.07	-0.02	0.13
NPOD	-0.34*	0.14	0.13	0.32	0.07
WPOD	-0.09	0.00	0.06	0.04	0.18
W100P	-0.07	0.01	0.07	0.01	0.17

*Significant after using Bonferroni correction (α= 0.05/ 55 = 0.0009090909). Days from emergence to first true leaf (D1L days); days from emergence to first flower (FLOR days); number of ramifications (NR); number of leaves (NL); aerial fresh weight (AFW g); aerial dry weight (ADW g); root dry weight (RDW g); aerial dry weight at second harvest (ADWh2 g); number of pods (NPOD); weight of pods (WPOD g); weight of 100 pods (W100P g). Electro-conductivity (EC); mean annual rainfall (An rain); temperature (T); relative humidity (RH).

DISCUSSION

One hundred and twenty lines of *M. polymorpha*, sampled from different eco-geographical regions in Tunisia, were used to quantify phenotypic diversity. Sixteen vegetative and reproductive characters were measured to generate valuable information on genetic variation within and among populations of this species. Results from ANOVA support that the variation of the traits was explained by population and lines nested within population effects. The largest effect was recorded for population. Decomposition of variation within and among populations indicated that most differentiation was recorded within populations. Such a result is not expected with regard to the self-fertilizing mating system of *M. polymorpha*. Accordingly, highest phenotypic variance within populations were reported for natural Tunisian populations of *M. truncatula*, *M. laciniata* and *M. ciliaris* (Badri et al., 2007, 2008, 2010; Arraouadi et al., 2009) and for *Brachypodium distachyon* (Neji et al., 2014). Friesen et al. (2014) detected evidence of substantial migration between all pairs of Tunisian populations of *M. truncatula*. We expect that migration occurs largely by migration of seed pods in the wool or hair of animals.

In the current study, a substantial variation was detected among populations for 11 of the 16 investigated traits. These differences between populations were essentially related to flowering time, plant vigor, and biomass and pods production. This agrees with previous studies which revealed that *M. truncatula*, *M. laciniata* and *M. ciliaris* were highly variable for morpho-phenological characters (Badri et al., 2007, 2008, 2010, 2015; Arraouadi et al., 2009). The seed hardness and dormancy of seeds of *M. polymorpha* coupled with highly variable flowering time, allowed this species to survive unfavorable periods in a wide variety of bioclimatic zones. Among the 120 studied lines of *M. polymorpha*, there was no spineless line. Similarly, no spineless ecotype of this species has ever been found in Sardinia (Loi et al., 1995). Nevertheless, populations with non-spiny pods are very common in Chile (Del Pozo et al., 2002). This feature is of great interest to breeders and farmers, since the spiny pods of *M. polymorpha* are frequently caught in large numbers in sheep's hair, and drastically reduce the commercial value of the wool.

Our results showed that environmental variance was higher than genetic variance for most traits and consequently had a relatively low average of heritability. In addition, low levels of Q_{ST} were registered for most investigated characters. There was no significant association between population differentiation and geographical distances. These results are consistent with previous findings showing an absence of significant correlation between geographical distance and population differentiation in annual *Medicago* species (Badri et al., 2008, 2010) and *Brachypodium hybridum* (Neji et al., 2014). Nevertheless, Neji et al. (2013) observed that geographical distance partially explained the genetic distance among populations of *Sulla carnosa*.

The flowering time was positively correlated with NL, AFW, and ADW. Indeed, the latest flowering plants invest most of their effort in the aerial growth. These results are consistent with those found in natural populations of *M. truncatula*, *M. laciniata* and *M. ciliaris* (Badri et al., 2007, 2008, 2010). The induction of flowering is a central event in the life cycle of plants. Flowering is controlled by environmental conditions and developmental regulation (Mouradov et al., 2002). The harvest index (HI) was negatively correlated with LS, NL, AFW, ADW, and ADWh2, indicating that plants allocated higher effort to the vegetative growth produce lower number of pods. Clustering of studied lines into three groups differing in the LS and NL has implications with regard to choice of parents for creating segregating populations so as to maintain genetic diversity in a breeding program.

Most correlations were found between the altitude and measured traits followed by relative humidity and temperature, suggesting that the degree of trait variation differs according to the region where seeds were collected. Accordingly, Del Pozo et al. (2002) demonstrated that days to first flower were positively correlated with both latitude and longitude, as well as mean annual precipitation of collecting site of 69 Chilean accessions of *M. polymorpha*. Ecotypic differentiation related to environmental parameters along an aridity gradient in Syria or in a heterogeneous area of distribution such as Sardinia have been found in numerous annual legumes, for traits such as flowering time and degrees of hard seededness (Ehrman and Cocks, 1990; Piano et al., 1996) and shoot growth (Loi et al., 1993; Prosperi et al., 1991; Ovalle et al., 1993; Norman et al., 1998). Adaptive genetic variation is defined as the variation in genes that affects the fitness of an organism (Holderegger et al., 2006). Adaptation may be facilitated by co-adapted gene complexes, which are multi-locus genotypes favored by selection (Schemske, 2010).

In conclusion, our study demonstrates that morpho-phenological traits are useful tools for detecting variation within and among populations of *M. polymorpha*. Higher levels of variability for most traits were found to occur within populations. Our results suggest a prominent role for natural selection in accounting for patterns of genetic differentiation at quantitative traits among natural populations of *M. polymorpha.* Further study is needed to genotype lines of this species using simple sequence repeat (SSR) markers developed for the model legume *M. truncatula*. This will radically improve genetic characterizations and breeding programs of *M. polymorpha*.

Conflict of interests

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

We thank Ken Moriuchi for constructive comments on the manuscript, Naceur Djébali for the collection of pods in the field, and Saoussen Mahfoudh and Henda Darine for assistance in the greenhouse. This research was supported by the Tunisian Ministry of Higher Education and Scientific Research (LR10 CBBC 02).

REFERENCES

Abdelkefi A, Boussaid M, Biborchi A, Haddioui A, Salhi Hannachi A, Marrakchi M (1996). Genetic diversity inventory and valuation of spontaneous species belonging to *Medicago* L. genus in Tunisia. Cah. Options Mediterr. 18:143-150.

Aitken Y (1981). Temperate herbage grasses and legumes. In Handbook of Flowering. Halevy, CRC, Boca Raton, Florida.

Arraouadi S, Badri M, Abdul Jaleel C, Djébali N, Ilahi H, Huguet T, Aouani ME (2009). Analysis of genetic variation in natural populations of *Medicago truncatula* of southern Tunisian ecological areas using morphological traits and SSR markers. Trop. Plant Biol. 2:122-132.

Arraouadi S, Chardon F, Huguet T, Aouani ME, Badri M (2011). QTL mapping of morphological traits related to salt tolerance in *Medicago truncatula*. Acta Physiol. Plant. 33:917-929.

Badri M, Arraouadi S, Huguet T, Aouani ME (2010). Comparative effects of water deficit on *Medicago laciniata* and *M. truncatula* lines sampled from sympatric populations. J. Plant Breed. Crop Sci. 2:192-204.

Badri M, Bouhaouel I, Arraouadi S, Taamalli W, Huguet T, Aouani ME (2015). Variation in tolerance to drought among Tunisian populations of *Medicago truncatula*. Plant Genet. Resour. pp. 1-9.

Badri M, Chardon F, Huguet T, Aouani ME (2011). Quantitative Trait Loci associated with drought tolerance in the model legume *Medicago truncatula*. Euphytica 181:415-428.

Badri M, Ilahi H, Huguet T, Aouani ME (2007). Quantitative and molecular genetic variation in sympatric populations of *Medicago laciniata* and *M. truncatula* (Fabaceae): relationships with eco-geographical geographical factors. Genet. Res. 89:107-122.

Badri M, Zitoun A, Soula S, Ilahi H, Huguet T, Aouani ME (2008). Low levels of quantitative and molecular genetic differentiation among natural populations of *Medicago ciliaris* Kroch. (Fabaceae) of different Tunisian eco-geographical origin. Conserv. Genet. 9:1509-1520.

De Kort H, Vandepitte K, Honnay O (2012). A meta-analysis of the effects of plant traits and geographical scale on the magnitude of adaptive differentiation as measured by the difference between Q_{ST} and F_{ST}. Evol. Ecol. 27:6.

Del Pozo A, Ovalle C, Aronson J, Avendaño J (2002). Ecotypic differentiation in *Medicago polymorpha* L. along an environmental gradient in central Chile. I. Phenology, biomass production and reproductive patterns. Plant Ecol. 159:119-130.

Ehrman T, Cocks PS (1990). Ecogeography of annual legumes in Syria: distribution patterns. J. Appl. Ecol. 27:578-591.

Escudero A, Iriondo JM, Torres ME (2003). Spatial analysis of genetic diversity as a tool for plant conservation. Biol. Conserv. 113:351-365.

Friesen ML, von Wettberg EJB, Badri M, Moriuchi KS, Barhoumi F, Chang PL, Cuellar-Ortiz S, Cordeiro MA, Vu WT, Arraouadi S, Djébali N, Zribi K, Badri Y, Porter SS, Aouani ME, Cook DR, Strauss SY, Nuzhdin SV (2014). The ecological genomic basis of salinity adaptation in Tunisian *Medicago truncatula.* BMC Genomics 15:1160.

Hahn MA, van Kleunen M, Müller-Schärer H (2012). Increased phenotypic plasticity to climate may have boosted the invasion success of polyploid *Centaurea stoebe.* PLoS One 7(11):e50284.

Hendry AP (2002). QST >=≠>FST? Trends Ecol. Evol. 17:502.

Heyn CC (1963). The Annual Species of *Medicago*. Scripta hierosolymitana Hebrew University Press Jerusalem.

Holderegger R, Kamm U, Gugerli F (2006). Adaptive vs. neutral genetic diversity: implications for landscape genetics. Landsc. Ecol. 21:797-807.

Lazrek F, Roussel V, Ronfort J, Cardinet G, Chardon F, Aouani ME, Huguet T (2009). The use of neutral and non-neutral SSRs to analyze the genetic structure of a Tunisian collection of *Medicago truncatula* lines and to reveal associations with eco-environmental variables. Genetica 135:391-402.

Loi A, Howieson JG, Cocks PS, Caredda S (1993). The adaptation of *Medicago polymorpha* to a range of edaphic and environmental conditions: effect of temperature on growth, and acidity stress on nodulation and nod gene induction. Aust. J. Agric. Res. 33:25-30.

Loi A, Porqeuddu C, Veronesi F, Cocks PS (1995). Distribution, diversity and potential agronomic value of *Medicago polymorpha* in Sardinia. J. Agric. Res. 124:419-426.

Mantel N (1967). The detection of disease clustering and a generalized regression approach. Cancer Res. 27:1055-1067.

Mouradov A, Cremer F, Coupland G (2002). Control of flowering time: interacting pathways as a basis for diversity. Plant Cell 14:S111-S130.

Neji M, Geuna F, Taamalli W, Ibrahim Y, Smida M, Badri M, Abdelly C, Gandour M (2014). Morpho-phenological diversity among Tunisian natural populations of *Brachypodium hybridum*. J Agric. Sci. 153(6):1006-1016.

Neji M, Taamalli W, Smida M, Abdelly C, Gandour M (2013). Phenotypic and molecular genetic variation in Tunisian natural populations of *Sulla carnosa.* Agron. J. 105:1094-1100.

Norman HC, Smith FP, Cocks PS, Nutt BJ (1998). Reproductive strategies in Mediterranean annual clovers: germination and hardseededness. Aust. J. Agric. Res. 49:973-982.

Ovalle C, Avendaño J, Del Pozo A, Crespo D (1993). Germplasm collection, description and selection of naturalized *Medicago polymorpha* in the Mediterranean zone of Chile. Proceedings XVII International Grassland Congress. Palmerston North, New Zealand, pp. 222-223.

Piano E, Pecetti L, Carroni AM (1996). Climatic adaptation in subterranean clover populations. Euphytica 92:39-44.

Prosperi JM, Boumard P, Angevain M, Mansat P (1991). Répartition et adaptation écotypique de *Medicago* annuelles en Méditerranée occidentale. In IVth International Rangeland Congress. CIRAD, Montpellier, France. pp. 413-416.

Salhi Hannachi A, Boussaid M, Marrakchi M (1998). Genetic variability organization and gene flow in natural populations of *Medicago polymorpha* L. prospected in Tunisia. Genet. Sel. Evol. 30:S121-S135.

Schemske DW (2010). Adaptation and the origin of species. Am. Nat. 176:S4-S25.

Seklani H, Zoghlami A, Mezni M, Hassen H (1996). Synthèse des travaux de recherche réalisés sur les *Medicago* à l'Institut National de la Recherche Agronomique de Tunisie. Cah. Options Mediterr. 18:31-37.

Small E (2010). Alfalfa and relatives: Evolution and classification of *Medicago*. NRC Research Press Ottawa Ontario Canada.

Small E, Jomphe M (1989). A synopsis of the genus *Medicago* (Leguminosae). Can. J. Bot. 67:3260-3294.

Tar'an B, Zhang C, Warkentin T, Tullu A, Vanderberg A (2005). Genetic diversity among varieties and wild species accessions of pea (*Pisum sativum*) based on molecular markers and morphological and physiological characters. Genome 48:257-272.

academicJournals

Vol. 15(25), pp. 1339-1349, 22 June, 2016
DOI: 10.5897/AJB2015.15133
Article Number: 1C6A47B59053
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Morph-physiological responses to water deficit in parental genotypes of *Medicago truncatula* recombinant inbred lines

Mounawer Badri*, Saoussen Mahfoudh and Chedly Abdelly

Laboratory of Extremophile Plants, Centre of Biotechnology of Borj Cedria, B.P. 901, Hammam-Lif 2050, Tunisia.

Received 29 November 2015, Accepted 29 April, 2016.

Medicago truncatula **is an omni-Mediterranean species grown as an annual forage legume. In addition to its small genome size and simple genetics, *M. truncatula* harbors several attributes which make it an attractive model legume. In this study, we investigated morphological and photosynthetic responses to water deficit in parental genotypes of *M. truncatula* recombinant inbred lines. Ten parental lines were cultivated under three water regimes (100% of field capacity (FC), 50% FC and 30% FC) and were harvested at flowering time and at the end of their lifecycle. Results from ANOVA showed that variability of measured parameters was explained by the effects of line, treatment and their interaction with treatment factor recorded the highest values. Out of the 27 traits, 14 were influenced by the line x treatment interaction. High to moderate broad-sense heritability (H^2) were observed for most of the traits under control treatment and drought stress. Most of the correlations between measured traits were positive under the three water regimes. The flowering time was positively correlated with aerial and root growth rate. The tolerance of lines to water deficit seems to depend, in particular, on their ability to maintain higher photosynthetic activity. In 30% FC, principal component analysis clustered lines into two groups. The Jemalong A17 line was the least affected for most phenotypic parameters. Hence, all populations of recombinant inbred lines derived from crosses between Jemalong A17 and the remaining lines were useful for the identification of the genetic determinants for tolerance to water deficit in *M. truncatula*.**

Key words: *M. truncatula*, parental lines, recombinant inbred lines, water deficit, morphological traits, photosynthetic parameters.

INTRODUCTION

Environmental constraints are the main factors limiting agricultural productivity and play a major role in the distribution of plant species. Among the abiotic stresses, water deficit is considered the most important factor in limiting crop production (Harb et al., 2010). Some adaptive responses to water deficit correspond to

*Corresponding author. Email: mounawer_badri@yahoo.fr, mounawer.badri@cbbc.rnrt.tn. Tel: +216 79 325 848.

changes in the structure of plants (Shao et al., 2008), the growth rate and gas exchange at the leaf level (Slama et al., 2007), and overproduction of compatible compounds (Nunes et al., 2008). One of the first responses to drought stress is stomatal closure (Anjum et al., 2011). As a result, photosynthetic assimilation is unavoidably reduced due to decreased CO_2 concentrations at the chloroplast level (Cornic, 1994). Furthermore, a reduced leaf area and decreased transpiration rate is often the result of drought stress (Dong and Zhang, 2000). Consequently, gas exchange characteristics and chlorophyll concentrations are reduced as stress intensity increases.

Legumes are very important sources of vegetable protein for human food and animal feed and these plants do not require nitrogen fertilization. Drought represents one of the main factors which limits legume productivity in the Mediterranean basin. A better understanding of the mechanisms for adaptation to water deficit in legumes is a prerequisite for any research aiming to improve legume yields. The slowness of genetic improvements in most legumes of economic interests is due to some unfavorable traits such as their large genome, their complex ploidy, and the difficulties of their transformation and regeneration. *M. truncatula* thus emerged as a model legume because of several unique characteristics (Barker et al., 1990). It is diploid (2n=16), self-pollinating, has a low DNA content per haploid genome (~465 Mbp), is suitable for cultivation in the laboratory (size, growth habit, flowering, etc.) and has a rapid lifecycle. Furthermore, several studies on *M. truncatula* have contributed to an expanded genetic and genomics tools (Tang et al., 2014). It is also an important forage crop species in several countries especially Australia.

In recent years, molecular mechanisms for tolerance to drought stress in plants have become an active area of investigation and many genes regulated by drought stress have been reported in a variety of plants (Sehgal et al., 2012; Thudi et al., 2014; Xu et al., 2014). Very little is known about the physiological and molecular mechanisms by which the model legume *M. truncatula* responds to drought stress. In most of the previous experiments plants were terminated at the vegetative stage (Badri et al., 2011; Wang, 2014; Zhang et al., 2014). Furthermore, these previous studies have used only one population of RILs to analyze the genetic determinants for tolerance to drought stress. Therefore, it is important to analyze the genetic bases of tolerance to this constraint by using more than one population of RILs in addition to studying their responses at both the vegetative and reproductive stages.

The objective of this study was to evaluate the effects of water deficit on photosynthetic and growth characteristics in parental genotypes of RILs of *M. truncatula* at flowering and end of lifecycle stages. This as well as similar investigations can lead to the selection of more than one appropriate RIL population useful for the analysis of genetic determinism to drought. Furthermore, knowledge obtained by studying *M. truncatula* can be transferred to other legumes.

MATERIALS AND METHODS

Plant material and experimental conditions

Ten parental lines of *M. truncatula* recombinant inbred lines (RILs) collected in different regions that varied in soil composition, salinity and water availability were used in this study. These lines were intercrossed in order to provide useful material for the analysis of genetic determinism of traits of agronomical interest (Figure 1). These lines are DZA315.16 and DZA45.5 from Algerian populations, Jemalong A17 from the Australian collection, F83005.5 line from the Var region in France, A20 line of Moroccan origin and Tunisian lines TN1.11, TN1.21, TN8.3, TN8.20 and TN6.18 collected respectively from Enfidha (TN1), Soliman (TN8) and Thala (TN6) sites. The nomenclature of local lines includes “TN” to signal their Tunisian origin, the first number to refer to their collection site, and the second number is added to indicate the number of the pod.

The experiment was performed under greenhouse conditions at the Centre of Biotechnology of Borj Cedria (CBBC) in the Tunisian winter of 2012. Because the seeds of *M. truncatula* are dormant, a mechanical scarification was carried out by using sandpaper Q600. Once the seeds were scarified, they were directly transplanted into black pots (diameter = 17 cm; deep = 13cm) containing two liters of sandy loam soil (Rouached et al., 2013) consisting of (cmol kg^{-1} dry soil) 0.23 Na^+, 0.94 K^+, 0.64 Ca^{2+}, and 0.05 Cl^-, and (g kg^{-1} dry soil) 0.23 P_2O_5 and 0.44 total nitrogen. The pH and the electrical conductivity of the aqueous extract (1/10) were 6.68 and 0.05 dS m^{-1} respectively, with three seeds per pot. At the stage of the sixth leaf all the plants were removed while only a single plant per pot was kept for treatments. Lines were grown in a randomized complete block design. Eight replicates per line and per treatment were used. Plants were submitted to three water regimes (i) control treatment without water deficit where the soil is maintained at 100% field capacity (FC), (ii) moderate (50% FC) and severe (30% FC) water deficit which were applied 21 days after seed germination. The equivalent soil moisture was determined by the method of Bouyoucos (1983). Watering of plants was done every two days using a nutrient solution (Hewitt, 1966). To maintain the constant water regime during the culture period, the amount of water absorbed by the plant or lost by evaporation was compensated regularly every two days for each level of irrigation. This quantity was determined in advance, for each pot and weighed to calculate the weight lost from the initial weight (soil in pot without any plants) for each treatment, and this difference corresponded to the amount of water lost. Eight replicates of each genotype were harvested at flowering time and eight replicates at the end of their lifecycle.

Morphological measured traits

Twenty three morphological traits related to aerial and root growth were measured for the 10 lines of *M. truncatula* at flowering and end of lifecycle stages (Table 1). These traits showed high levels of polymorphism and broad-sense heritability (H^2) as observed in previous studies (Badri et al., 2007, 2011; Arraouadi et al., 2011). Plants were harvested at flowering stage and at the end of their lifecycle; plants were divided into leaves, stems and roots. Dry weight was obtained after oven-drying leaf, stem and root samples at 60ºC for 48 h. For each water regime, the leaf water content (LWC), the aerial water content (AWC), and the root water content (RWC) were estimated as follows:

LWC = 100(FWL-LDW)/FWL

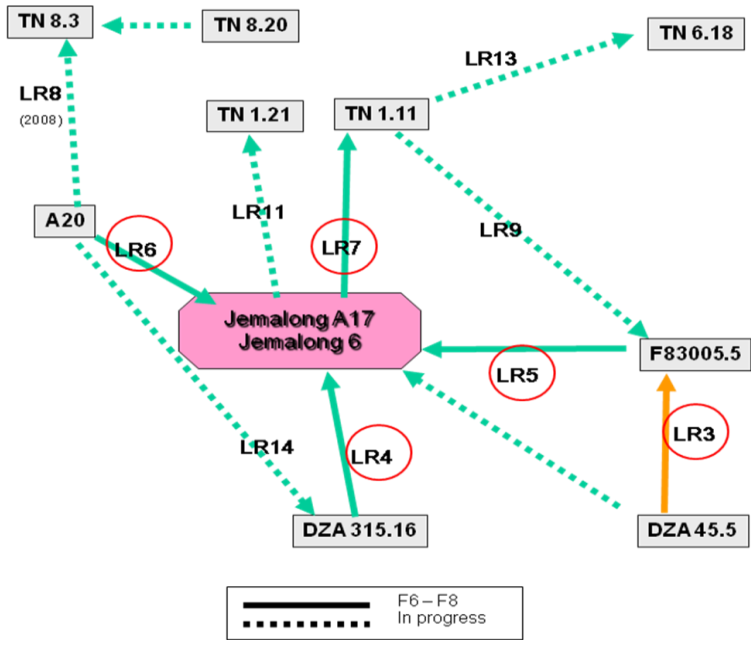

Figure 1. Scheme of crosses performed between lines of *Medicago truncatula* in the Centre of Biotechnology of Borj Cedria (CBBC), Tunisia and Institut National Polytechnique-Ecole Nationale Supérieure Agronomique de Toulouse, France.

AWC = 100(AFW-ADW)/AFW
RWC = 100(RFW-RDW)/RFW

Where FWL, AFW and RWC are the fresh weights of leaves, aerial part and roots, respectively, while LDW, ADW and RDW are the dry weights of leaves, aerial part and roots, respectively. The aerial part is the leaves plus stems.

The leaf water potential (Ψh) was measured using a pressure chamber (Soil Moisture Equipment, Santa Barbara, CA, USA) (Scholander et al., 1965).

Photosynthesis measurements

Gas exchange measurements (net CO_2 assimilation rate (A), transpiration rate (E), stomatal conductance (gs), and instantaneous water-use efficiency (WUEi) as the ratio A/E were done on mature leaves using a CO_2 and H_2O infrared gas analyzer (Li-Cor 6200, Li-Cor Nebraska, USA). Measurements were performed between 10 and 12 am on the leaves of plants subjected to various treatments as described by Hessini et al. (2013).

Statistical analyses

A three-way analysis of variance was used to test for line, drought treatment differences, and line x treatment interaction effects. Only characters that showed a significant line x treatment interaction were used for further analysis. Means were compared using Duncan's multiple range test at 5%. Correlations between parameters were calculated using Pearson's correlation method. The significance level of associations between morphological traits and photosynthetic parameters was set to 0.05, and adjusted for multiple comparisons by Bonferroni corrections. All analyses were performed using SPSS software (SPSS Inc. Released 2007 SPSS for Windows, Version 16.0. Chicago, SPSS Inc.). Drought response index (DRI) is considered as an indicator of tolerance to water deficit (Chen et al., 2007) and is estimated as the ratio between the values observed under water deficit conditions and those obtained in the control treatment.

Broad-sense heritability (H^2) for each trait was estimated as:

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_e^2}$$

Where, σ^2_g is the genetic variance observed between the lines and σ^2_e is the environmental variance corresponding to the residual error between the eight replicates of the same genotype (=line).

Drought response indices (DRI) for measured traits in lines of *M. truncatula* grown under 50 and 30% of field capacity were subjected to principal component analysis (PCA). This analysis was carried out using the XLSTAT software version 7.5 (Addinsoft, 2007, New York, USA).

RESULTS

Morph-physiological variation among lines

Results from ANOVA showed that variation of morph-physiological traits was explained by the effects of line, treatment and their interaction (Table 1). The parameters were mostly influenced by the treatment factor. Out of the 27 measured characters, 14 were significantly explained by the line x treatment interaction. They include the

Table 1. Contribution of line, treatment, and line x treatment interaction to the total variance of measured traits for lines of *M. truncatula.*

Trait	Line (L)		Treatment (T)		L x T	
	F	*P*	F	*P*	F	*P*
†FLOR	67.36	0.000	6.89	0.001	2.29	**0.003**
LS	6.41	0.000	557.47	0.000	0.90	0.582
LR	2.38	0.014	64.22	0.000	0.96	0.512
NL	41.66	0.000	390.83	0.000	3.00	**0.000**
LA	12.90	0.000	197.28	0.000	5.15	**0.000**
AFW	4.60	0.000	464.14	0.000	1.27	0.211
ADW	1.68	0.097	191.07	0.000	0.49	0.960
AWC	2.88	0.003	163.46	0.000	0.69	0.823
FWL	6.33	0.000	344.87	0.000	1.76	**0.032**
DWL	7.94	0.000	390.08	0.000	1.48	0.101
LWC	5.26	0.000	31.76	0.000	0.97	0.495
RFW	20.62	0.000	402.97	0.000	3.20	**0.000**
RDW	10.44	0.000	145.52	0.000	2.80	**0.000**
RWC	1.52	0.141	30.08	0.000	1.02	0.441
RDW/ADW	8.65	0.000	1.57	0.211	1.14	0.315
NbNOD	10.69	0.000	227.70	0.000	2.30	**0.003**
Ψh	4.30	0.000	369.87	0.000	1.16	0.295
ADWh2	3647.00	0.000	892504.00	0.000	1538.00	0.080
NPOD	41244.00	0.000	239446.00	0.000	10581.00	**0.000**
NbSD/POD	44.74	0.000	24.70	0.000	1.06	0.411
WPOD	2461.00	0.011	283721.00	0.000	1784.00	**0.029**
W100P	133622.00	0.000	9711.00	0.000	1832.00	**0.024**
HI	3442.00	0.001	16787.00	0.000	1679.00	**0.045**
A	23.96	0.000	169.50	0.000	4.31	**0.000**
E	15.07	0.000	68.23	0.000	3.62	**0.000**
gs	9.72	0.000	60.52	0.000	1.85	**0.028**
WUEi (A/E)	2.92	0.004	2.59	0.079	1.64	0.063

F: Coefficient of Snedecor-Fisher, not significant ($P>0.05$), significant ($P\leq0.05$). FLOR, Date of the first stem flower bud stage (days); LS, length of stems (cm); LR, length of roots (cm); NL, number of leaves; LA, leaf area (cm^2); AFW, aerial fresh weight (g); ADW, aerial dry weight (g); AWC, aerial water content (%); FWL, fresh weight of leaves (g); DWL, dry weight of leaves (g); LWC, leaf water content (%); RFW, root fresh weight (g); RDW, root dry weight (g);RWC, root water content (%); RDW/ADW, root dry weight and aerial dry weight ratio; NbNOD, number of nodules; Ψh, osmotic potential (MPa); ADWh2, aerial dry weight at harvest 2; NPOD, number of pods;NbSD/POD, mean number of seeds per pod; WPOD, weight of pods; W100P, weight of 100 pods; HI, harvest index; A, µmol CO_2 $m^{-2}s^{-1}$, photosynthetic rate; E, mmol H_2O $m^{-2}s^{-1}$, transpiration rate; gs, stomatal conductance; WUEi, mol CO_2 $m^{-2}s^{-1}$ instantaneous water use efficiency

flowering time (FLOR), number of leaves (NL), leaf area (LA), leaf fresh weight (FWL), root fresh weight (RFW), root dry weight (RDW), number of nodules (NbNOD), number of pods (NPOD), weight of pods (WPOD), weight of 100 pods (W100P), harvest index (HI), net CO_2 assimilation (A) and transpiration (E), and stomatal conductance (gs).

In the control treatment, all the 14 parameters showed significant difference between lines (Table 2). The A20 line was the earliest flowering genotype while DZA45.5 was the latest flowering genotype. The largest number of pods (NPOD) was observed for TN6.18 while the lowest value was registered for A20. The net CO_2 assimilation (A), transpiration (E), and stomatal conductance (gs) showed significant difference between lines. The highest net CO_2 assimilation (A) and transpiration (T) were noted for the F83005.5 line while the lowest values were observed for DZA45.5.

Of the 14 parameters, 11 and 12 showed significant reductions between lines in 50 and 30% FC respectively. Reductions were more significant for most traits in lines under severe (30% FC) than moderate (50% FC) water deficit. In 50% FC, the TN6.18 line showed the greatest reduction for NPOD and WPOD while TN1.11 and TN8.20 exhibited the highest levels for leaf area (LA). Furthermore, the largest reductions for A, E and gs were recorded for the A20 line. In 30% FC, the highest reductions for A and E were registered for DZA315.16 and F83005.5.

The broad-sense heritability (H^2) of traits ranged from 0.13 to 0.93, from 0 to 0.92, and from 0.09 to 0.97 under the control treatment as well as for 50 and 30% FC

Table 2. Mean values of measured traits and drought indices (DRI) for lines of *M. truncatula* cultivated under control treatment (100% FC) and water deficit (50% and 30% FC).

Control treatment (100% FC)														
Trait\line	**FLOR**	**NL**	**LA**	**FWL**	**RFW**	**RDW**	**NbNOD**	**NPOD**	**WPOD**	**W100P**	**HI**	**A**	**E**	**gs**
A20	71.75^{e}	135.50f	38.22^{bcd}	5.76^{c}	18.46^{c}	1.30^{c}	113.12^{d}	40.88^{e}	9.61^{a}	23.44^{a}	0.64^{a}	44.13^{ab}	5.36^{bc}	0.40^{a}
DZA315	83.19^{c}	315.87^{cd}	48.66^{bc}	9.55^{a}	41.87^{a}	3.38^{a}	213.00^{ab}	119.62^{b}	9.51^{ab}	7.98^{e}	0.55^{ab}	29.47^{c}	4.48^{bc}	0.27^{a}
DZA45	94.94^{a}	352.00^{bc}	27.23^{d}	6.22^{bc}	21.50^{bc}	1.40^{c}	223.12^{a}	46.00^{de}	10.53^{a}	22.64^{a}	0.57^{ab}	10.45^{d}	1.92^{d}	0.10^{b}
F83	83.88^{c}	242.88^{e}	47.22^{bc}	9.31^{a}	24.27^{bc}	1.62^{c}	133.62^{cd}	74.00^{cd}	8.57^{ab}	11.72^{d}	0.54^{ab}	46.48^{a}	8.72^{a}	0.42^{a}
JA17	86.12^{c}	281.75^{cde}	42.86^{bcd}	7.49^{abc}	24.50^{bc}	1.57^{c}	234.12^{a}	41.25^{e}	6.25^{b}	14.18^{c}	0.41^{c}	27.54^{c}	5.39^{bc}	0.33^{a}
TN1.11	77.69^{d}	225.00^{e}	52.53^{ab}	8.39^{ab}	22.64^{bc}	1.68^{c}	131.50^{cd}	61.88^{cde}	10.84^{a}	17.31^{b}	0.60^{ab}	42.04^{ab}	5.10^{bc}	0.34^{a}
TN1.21	77.00^{d}	296.00^{cde}	43.81^{bcd}	8.28^{abc}	21.35^{bc}	1.60^{c}	184.50^{abc}	62.25^{cde}	8.34^{ab}	13.44^{c}	0.49^{bc}	36.77^{abc}	5.28^{bc}	0.35^{a}
TN6.18	83.69^{c}	423.25^{a}	39.70^{bcd}	8.01^{abc}	26.21^{b}	1.91^{bc}	178.75^{abc}	191.62^{a}	11.90^{a}	6.27f	0.60^{ab}	39.46^{ab}	6.94^{ab}	0.35^{a}
TN8.20	90.56^{b}	389.62^{ab}	33.77^{cd}	8.69^{ab}	27.75^{b}	2.51^{b}	236.62^{a}	69.75^{cde}	8.80^{ab}	13.04^{cd}	0.53^{a}	28.52^{c}	4.00^{cd}	0.29^{a}
TN8.3	75.25^{d}	271.62^{de}	66.39^{a}	7.95^{abc}	21.93^{bc}	1.46^{c}	157.12^{bcd}	74.88^{c}	10.87^{a}	14.64^{c}	0.61^{a}	34.67^{bc}	4.90^{bc}	0.35^{a}
DRI 50% FC														
Trait\line	**FLOR**	**NL**	**LA**	**FWL**	**RFW**	**RDW**	**NbNOD**	**NPOD**	**WPOD**	**W100P**	**HI**	**A**	**E**	**gs**
A20	102.28^{a}	66.70^{a}	89.88^{a}	69.53^{ab}	56.33^{a}	63.22^{a}	60.78^{a}	62.07^{abc}	52.88^{abc}	86.46^{b}	82.68^{bc}	53.30^{c}	53.63^{e}	39.70^{c}
DZA315	103.44^{a}	72.18^{ab}	83.77^{ab}	67.99^{abc}	64.17^{a}	55.18^{a}	82.69^{a}	58.62^{abc}	61.28^{abc}	103.24^{a}	88.96^{bc}	71.76^{b}	66.49^{cde}	75.46^{bc}
DZA45	99.18^{ab}	55.86^{b}	57.70^{cd}	58.16^{bc}	62.46^{a}	63.40^{a}	62.07^{a}	61.41^{abc}	54.71^{abc}	88.96^{ab}	92.27^{abc}	74.24^{b}	74.22^{bcde}	66.25^{b}
F83	99.90^{a}	64.28^{ab}	83.10^{ab}	58.23^{abc}	70.45^{a}	73.19^{a}	76.34^{a}	72.13^{ab}	72.29^{a}	100.71^{ab}	99.26^{ab}	61.89^{bc}	103.15^{a}	76.67^{ab}
JA17	97.56^{ab}	67.28^{ab}	65.45^{c}	71.06^{abc}	79.36^{a}	81.10^{a}	79.23^{a}	76.36^{abc}	69.13^{ab}	95.80^{ab}	107.60^{a}	75.39^{b}	60.58^{de}	58.73^{b}
TN1.11	99.58^{a}	63.33^{ab}	50.04^{de}	56.78^{abc}	56.15^{a}	54.47^{a}	73.10^{a}	54.34^{abc}	50.83^{abc}	96.80^{ab}	87.00^{bc}	79.96^{ab}	95.74^{ab}	80.12^{ab}
TN1.21	102.68^{a}	53.29^{ab}	77.84^{b}	49.17^{c}	54.48^{a}	51.26^{a}	77.57^{a}	68.88^{ab}	61.33^{abc}	89.94^{ab}	90.37^{abc}	83.05^{ab}	90.44^{abc}	81.18^{ab}
TN6.18	102.38^{a}	76.99^{a}	77.41^{b}	86.92^{a}	74.04^{a}	66.40^{a}	66.36^{a}	41.36^{c}	40.82^{c}	98.57^{ab}	76.77^{c}	67.58^{bc}	96.88^{ab}	101.98^{a}
TN8.20	98.92^{b}	69.43^{ab}	45.68^{e}	51.16^{abc}	57.15^{a}	47.21^{a}	61.86^{a}	66.85^{ab}	68.08^{ab}	99.69^{ab}	95.75^{ab}	98.01^{a}	101.30^{ab}	75.09^{ab}
TN8.3	102.09^{ab}	64.11^{a}	51.18^{de}	51.14^{abc}	64.48^{a}	69.07^{a}	70.09^{a}	49.75^{bc}	46.49^{bc}	93.31^{ab}	76.60^{c}	68.60^{bc}	83.55^{abcd}	68.10^{b}
DRI 30% FC														
Trait\line	**FLOR**	**NL**	**LA**	**FWL**	**RFW**	**RDW**	**NbNOD**	**NPOD**	**WPOD**	**W100P**	**HI**	**A**	**E**	**gs**
A20	106.29^{ab}	25.18^{abc}	62.54^{a}	18.76^{abc}	19.19^{a}	29.10^{ab}	23.65^{a}	22.63^{b}	16.71^{bc}	76.10^{c}	93.32^{d}	40.77^{ab}	41.27^{c}	34.67^{bc}
DZA315	105.62^{abc}	35.18^{abc}	42.43^{cd}	24.05^{abc}	20.11^{a}	20.47^{abc}	22.01^{a}	19.44^{b}	18.98^{bc}	98.52^{ab}	92.47^{d}	17.67^{c}	16.89^{d}	15.18^{c}
DZA45	96.22^{d}	22.08^{c}	58.61^{a}	21.58^{bcd}	27.19^{a}	39.53^{a}	10.48^{a}	26.36^{ab}	22.06^{bc}	84.75^{bc}	108.13^{bcd}	42.97^{a}	52.28^{abc}	40.00^{ab}
F83	99.00^{c}	23.93^{bc}	50.87^{b}	13.47^{d}	17.11^{a}	22.67^{bc}	20.39^{a}	24.16^{ab}	23.16^{ab}	98.38^{ab}	107.56^{bc}	14.29^{c}	7.68^{d}	18.25^{c}
JA17	101.70^{bcd}	31.50^{ab}	27.35fg	27.59^{a}	23.38^{a}	38.80^{abc}	26.43^{a}	32.12^{a}	30.27^{a}	101.99^{a}	146.37^{a}	43.21^{ab}	36.46^{c}	43.17^{ab}
TN1.11	103.44^{abc}	26.28^{abc}	32.73^{e}f	15.61^{bcd}	20.25^{a}	26.02^{abc}	29.37^{a}	25.65^{ab}	24.41^{abc}	98.34^{ab}	107.50^{bcd}	33.80^{bc}	45.37^{ab}	32.64^{b}
TN1.21	109.98^{a}	21.58^{c}	44.98^{bc}	15.62^{d}	15.25^{a}	20.24^{bc}	17.89^{a}	18.47^{b}	17.92^{bc}	96.46^{ab}	103.46^{bcd}	46.67^{ab}	55.92^{a}	39.40^{ab}
TN6.18	104.99^{abc}	42.91^{abc}	22.18gh	28.97^{ab}	21.20^{a}	19.67^{bc}	23.29^{a}	18.33^{b}	17.12^{bc}	92.69^{ab}	97.76^{cd}	35.25^{ab}	41.62^{bc}	42.49^{ab}
TN8.20	97.34^{d}	25.12^{abc}	37.52^{de}	14.41^{bcd}	16.65^{a}	16.42^{c}	10.20^{a}	23.84^{ab}	21.96^{bc}	90.51^{abc}	119.73^{b}	45.32^{a}	48.61^{abc}	47.78^{ab}
TN8.3	103.01^{bcd}	26.60^{abc}	16.36h	11.88^{cd}	16.25^{a}	26.81^{abc}	22.36^{a}	16.36^{b}	14.30^{c}	85.27^{bc}	88.38^{cd}	43.92^{a}	46.27^{abc}	52.57^{a}

FLOR, Date of the first stem flower bud stage (days); NL, number of leaves; LA, leaf area (cm^2); FWL, fresh weight of leaves (g); RFW, root fresh weight (g); RDW, root dry weight NbNOD (number of nodules, NPOD number of pods; WPOD, weight of pods; W100P, weight of 100 pods; HI, harvest index; A, photosynthetic rate (µmol CO_2 $m^{-2}s^{-1}$); E, transpiration rate (mmol H_2O $m^{-2}s^{-1}$); gs, stomatal conductance. Means of each trait followed by the same letters are not significantly different between lines of *M. truncatula* at P = 0.05 based on Duncan's multiple range test. DZA315.16 (DZA315), DZA45.5 (DZA45), F83005.5 (F83), Jemalong A17 (JA17).

Table 3. Genetic (σ^2_g) and environmental (σ^2_e) variances, and heritabilities (H^2) for measured traits in lines of *M. truncatula* under control treatment (100% FC) and drought stress (50 and 30% FC).

Treatment/trait	Control (100% FC)			50% FC			30% FC		
	σ^2_g	σ^2_e	H^2	σ^2_g	σ^2_e	H^2	σ^2_g	σ^2_e	H^2
FLOR	49.30	12.76	0.79	39.07	9.26	0.81	23.43	13.83	0.63
NL	6440.01	4337.46	0.60	4362.95	1829.53	0.70	1589.72	465.32	0.77
LA	87.85	84.95	0.51	74.21	6.49	0.92	29.46	1.00	0.97
FWL	0.82	5.26	0.13	1.06	1.56	0.40	0.23	0.25	0.48
RFW	37.03	39.63	0.48	22.21	12.54	0.64	2.04	2.73	0.43
RDW	0.34	0.56	0.37	0.09	0.11	0.45	0.01	0.04	0.22
NbNOD	1838.22	1829.23	0.50	1144.82	1424.67	0.45	65.10	633.36	0.09
NPOD	2020.00	724.88	0.74	293.06	249.97	0.54	54.03	29.67	0.65
WPOD	1.48	9.33	0.14	0.00	3.32	0.00	0.07	0.43	0.15
W100P	30.29	2.38	0.93	20.21	4.28	0.83	17.47	4.06	0.81
HI	0.00	0.01	0.23	0.00	0.01	0.00	0.00	0.01	0.10
A	110.305	55.951	0.66	49.793	19.436	0.72	24.963	10.434	0.71
E	2.262	2.748	0.45	3.716	1.044	0.78	0.675	0.191	0.78
gs	0.007	0.013	0.35	0.007	0.004	0.64	0.002	0.001	0.67

FLOR, Date of the first stem flower bud stage (days); NL, number of leaves; LA, leaf area (cm^2); FWL, fresh weight of leaves (g); RFW, root fresh weight (g); RDW, root dry weight; NbNOD, number of nodules; NPOD, number of pods; WPOD, weight of pods; W100P, weight of 100 pods; HI, harvest index; A, photosynthetic rate (µmol CO_2 $m^{-2}s^{-1}$); E, transpiration rate (mmol H_2O $m^{-2}s^{-1}$); gs, stomatal conductance.

respectively (Table 3). Of the 14 traits, 9, 11 and 10 showed high heritability values under control treatment, 50 and 30% FC respectively. In the control treatment, high heritability (H^2>0.4) values were recorded for FLOR, NL, LA, RFW, NbNOD, NPOD, W100P, A, and E. Moderate values (0.2≤H^2≤0.4) were recorded for RDW, HI and gs, and low levels of heritability (H^2<0.2) were registered for FWL and WPOD.

On the other hand, high broad-sense heritability (H^2>0.4) under moderate water deficit (50% FC) were observed for FLOR, NL, LA, RFW, RDW, NbNOD, NPOD, W100P, A, E, and gs. Moderate value was recorded for FWL while low levels of heritability were registered for WPOD and HI characters. Lastly, under severe water stress (30% FC), high heritability levels were observed for FLOR, NL, LA, FWL, RFW, NPOD, W100P, A, E, and gs. Moderate value was recorded for RDW while low values were registered for NbNOD, WPOD and HI traits.

Of the 252 possible correlations between measured parameters, 34, 41 and 33 were significant for the control treatment, as well as for 50 and 30% FC respectively (Table 4). Among these correlations, 23, 30, and 23 are positive in control treatment, 50 and 30% FC, respectively. Flowering time is positively correlated with NL and NbNOD, while it is negatively correlated with LA and A. The NL is positively correlated with root growth and pods production. Photosynthetic parameters (A, E and gs) are positively correlated with LA in 50% FC while they are negatively correlated with RFW in 30% FC. Comparison between the three matrices between parameters measured in lines of *M. truncatula* under control treatment, 50% FC and 30% FC (Table 4) showed specific correlations. In the control treatment, the NbNOD is negatively correlated with HI and A. In 50% FC, the LA is negatively correlated with NbNOD and W100P while the NPOD is positively correlated with E and gs, and the W100P is negatively correlated with A and gs. In 30% FC, the FLOR is positively correlated with FWL and RDW while NL is negatively correlated with LA as well as the RFW is negatively correlated with A and gs. Furthermore, the RDW is negatively correlated with HI.

Principal component analysis (PCA)

Principal component analysis showed that the first three principal components with eigenvalues >1 explained 72.40 and 71.31% of the total variability among genotypes grown under 50 and 30% FC, respectively, for the 15 measured parameters (Table 5). In 50% FC, the relative magnitude of the eigenvectors from the first principal component (28.15%) indicated that the flowering time, the number and weight of pods and the harvest index were the most important contributing traits. For the second principal component which explained 24.01% of the total variation, the most contributing characters were the root fresh and dry weight. The third principal component explained 20.25% of the total variation with weight of 100 pods and stomata conductance as the major contributing characters. In PCA three-dimensional graph, the 10 lines were clustered into three groups

Table 4. Matrices of correlations between measured traits for lines of *M. truncatula* grown under control treatment (100% FC), 50% and 30% of field capacity (FC).

Trait	FLOR	NL	LA	FWL	RFW	RDW	NbNOD	NPOD	WPOD	W100P	HI	A	E	gs
FLOR	1.00													
NL	0.48*	1.00												
LA	-0.55*	-0.06	1.00											
FWL	0.02	0.41*	0.38*	1.00										
RFW	0.20	0.43*	-0.06	0.47*	1.00									
RDW	0.16	0.44*	0.02	0.45*	0.89*	1.00								
NbNOD	0.45*	0.45*	-0.22	0.22	0.27*	0.21	1.00							
NPOD	0.05	0.43*	0.02	0.13	0.34*	0.26*	0.01	1.00						
WPOD	-0.07	0.11	0.02	-0.08	0.06	0.01	-0.17	0.58*	1.00					
W100P	-0.11	-0.40*	-0.22	-0.36*	-0.41*	-0.35*	-0.18	-0.66*	0.09	1.00				
HI	-0.20	-0.11	0.03	-0.15	-0.05	-0.01	-0.27*	0.34*	0.73*	0.23*	1.00			
A	-0.68**	-0.21	0.61**	0.29	0.00	-0.00	-0.42**	0.09	-0.06	-0.17	0.08	1.00		
E	-0.21	-0.10	0.46**	0.32	0.27	-0.04	-0.20	0.20	-0.05	-0.28	0.07	0.66**	1.00	
gs	-0.51**	-0.16	0.52**	0.15	-0.02	-0.01	-0.21	0.05	-0.06	-0.14	0.02	0.79**	0.59**	1.00
	FLOR	**NL**	**LA**	**FWL**	**RFW**	**RDW**	**NbNOD**	**NPOD**	**WPOD**	**W100P**	**HI**	**A**	**E**	**gs**
FLOR	**1.00**	0.45*	-0.37*	0.08	0.24*	0.15	0.30*	0.09	-0.04	-0.16	-0.07	-0.56**	-0.21	-0.38
NL	0.34*	**1.00**	-0.14	0.49*	0.48*	0.44*	0.27*	0.47*	0.01	-0.53*	-0.13	-0.03	0.04	0.19
LA	-0.18	-0.64*	**1.00**	0.34*	0.15	0.22	-0.31*	0.17	-0.03	-0.39*	-0.09	0.42**	0.62**	0.56**
FWL	0.26*	0.71*	-0.17	**1.00**	0.51*	0.47*	0.23*	0.40*	0.00	-0.47*	-0.00	0.17	0.19	0.26
RFW	0.29*	0.54*	-0.12	0.58*	**1.00**	0.83*	0.41*	0.43*	0.04	-0.54*	-0.03	-0.01	0.10	0.15
RDW	0.28*	0.24*	-0.02	0.34*	0.69*	**1.00**	0.30*	0.42*	0.11	-0.45*	0.00	0.01	-0.01	0.05
NbNOD	-0.07	0.34*	-0.17	0.52*	0.34*	0.24*	**1.00**	0.16	0.04	-0.22	-0.05	-0.11	-0.21	-0.13
NPOD	0.14	0.62*	-0.15	0.46*	0.25*	-0.00	0.08	**1.00**	0.59*	-0.66*	0.30*	0.21	0.46**	0.48**
WPOD	-0.07	0.04	-0.06	0.14	0.09	-0.07	-0.03	0.54*	**1.00**	0.09	0.79*	-0.05	-0.04	-0.04
W100P	-0.19	-0.61*	0.12	-0.39*	-0.28*	-0.07	-0.16	-0.65*	0.15	**1.00**	0.32*	-0.41	-0.54**	-0.58**
HI	-0.09	-0.08	-0.14	-0.19	-0.14	-0.23*	-0.18	0.12	0.43*	0.25*	**1.00**	-0.10	-0.09	-0.08
A	-0.46**	-0.15	0.05	-0.14	-0.48**	-0.30	0.11	-0.11	-0.09	-0.05	-0.14	**1.00**	0.66**	0.82**
E	-0.31	0.03	-0.16	-0.15	-0.43**	-0.28	0.17	-0.03	0.00	0.03	0.04	0.81**	**1.00**	0.77**
gs	-0.41	-0.01	-0.21	-0.29	-0.42**	-0.23	0.11	-0.11	-0.19	-0.14	-0.04	0.84**	0.79**	**1.00**

*Significant ($P \leq 0.05$), **significant after using Bonferroni correction at $\alpha = (0.05/33 = 0.00151)$. FLOR, Date of the first stem flower bud stage (days); NL, number of leaves; LA, leaf area (cm2); FWL, fresh weight of leaves (g); RFW, root fresh weight (g); RDW, root dry weight NbNOD (number of nodules, NPOD number of pods; WPOD, weight of pods; W100P, weight of 100 pods; HI, harvest index; A, photosynthetic rate (µmol CO2 m−2s−1); E, transpiration rate (mmol H2O m−2s−1); gs, stomatal conductance.

(Figure 2). The first group consisted of TN8.20, TN1.21, TN1.11, TN8.3 and DZA45.5. The second group was formed by Jemalong A17, A20, and F83005.5. The third group composed of DZA315.16 and TN6.18. The A20, TN6.18 and TN8.3 are positively associated with PC1 (Table 5). Jemalong A17, TN8.20 and F83005.5 are negatively correlated with PC1. TN1.11, TN1.21 and TN8.20 are positively correlated with PC2. A20 and DZA45.5 are positively associated with PC3. F83005.5 is negatively correlated with PC1 and PC2, Jemalong A17 is negatively associated with PC2, and TN6.18 and TN8.20 are negatively associated with PC3.

In 30% FC, the first component accounted for 29.58% of the total variation between studied lines while the most contributing parameters are the root fresh and dry weights, the number of pods and the harvest index. The second component explained 24.47% of the total variation among the 10 lines while the most contributing parameters are the photosynthetic rate, the transpiration rate and the stomata conductance. The third component accounted for 17.26% of the total variation among lines with the number and area of leaves being the most contributing characters. The scatter diagram of studied lines defined by the three first principal components shows three groups (Figure 2). The first group consisted of Jemalong A17 while the second group was formed by DZA45.5 and F83005.5, and the third group was composed by the remaining lines. The lines DZA45.5 and Jemalong A17 are negatively associated with PC1 while TN1.21 and TN8.3 are positively associated with PC1

Table 5. Eigenvalues, percentage of total variance and cumulative percentage of variance for the first three principal components (F1, F2 and F3), and the coordinates of 14 measured parameters for lines of *M. truncatula.*

Factor\parameter	DRI 50% FC			DRI 30% FC		
	F1	F2	F3	F1	F2	F3
Eigenvalue	3.94	3.36	2.83	4.14	3.43	2.42
Percentage of total variance	28.15	24.01	20.25	29.58	24.47	17.26
Cumulative percentage of variance	28.15	52.15	72.40	29.58	54.05	71.31
FLOR	**0.71**	0.13	0.13	0.54	-0.24	0.42
NL	0.48	-0.38	-0.53	0.00	-0.52	**0.71**
LA	0.31	-0.57	0.21	-0.08	0.08	**-0.79**
FWL	0.59	-0.64	-0.20	-0.46	-0.29	0.55
RFW	0.04	**-0.80**	-0.43	**-0.72**	0.08	0.11
RDW	0.00	**-0.81**	0.06	**-0.70**	0.32	0.00
NBNOD	-0.26	-0.37	-0.35	-0.03	-0.52	0.47
NPOD	**-0.89**	-0.30	0.20	**-0.95**	0.05	-0.16
WPOD	**-0.87**	-0.27	-0.12	**-0.92**	-0.15	-0.06
W100P	-0.06	-0.17	**-0.89**	-0.36	-0.62	0.19
HI	**-0.90**	-0.38	-0.13	**-0.86**	0.10	0.12
A	-0.52	0.60	-0.42	-0.04	**0.90**	0.37
E	-0.07	0.51	-0.65	0.08	**0.86**	0.34
gs	0.27	0.29	**-0.82**	0.01	**0.81**	0.50

FLOR, Date of the first stem flower bud stage (days); NL, number of leaves; LA, leaf area (cm2); FWL, fresh weight of leaves (g); RFW, root fresh weight (g); RDW, root dry weight NbNOD (number of nodules, NPOD number of pods; WPOD, weight of pods; W100P, weight of 100 pods; HI, harvest index; A, photosynthetic rate (µmol CO2 m−2s−1); E, transpiration rate (mmol H2O m−2s−1); gs, stomatal conductance.

(Table 5). DZA45.5, TN8.20 and TN8.3 are positively correlated with PC2 while DZA315.15, F83005.5 are negatively associated with PC2. Jemalong A17 and TN6.18 are positively associated with PC3 while DZA45.5 and F83005.5 are negatively correlated with PC3. Overall, our results showed that similarities between lines of *M. truncatula* were dependent on the treatment effects (50 and 30% FC).

DISCUSSION

Lines of *M. truncatula* are potentially subjected to different selection pressures (Badri et al., 2007; Lazrek et al., 2009) in their natural areas of distribution therefore variability is expected in response to water deficit in the different genotypes used in this study. Lines may have developed an adaptive response to drought that can be exploited later to identify the genetic bases of tolerance to drought. The analysis of 27 morph-physiological characters showed significant variation among the 10 lines of *M. truncatula*. The treatment factor explains the maximum of variation of measured traits. Out of the 27 characters, 14 were explained by the interaction treatment x line indicating an involvement of these parameters in the adaptation of lines to water stress.

Positive correlations between flowering time and growth traits showed that late flowering lines have well-developed vegetative organs. The same observation was noted in chickpea (Soltani et al., 2006) and populations of *M. laciniata* and *M. truncatula* (Badri et al., 2007). Indeed, it has been shown that the genotype of plants, temperature and photoperiod factors affect flowering time in chickpea (Berger et al., 2006; Soltani et al., 2006) and rice (Ouk et al., 2006). For some accessions of *M. truncatula*, vernalization is a significant factor in reducing time to flowering when days are long and temperatures are moderate (e.g. 16 h/8 h and 21-25°C) (Chabaud et al., 2006). Under greenhouse condition, with seasonal changes in day-length and temperature, the effect of vernalization on time to flowering is secondary to the photoperiodic response. Annual *Medicago* grow best under long days and warm temperatures (De Ruiter and Taylor, 1979) and on neutral to alkaline soils (Nichols et al., 2010). Early flowering is an adaptation to escape adverse conditions, as observed in populations of chickpea in India (Berger et al., 2006). This adaptive characteristic allows lines of *M. truncatula* to survive in unfavorable regions for growth by restricting their development to short rainy periods of the year. This result is consistent with results observed in Algerian populations of *M. truncatula* (Si Ziani and Abdelguerfi, 1995), where flowering time was correlated with altitude and annual rainfall of collection sites. A high growth rate

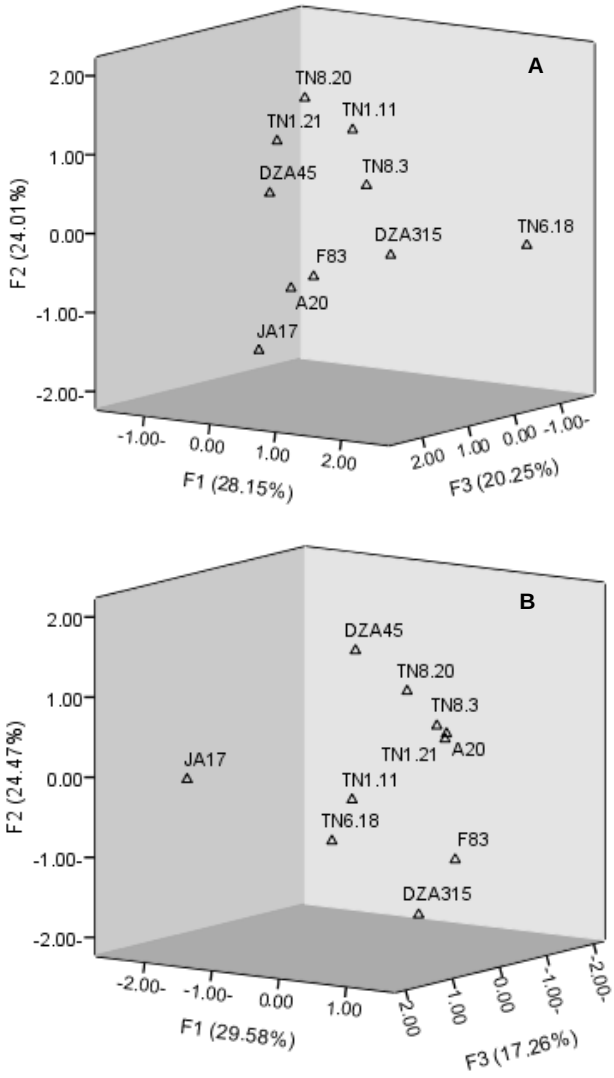

Figure 2. Three-dimensional diagram showing the relationships among the 10 lines of *M. truncatula* based on the first three principal components (F1, F2 and F3) under 50% FC (A) and 30% FC (B).

can potentially compensate for low plant vigor at flowering, but this reduces their tolerance to drought (Mitchell-Olds, 1996).Our results showed that DZA315.16 line allocates more effort to root growth under both irrigation regimes (50 and 30% FC). The importance of the root system in the acquisition of water has long been recognized (Lynch et al., 2014). The development of the root system increases the water absorption and maintains the osmotic pressure. An increase in the root system under water stress has been reported in sunflower (Tahir et al., 2002), and in *Sesuvium portulacastrum* (Slama et al., 2007). Water deficit reduces the growth of leaves more compared to roots because the growth of roots and leaves exhibits different sensitivities to reduced water availability (Verslues et al., 2006). This implies a reallocation of carbon (C) to the roots, even if the rate of net assimilation of C is reduced due to the low diffusion of CO_2 caused by the limited stomata opening under impeding water loss (Cornic, 2000). The water deficit potentially increased availability of C for roots (Hummel et al., 2010).

In the current study, the reduction of growth is associated with a decrease in the number of leaves. Strong positive correlations were recorded between the biomass of whole plant and number of leaves (NL) for studied lines under control treatment and drought stress. Indeed, plants reduce water loss by restricting their leaf area transpiration.

The decrease of plants growth under water deficit is also due to an inhibition of photosynthesis (Cornic, 2000). In this study, lines of *M. truncatula* showed a decrease in net CO_2 assimilation under water deficit, which is the highest value recorded for F83005.05. This decrease is mainly due to the closure of stomata and fewer leaves and/or leaf area as part of a strategy adopted by plants to reduce their transpiring surface. The reduction in the number as well as the biomass of leaves suggest that net CO_2 assimilation (A) per unit of leaf area is higher in plants subjected to drought stress than those grown in non-limiting conditions. Accordingly, Slama et al. (2007) reported a decrease of number and size of leaves and an increase in net CO_2 assimilation in leaf growth rate per unit under water deficit in *S. portulacastrum*. This reduction saves water for the plant and is therefore considered as a criterion for adaptation to drought. Our results showed negative correlations between net CO_2 assimilation (A) and flowering time (FLOR), indicating that plant growth was affected under water deficit due to reduced capacity of photosynthetic organs (that is, the amount of assimilates). This reduction can be harmful, from an agronomic point of view, because it is irreversible when the water conditions become favorable after droughts (Flexas and Medrano, 2002). According to Chaves et al. (2003), most plants tend to increase their water use efficiency (WUE) when they are subjected to moderate drought stress. This increase is due to a non-linear relationship between carbon assimilation and stomata conductance. Stomata conductance is one of the main factors affecting photosynthesis of plants (Medrano et al., 2002). It appears that plants use efficient mechanisms to limit the loss of water before the inhibition photosynthesis.

High to moderate values of heritability (H^2) were registered for most measured parameters in the control treatment, 50 and 30% FC, indicating that much of the variation of these traits is under genetic control. The spatial structure of studied lines in the three first principal component based on their responses to water deficit in 50 and 30% FC showed that lines were differently affected by drought stress (Figure 2). In 30% FC,

Jemalong A17 exhibited the lowest reductions for FWL, NPOD, W100P, and HI while it showed the highest reduction for LA. Hence, all populations of recombinant inbred lines derived from crosses between Jemalong A17 and the remaining lines (TN1.21, TN1.11, A20, DZA315, DZA45 and F83) (Figure 1) are useful for the identification of the genetic determinants for tolerance to water deficit in *M. truncatula*.

Overall, the variability of measured parameters was mainly explained by the treatment factor. High to moderate broad-sense heritability (H^2) were registered for most traits under control treatment and drought stress. The high heritability of most traits indicates that genetic factors are most likely controlling these characteristics even in water deficit conditions. Further genetic studies will be required to dissect the specific genetic factors that are responsible for these traits. Most of the correlations between the characters are positive under the three water regimes. The tolerance of lines to water deficit seems to depend, in particular, on their aptitude to maintain a good photosynthetic activity. Knowledge obtained by studying this model plant can be transferred to other crop and forage legumes.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank Ken Moriuchi and Marshall Keyster for the helpful comments on the manuscript, and Kamel Hessini and Fethia Zribi for the photosynthesis measurements. They are grateful to Mohamed Elarbi Aouani for the useful discussion, Mohamed Salhi and Najah Ben Cheikh for assistance in the greenhouse. This work was supported by the Tunisian Ministry of Higher Education and Scientific Research (LR10 CBBC 02), and the FP6 European Grain Legume Integrated Project.

REFERENCES

Anjum SA, Xie XY, Wang LC, Saleem MF, Man C, Lei W (2011). Morphological, physiological and biochemical responses of plants to drought stress. Afr. J. Agric. Res. 6:2026-2032.

Arraouadi S, Chardon F, Huguet T, Aouani ME, Badri M (2011). QTLs mapping of morphological traits related to salt tolerance in *Medicago truncatula*. Acta Physiol. Plant. 33:917-929.

Badri M, Chardon F, Huguet T, Aouani ME (2011). Quantitative Trait Loci associated with drought tolerance in the model legume *Medicago truncatula*. Euphytica 181:415-428.

Badri M, Ilahi H, Huguet T, Aouani ME (2007). Quantitative and molecular genetic variation in sympatric populations of *Medicago laciniata* and *M. truncatula* (Fabaceae): Relationships with ecogeographical factors. Genet. Res. 89:107-122.

Barker DG, Bianchi S, Blondon F, Datté Y, Duc G, Essad S, Fament P, Gallusci P, Génier G, Guy P, Muel X, Tourneur J, Dénarié J, Huguet T (1990) *Medicago truncatula*, a model for studying the molecular genetics of the *Rhizobium*-legume symbiosis. Plant Mol. Biol. Rep. 8:40-49.

Berger JD, Ali M, Basu PS, Chaudhary BD, Chaturvedi SK, Deshmukh PS, Dharmaraj PS, Dwivedi SK, Gangadhar GC, Gaur PM, Kumar J, Pannu RK, Siddique KHM, Singh DN, Singh DP, Singh SJ, Turner NC, Yadava HS, Yadav SS (2006). Genotype by environment studies demonstrate the critical role of phenology in adaptation of chickpea (*Cicer arietinum* L.) to high and low yielding environments of India. Field Crop Res. 98:230-244.

Bouyoucos C (1983). Les propriétés physiques du sol dépendent de sa texture et de sa structure. In Les bases de la production végétale. Tome 1. Collection Sciences et Technique agricoles, pp67-87.

Chabaud M, Lichtenzveig J, Ellwood S, Pfaff T, Journet EP (2006). Vernalization, crossings and testing for pollen viability. In The *Medicago truncatula* handbook. Mathesius U, Journet EP, Sumner LW (eds). ISBN 0-9754303-1-9.

Chaves MM, Maroco JP, Pereira JS (2003). Understanding plant responses to drought - from genes to the whole plant. Funct. Plant Biol. 30: 239-264.

Chen C, Tao C, Peng H, Ding Y (2007). Genetic analysis of salt stress responses in Asparagus Bean (*Vigna unguiculata* (L) ssp Sesquipedalis Verdc). J. Hered. 98(7):655-665.

Cornic G (1994). Drought stress and high light effects on leaf photosynthesis. Photoinhibition of photosynthesis 17:297-313.

Cornic G (2000). Drought stress inhibits photosynthesis by decreasing stomatal aperture–not affecting ATP synthesis. Trends Plant Sci. 5:187-188.

De Ruiter JM, Taylor AO (1979). Effects of temperature, photoperiod, and vernalization on flowering. New Zeal. J. Exp. Agr. 7(2): 153-156.

Dong XJ, Zhang XS (2000). Special stomatal distribution in *Sabina vulgaris* in relation to its survival in a desert environment. Trees-Struct. Funct. 14:369-375.

Flexas J, Medrano H (2002). Drought-inhibition of photosynthesis in C3 plants: Stomatal and non-stomatal limitations revisited. Ann. Bot. 89(2):183-189.

Harb A, Krishnan A, Ambavaram MMR, Pereira A (2010). Molecular and physiological analysis of drought stress in *Arabidopsis* reveals early responses leading to acclimation in plant growth. Plant Physiol. 154(3):1254-1271.

Hessini K, Ben Hamed K, Gandour M, Mejri M, Abdelly C, Cruz C(2013). Ammonium nutrition in the halophyte *Spartina alterniflora* under salt stress: Evidence for a priming effect of ammonium: Plant Soil 370:163-173.

Hewitt EJ (1966). Sand and water culture methods used in the study of plant nutrition. Commonw. Agric. Bur. 22:431-446.

Hummel I, Pantin F, Sulpice R, Piques M, Rolland G, Dauzat M, Christophe A, Pervent M, Bouteillé M, Stitt M, Gibon Y, Muller B (2010). *Arabidopsis* plants acclimate to water deficit at low cost through changes of carbon usage: an integrated perspective using growth, metabolite, enzyme, and gene expression analysis. Plant Physiol. 154(1):357-372.

Lazrek F, Roussel V, Ronfort J, Cardinet G, Chardon F, Aouani ME, Huguet T (2009). The use of neutral and non-neutral SSRs to analyze the genetic structure of a Tunisian collection of *Medicago truncatula* lines and to reveal associations with eco-environmental variables. Genetica 135:391-402.

Lynch JP, Chimungu JG, Brown KM (2014). Root anatomical phenes associated with water acquisition from drying soil: targets for crop improvement. J. Exp. Bot.

Medrano H, Escalona JM, Bota J, Gulias J, Flexas J (2002). Regulation of photosynthesis of C-3 plants in response to progressive drought: Stomatal conductance as a reference parameter. Ann. Bot. (Lond) 89:895-905.

Mitchell-Olds T (1996). Genetic constraints on life-history evolution: Quantitative-trait loci influencing growth and flowering in *Arabidopsis thaliana*. Evolution 50:140-145.

Nichols P, Loi A, Nutt B, Snowball R, Revell C (2010). Domestication of new Mediterranean annual pasture legumes. In Sustainable use of genetic diversity in forage and turf breeding, Huyghe C (ed.). pp. 137-

142.
Nunes C, Araújo SS, da Silva JM, Fevereiro MPS, da Silva AB (2008). Physiological responses of the legume model *Medicago truncatula* cv. Jemalong to water deficit. Environ. Exp. Bot. 63:289-296.
Ouk M, Basnayake J, Tsubo M, Fukai S, Fischer K, Cooper M, Nesbitt H (2006). Use of drought response index for identification of drought tolerant genotypes in rainfed lowland rice. Field Crop. Res. 99:48-58.
Rouached A, Slama I, Zorrig W, Jdey A, Cukier C, Rabhi M, Talbi O, Limami AM, Abdelly C (2013). Differential performance of two forage species: *Medicago truncatula* and *Sulla carnosa* under water deficit stress and recovery. Crop Pasture Sci. 64(3):254-264.
Si Ziani Y, Abdelguerfi A (1995). Comportement de 54 populations de *Medicago truncatula* Gaertn. dans deux sites différents; relation avec les facteurs du milieu d'origine. Meeting of FAO European sub-Network on Mediterranean Pastures and fodder Crops, Avignon (Fr.). Cah. Opt. Méditerranéennes 72:29-32.
Slama I, Ghnaya T, Messedi D, Hessini K, Labidi N, Savouré A, Abdelly C (2007). Effect of sodium chloride on the response of the halophyte species *Sesuvium portulacastrum* grown in mannitol-induced water stress. J. Plant Res. 120:291-299.
Soltani A, Hammer GL, Torabi B, Robertson MJ, Zeinali E (2006). Modeling chickpea growth and development: Nitrogen accumulation and use. Field Crop. Res. 99:24-34.
Tahir MHN, Imran M, Hussain MK (2002). Evaluation of sunflower (*Helianthus annuus* L) inbred lines for drought tolerance. Int. J. Agric. Biol. 3:398-400.
Tang H, Krishnakumar V, Bidwell S, Rosen B, Chan A, Zhou S, Gentzbittel L, Childs KL, Yandell M, Gundlach H, Mayer KFX, Schwartz DC, Town CD (2014). An improved genome release (version Mt4.0) for the model legume *Medicago truncatula*. BMC Genom. 15:312.
Wang TZ (2014). Recent research progress on MicroRNA's in *Medicago truncatula.* Clon. Transgen. 3:2.
Thudi M, Upadhyaya HD, Rathore A, Gaur PM, Krishnamurthy L, Roorkiwal M, Nayak SN, Chaturvedi SK, Basu PS, Gangarao NVPR, Fikre A, Kimurto P, Sharma PC, Sheshashayee MS, Tobita S, Kashiwagi J, Ito O, Killian A, Varshney RK (2014). Genetic dissection of drought and heat tolerance in chickpea through genome-wide and candidate gene based association mapping approaches. PloS one 9(5):1-12.
Verslues PE, Agarwal M, Katiyar-Agarwal S, Zhu J, Zhu JK (2006). Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant J. 45(4):523-539.
Xu J, Yuan Y, Xu Y, Zhang G, Guo X, Wu F, Wang Q, Rong T, Pan G, Cao M, Tang Q, Gao S, Liu Y, Wang J, Lan H, Lu Y (2014). Identification of candidate genes for drought tolerance by whole-genome resequencing in maize. BMC Plant Biol. 14:83.
Zhang J-Y, Cruz de Carvalho MH, Torres-Jerez I, Kang Y, Allen SN, Huhman DV, Tang Y, Murray J, Sumner LW, Udvardi MK (2014). Global reprogramming of transcription and metabolism in *Medicago truncatula* during progressive drought and after rewatering. Plant Cell Environ. 37(11):2553-2576.

academicJournals

Vol. 15(25), pp. 1350-1362, 22 June, 2016
DOI: 10.5897/AJB2016.15371
Article Number: 29B0DD059055
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Drought tolerance in transgenic tropical maize (*Zea mays* L.) by heterologous expression of *peroxiredoxin2* gene-*XvPrx2*

Miccah S. Seth[1,3]*, Leta T. Bedada[1], Steven M. Runo[1], Mathew P. Ngugi[1], Revel Iyer[2], Jennifer A. Thomson[2], Emmarold E. Mneney[3] and Richard O. Oduor[1]

[1]Plant Transformation Laboratory, Department of Biochemistry and Biotechnology, Kenyatta University, P. O. Box 43844, Nairobi, Kenya.
[2]Plant Stress Laboratory, Department of Cell and Molecular Biology, University of Cape Town, Private Bag, Cape Town, South Africa.
[3]Mikocheni Agricultural Research Institute, P. O. Box 6226, Dar Es Salaam, Tanzania.

Received 30 March, 2016; Accepted 26 May, 2016

Transformation of a tropical maize inbred genotype (CML144) with the *Xerophyta viscosa* peroxiredoxin2 (*XvPrx2*) gene was reported. The protective role of peroxiredoxin2 against the damage resulting from reactive oxygen species (ROS) under dehydration stress was further determined. Successful integration of *XvPrx2* gene into maize we achieved and recovered 10 independent transgenic events. Transformation and regeneration frequencies were 12.9 and 31.3%, respectively. Reverse transcription polymerase chain reaction (PCR) revealed the expression of the *XvPrx2* gene in transformed plants under dehydration. Stressed transgenic plants had higher relative water content (RWC) as compared to the conventional plants. Recovery irrigation showed higher RWC in transgenics than in conventional plants. Unlike in conventional plants, rapid morphogenic recovery was observed in transgenics within 24 h. Chlorophyll contents decreased faster in conventional plants than in transgenics with prolonged drought. Generally, transgenic plants were more tolerant to dehydration stress than conventional plants. This tolerance may be associated with the over expression of peroxiredoxin2 playing a role in managing ROS generated in plant cells.

Key words: *Xerophyta viscosa,* reactive oxygen species (ROS), transgenic drought tolerant maize, plant breeding.

INTRODUCTION

Drought is one of the leading environmental stresses that accounts for severe losses of grain yield in the Eastern and Central Africa sub regions. Relative water content (RWC) has been reported to be a relevant physiological

*Corresponding author. E-mail: micahgunah@yahoo.co.uk.

measure for plant water loss due to drought (Blum et al., 1998; Rodriguez-Maribona et al., 1992). RWC gives the current status of water content in the tissue of a leaf relative to its optimum amount of water that can be held at full turgidity. Ideal values of RWC when the cells are turgid have been estimated to range between 98% in transpiring leaves to about 40% in severely desiccated and dying leaves. Further, RWCs of most crop species at wilting have been estimated to be about 60 to 70% (Lugojan and Ciulca, 2011).

Under mild desiccations, the internal leaf CO_2 concentration is reduced due to stomatal closure, which results in reduction in photosynthetic activity (Cornic, 2000; Flexas et al., 2004). Severe drought on the other hand results in reduction of chlorophyll content and thereby destruction of the photosynthetic apparatus.

Chlorophyll content is one of the key indicators of the availability of nitrogen in crop plants and plays an important role as light harvesting machinery essential for carbon assimilation. Severe dehydration decreases photosynthetic pigments resulting in increased levels of carotenoids (xanthophylls and carotene). Carotenoids are important in plants because they tend to reflect back the heavy surge of light thereby protecting chlorophylls from further destruction. The reduction in chlorophylls (*a*, *b* and *total*) is reported to be caused mainly by oxidative stress due to excessive accumulation of reactive oxygen species (ROS) under drought stress (Smirnoff, 1995).

Plants can protect themselves against harmful ROS by undergoing a variety of biochemical and physiological responses. One of such response is the generation of antioxidant enzymes, such as peroxiredoxin2, which plays a fundamental role in scavenging ROS and converting them to harmless molecules, thereby protecting plant cell membranes and DNA from damage. Peroxiredoxins are post-translationally targeted to chloroplasts where they protect the photosynthetic membrane from photo-oxidative destruction (Baier and Dietz, 1997, 1999).

Xerophyta viscosa is a resurrection plant, native to South Africa, which has evolved mechanisms of withstanding prolonged drought stresses. This plant can be dehydrated to an air dry state, but upon rewatering, can rehydrate within 24 to 80 h (Mundree et al., 2002). This phenomenon has been predicted to be controlled by a number of genes, including the *XvPrx2* which encodes peroxiredoxin2 (Govender, 2006).

A type II peroxiredoxin gene (*XvPrx2*) polypeptide has been shown to display significant similarity with other plant type II peroxiredoxins, with the conserved amino acid motif (PGAFTPTCS) proposed to constitute the active site of the enzyme (Govender et al., 2016). The *XvPrx2* gene is stress-inducible in response to abiotic stresses and it has been revealed that XvPrx2 homologues exist within the *X. viscosa* proteome (Govender et al., 2016). *In vitro* DNA protection assay has shown that, in the presence of XvPrx2, protection of DNA occurs. *In vitro* assays have also revealed maximum activity of the XvPrx2 with DTT as electron donor and H_2O_2 as substrate (Govender et al., 2016), implying that the gene is responsible for managing the ROS generated by plants under stress.

Thus, the objective of the present study was to transform CML 144 inbred maize lines using *XvPrx2* gene construct and evaluate the performance of transformed maize lines against their non- transgenic counterparts under drought stress condition. Hence, the relative water content (RWC), chlorophylls (*a*, *b* and *total* chlorophyll) contents, and the chlorophyll a/b ratio in the transgenic and conventional maize plants were measured to determine the ability of the former to overcome the effects of dehydration stress.

MATERIALS AND METHODS

Vector development and *Agrobacterium* strain

The *XvPrx2* gene and the drought inducible promoter XvPsap1 were kindly obtained from the Department of Molecular and Cell Biology, UCT, Cape Town. The gene was previously isolated and cloned into the pTF101.1 vector. The construct harbours an herbicide resistant *bar* gene for selection of transformed plant cells. Given the rising concern about the use of antibiotics and herbicides to generate transgenics, the expression cassette of the gene was sub-cloned into the plant expression vector, pNOV2819 to take advantage of the *manA* gene as a plant selectable marker and allow the use of mannose as a selective agent. The construct contains the right and left T-DNA borders from *nopaline* strains of the *A. tumefaciens*, a broad host origin of replication (pVS1) and a spectinomycin-resistant marker gene (*aadA*) for bacterial selection (Figure 1). The XvPsap1promoter (Oduor et al., 2009, WIPO patent No.WO/2009/060402) was used to drive the expression of the *XvPrx2* and terminated by the *nopaline synthase* (*nosT*), whereas the expression of the *PMI* (*manA*) selectable marker gene was under the CMPS promoter from cestrum yellow leaf curling virus and terminated by the *nosT*. The resulting gene construct was transformed into *Agrobacterium tumefaciens* strain EHA101 (Hood et al., 1986) and used to transform immature zygotic embryos of tropical CML 144 maize adapted to East and Central African countries.

Bacterial growth and maintenance media

The EHA101 carrying the gene construct was maintained in Luria-Bertani, LB (10 g Bacto-Tryptone; 5 g yeast extract; 5 g sodium chloride NaCl and 10 g Bacto-Agar) medium supplemented with 100 mg/L spectinomycin (for plasmid), 100 mg/L kanamycin and 25 mg/L chloramphenicol (for EHA101) [pH 6.8].

Plant transformation and regeneration media

All media and vitamin composition used in this study are shown in Table 1. Infection medium (LS-Inf), co-cultivation (LSAc), resting (RM) and selection (SEM) were prepared based on Linsmaier and Skoog (1965) salts and regeneration (REGI) for embryo maturation, REGII for shoot induction and REGIII (for root induction) media were prepared based on Murashige and Skoog (1962) basal salts. Except for infection medium whose pH was adjusted to 5.2 and

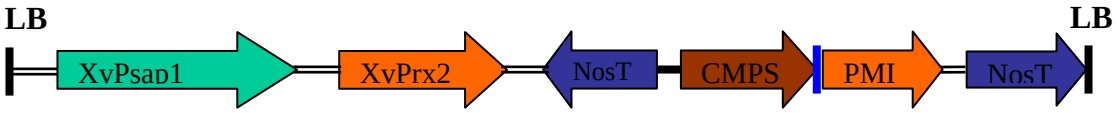

Figure 1. A T-DNA region of pNOV2819 vector harbouring *XvPrx2* gene; LB: Left border, RB: Right border, NosT: Termination sequence of the *nopaline synthase* gene, isolated from *Agrobacterium tumefaciens*. The function of this sequence is to signal the termination of the gene expression. CMPS: A strong constitutive promoter from cestrum yellow leaf curling virus which can be used for regulating transgene expression in a wide variety of plant species. Here, it was used to drive the PMI gene, Xvpsap1 a promoter sequence native to *Xerophyta viscosa* used to drive the *XvPrx2* gene.

Table 1. Media composition for transformation and regeneration.

Medium	Composition
LS-Inf	Macro- and micro-salts (Linsmaier and Skoog, 1965), LS-vitamins[a], 1.5 mg/L 2,4-D, 1 g/L casein hydrolysate, 34.25 g/L sucrose, 18 g/L glucose, 100 µM acetosyringone (As), pH 5.2
LSAc	Macro- and micro-salts, LS-vitamins[a], 1.5 mg/L 2, 4-D, 700 mg/L proline, 500 mg/L MES, 100 µM As, 30 g/L sucrose, 10g/L glucose, 8 g/L agar, pH 5.8
RM	Macro- and micro-salts, LS vitamins[a], 2 mg/L 2, 4-D, 700 mg/L praline, 500 mg/L MES, 30 g/L sucrose, 1.6 mg/L silver nitrate, 8 g/L agar, 250 mg/L Carbenicillin, pH 5.8
SEM	RM with 2 mg/L 2, 4-D, 5 g/L mannose, 25 g/L sucrose, no silver nitrate
REG I	MS macro- and micro- salts, vitamins[β], 0.5 mg/L kinetin,700 mg/L proline, 500 mg/L MES, 25 g/L sucrose, 2.5 g/L mannose, 8 g/L agar, 250 mg/L carbenicillin, pH 5.8
REG II	MS macro- and micro- salts, vitamins[β], 25 g/L sucrose, 2.5 g/L mannose, 8 g/L agar, 250 mg/L carbenicillin, pH 5.8
REG III	½ strength MS macro- and micro- salts, vitamins[β], 20 g/L sucrose, 5 g/L mannose, 8 g/L agar, 250 mg/L carbenicillin, pH 5.8

[a]LS Vitamins contains: 100 mg myo-Inositol and 0.1 mg Thiamine HCl; [β]Vitamins: 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 0.1 mg/L thiamine HCl, 100 mg/L mayo-inostol, 2 mg/L glycine†; †Glycine is an amino acid but it was included in vitamin preparations.

filter sterilized, the pH of all media were adjusted to 5.8 before autoclaving.

Source of seeds and plant establishment

Maize seeds of CML144 (QPM) were provided by Dr. Dan Makumbi (CIMMYT, Nairobi). CML 144 was chosen based on consultation with Kenya Agricultural Research Institute (KARI) and CIMMYT breeders where it was agreed that besides being quality protein maize (QPM), it is also sensitive to drought, therefore it should be easy to see the improvement. Maize plants were grown in pots (150 mm radius with 330 mm depth) containing sterile forest loam soils mixed with manure and sands in the ratio of 2:2:1 and bulked in the greenhouse at Plant Transformation Laboratory (PTL), Kenyatta University, Nairobi, Kenya.

Surface sterilization and immature embryo excision

Ears harvested 12-15 days post pollination were surface sterilized in 3% (v/v) commercial bleach (JIK) with 2 drops of Tween®20 for 15 to 20 min and rinsed three times with sterile distilled water. Immature zygotic embryos (1-1.5 mm) were aseptically excised from kernels and placed in Petri-plates containing infection medium

Agrobacterium pre-induction procedures

A. tumefaciens (EHA 101) containing the single gene construct pNOV2819-X*vPsap1-XvPrx2-nosT* was cultured at 28°C for 2 days in darkness on LB agar supplemented with the same antibiotics used for bacterial maintenance. One loop of bacteria was scooped from a freshly grown plate and suspended in 10 mL LB broth supplemented with 100 mg/L spectinomycin, 100 mg/L kanamycin and 25 mg/L chloramphenicol and shaken at 75 rpm overnight at 28°C. The overnight grown culture was centrifuged and the medium discarded. *Agrobacterium* cells were then resuspended in a fresh LB broth (without antibiotics) and centrifuged to remove any remaining antibiotics. Thereafter, the *Agrobacterium* cells were resuspended in 5 mL of liquid infection medium (LS-Inf) supplemented with 100 µM acetosyringone, AS (LS-Inf + AS) in sterile 50 ml falcon tube. The bacterial cell densities were then adjusted to an optical density (OD_{550}) of 0.3 to 0.4 using 722 N Visible spectrophotometer (EVERICH MEDCARE IMPORT AND EXPORT Co. LTD, Nanjing, China) before infecting embryos. The pre-induction step was carried out for all experiments.

Infection, co-cultivation and resting

The infection medium (LS-Inf) was drained off from the Petri-plates

before the pre-induced *A. tumefaciens* was poured onto the plates containing excised embryos and incubated for 5 min in darkness. The infected embryos were then poured onto the co-cultivation medium (LSAc) and the excess infection medium drained off using a micropipette. Embryos were then aligned in such a way that the axis faced the medium. The plates were wrapped with parafilm and incubated at 20±2°C for 3 days in darkness. Thereafter, embryos were transferred to resting medium (RM) and incubated at 28±2°C in the dark for 10 days.

Selection for transformed events

From RM, all the embryos were transferred to selection medium (SEM) containing 5 g/L mannose. Embryos (35 embryos per plate) were incubated on this medium for 4 weeks in darkness with fortnightly sub-culturing onto fresh medium.

Regeneration of shoots from calli

Embryogenic calli that survived selection were regenerated by maturing them for 1-2 weeks on REGI medium in darkness followed by transfer to light for shoot induction on REGII as described by Negrotto et al. (2000). Putative transgenic maize plantlets with well-developed root systems were removed from culture bottles and washed with sterile distilled water to remove adhering agar from the roots. The plantlets were then hardened in small pots (100 x 100 x 100 mm) containing peat moss (KEKKILA Co. Ltd, Tuusula, Finland). Plantlets were covered with transparent polyethylene bags for 2 - 3 days and allowed to grow in these pots to about 3 leaves prior to transplanting to bigger pots (150 mm radius with 330 mm depth) containing sterile forest loam soils mixed with manure and sands in the ratio of 2:2:1. The putative transgenic maize plants were allowed to grow to full maturity and self-pollinated at the flowering stage to give T_0 seeds.

DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted from the transgenic CML144 and non-transformed control maize lines as per the CTAB method (Allen et al., 2006). The PCR amplification was performed in 50 µL reaction volume containing 100 ng of genomic DNA, 1X PCR buffer (supplied with the Taq polymerase enzyme) with 1.5 mM $MgCl_2$, 20 pmoles primers each of either (*PMI-U*-1:5'-ACA GCC ACT CTC CAT TCA-3'; *PMI*-L: 5' GTT TGC CAT CAC TTC CAG-3') or promoter specific (XvPsap1-F1 5'-GGA CTT CAT GGC ATC CAT GTG C-3'; XvPsap1-R1 5'-ATT TGC CCC ATG GAA AGT GAC G-3'), 200 µM each of dNTPs, and 1 unit of Taq DNA polymerase enzyme. The reaction was carried out using Eppendorf Vapo Protect thermal cycler (EPPENDORF AG 22331 Hamburg Germany). The following reaction condition was used: initial denaturation step for 10 min at 95°C followed by 45 cycles of 95°C for 30 s; 55°C for 45 s; 72°C for 50 s, and a final elongation step at 72°C for 7 min. The amplification product (550 bp for PMI and 395 bp for XvPsap1 promoter) was resolved on 1% TAE agarose gel and photo was captured (SYNGENE BIOIMAGING model No. 55000, SYNOPTICS LTD, Cambridge, UK).

RNA isolation from drought stressed plants

Total RNA was isolated using the Trizol reagent (Gibco-BRL). Maize leaves (200 mg) from stressed plants were ground in liquid nitrogen and homogenized in 0.75 mL of the reagent. Following incubation for 5 min at room temperature, 0.2 mL chloroform was added followed by a further incubation at room temperature for 10 min. Samples were centrifuged at 12000 rpm for 10 min at 4°C and the RNA was precipitated using isopropanol. The RNA was treated with RNaseA free-DNase I (Invitrogen, USA). RNA was separated on a 1.2% agarose formaldehyde gel and stained with cyber green to verify the quantity.

cDNA synthesis using RT-PCR

cDNA was synthesized using a cDNA synthesis kit (Invitrogen, USA) according to the manufacturer's instruction. Standard PCR was carried out using either *XvPrx2* gene (XvPrx2-F 5'-ACG ATC CCA GAC GGA ACG CT-3' and XvPrx2-R 5'-CTT CAA GAT CTC ATC GGC ACC-3') along with an internal control maize actin (*Zm-actin*) gene primers (GenBank accession no: AY107106) (ZmAct-F 5'-ACC CAA AGG CTA ACC GTG AG-3' and ZmAct-R 5'-TAG TCC AGG GCA ATG TAG GC-3'). The PCR machine was programmed as follows, initial denaturation step of 5 min at 94°C followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, elongation at 72ºC for 50 s, and a final elongation step at 72°C for 7 min. The *XvPrx2*gene and maize *actin* gene transcripts were detected by amplifying a 458 and 426 bp fragment, respectively.

Drought stress experiments

From single transformation event T_0 maize seeds (2 seeds per pot) were planted in pots (17 cm diameter and 21 cm height) containing 3 kg of forest soil mixed with manure and sand at the ratio of 2:2:1. Although, the study was in response to drought, it was envisaged that in nature, plants face multiple stresses. Hence the glasshouse temperature was maintained at 43±2°C and relative humidity of 55%. After getting T1 seeds, they were planted in pots as described earlier. Germinated T_1 plants were thinned and transplanted into other pots such that the number remaining one plant per pot. Eight transgenic T_1CML144 maize plants and 8 conventional controls at the age of 6 weeks were selected for the experiment. From germination, plants were watered with half a litre of water once (every morning) daily before dehydration stress was imposed. From each group (transgenic and conventional plants) of maize plants, 4 maize plants were used for dehydration stress treatment while 4 were watered with half a litre once daily throughout the experiment. The experiment was arranged in 2 x 4 x 3 factorial (2 genotypes (transgenic and conventional maize) with 4 observation points under drought stress (days and recovery) and 3 replications) in a complete randomised design.

Determination of watering regime for plants during drought stress assays

Plant water requirement for daily irrigation was determined empirically as the difference between the wet soil and dry soil. This was done by first weighing the empty pot (W1). The pot was filled with 3 kg of oven dry soil and weighed again (W2). The soil was watered slowly until the first drip of water was seen at the bottom of the pot and then the soil was left until there was no more dripping. Thereafter, the weight of the pot containing wet soil (W3) was recorded. The difference between the weight of wet soil (W3-W1) and the weight of oven dry soil (W2-W1) was used as the volume of water required to water plants considering the density of water to be 1 g/cm^3.

Drought stress assay procedures

Before commencement of dehydration stress experiment, plants were equally watered at mid-day (at around 12 noon) to standardize

on a particular point the water content and metabolic fluctuation on their circadian rhythm. The following morning at 6 am, pieces of leaf samples representing day 0 were taken in triplicate for RWC and chlorophylls content determination from both transgenic and conventional plants. Plant photos were taken to mark as a reference for comparison of maize responses to dehydration stress. Thereafter, sampling was done from same plants at mid-day on days 7, 14 and 21 after withholding water in transgenic and conventional plants. Sampling was also done after 24 h following one time re-watering of plants at the end of dehydration treatment to determine the rate of plant recovery. Plant photos were also taken in each sampling day to compare the morphogenic response between the transgenic and the conventional plants.

Determination of leaf relative water content

To determine leaf relative water content (RWC), 3 pieces of leaf samples (3 x 4 cm) were cut from each experimental plant and weighed to obtain leaf fresh weight (FW). The leaves were immediately immersed in sterile distilled water and incubated overnight at 4°C to re-hydrate to full turgor. The following morning, leaves were drained and weighed to obtain saturation or leaf turgor weight (SW). The leaves were then dried at 80°C in the oven for 24 h or until a constant weight was repeatedly recorded. This weight was recorded as leaf dry weight (DW). The RWC was then calculated using the following formula by Turner (1981):

RWC = (FW – DW/SW – DW) x 100

Determination of total chlorophyll, chlorophyll *a*, *b* and chlorophyll ratios

Photosynthetic pigments were extracted using 2 ml of 100% (v/v) of acetone per sample as described by Lichtenthaler and Wellburn (1983). Ten leaf discs of paper punch size from the upper part of same leaf were prepared from each plant in triplicate and crushed separately with pestle in a motor. The 2 ml acetone leaf extract was placed into 2 ml Eppendorf tubes and centrifuged for 10 min at 14000 rpm. Then the supernatant (1.5 ml) was transferred to a new clean 2 ml Eppendorf tubes. Chlorophyll extracts were transferred into cuvettes for OD reading at A_{662nm} and $A_{645nm.}$ One cuvette with acetone served as a blank. Maximum absorbance for chlorophylls (Chl) *a* and *b* were recorded at A_{662nm} and $A_{645nm,}$ respectively. The respective pigments were calculated using the formula by Lichtenthaler and Wellburn (1983):

Chl*a* (µg/gfw) = $11.75A_{662}$ - $2.350A_{645}$; Chl*b* (µg/gfw) = $18.61A_{645}$ - $3.960A_{662}$; *Total* Chl (µg/gfw) = Chl*a*(µg/gfw) + Chl*b* (µg/gfw). Chlorophyll ratio was simply obtained by dividing Chl*a* (µg/gfw) by Chl*b*(µg/gfw).

Data management and analysis

Transformation frequencies (TF%) was calculated as number of mannose resistant calli events recovered per 100 embryos infected and transformation efficiencies (TE%) as number of PCR positive plant events per total numbers of embryos co-cultivated. The regeneration frequency (RF%) was computed as number of shoots regenerated per 100 embryogenic calli transferred to REGII medium. Data recorded on the leaf fresh weight, leaf turgor/saturated weight, and leaf dry weight were used to compute leaf relative water content (RWC) on each sampling days. Optical density (OD) of chlorophyll extract determined by reading absorbance at A_{662nm} and $A_{645nm,}$ were used to compute the amount/content of chlorophyll *a*, and chlorophyll *b*, respectively.

Analysis of variance (ANOVA) was carried out using GenStat Discovery Edition 4 (VSN International software for biosciences, (www.vsni.co.uk/software/genstat/) to test the statistical significance of differences among the transgenic and conventional plants and the days of exposure to dehydration stress. Pair wise comparison of means (for RWC, chlorophylls *a* content, chlorophylls *b* content, *total* chlorophylls content and chlorophyll *a/b* ratio) was carried out using least significance difference (LSD) test at 5% probability level.

RESULTS

Transformation of CML144 maize

The infected but untransformed cells (Figure 2A) grew slowly before turning necrotic and finally died. In contrast to this, putatively transformed cells grew quickly and increased in size forming white or creamy white friable embryogenic calli, which developed somatic embryos (Figure 2A).

Regeneration of putative transgenic plants

On regeneration I(REG I) medium, embryogenic calli were seen to develop somatic embryos (Figure 2B). Upon transfer to regeneration II (REG II) medium under 16 h light and 8 h dark photoperiods, the embryogenic calli turned green and formed shoot buds (Figure 2C), which continued photosynthesizing and grew into distinct shoots, and later developed roots. Shoots which did not form roots in REGII medium were transferred to regeneration III (REG III) for rooting (Figure 2D). Shoots with good root systems were hardened successfully in peat moss (Figure 2E). Plants transplanted into soil in buckets grew further developing to maturity (Figure 2F). Pollinated T_0 plants formed cobs with T_0 seed (Figure 2G). All putative transgenic T_0 events presented in (Table 2) were screened by PCR using PMI specific primers and results were presented in Figure 3. Transgenic (T_1) plants were also screened by PCR using the Psap1 specific primers and results were presented in Figure 4. Primers for XvPrx2 gene specific was not used for PCR to avoid possible amplifying a native Prx2. RT-PCR (Figure 5) revealed the expression of the *XvPrx2* gene transcripts in transgenic maize.

Effect of drought stress on morphological response of CML144 maize

Upon commencement of drought stress, leaf wilting was observed within three to five days in conventional and transgenic plants, respectively. However, photo documentation was taken on the seventh day after withholding water (Figure 6A and B). On day 14, transgenic maize plants were still morphologically active and up right whereas conventional maize plants had folded their leaves (Figure 6C). On day 21, both transgenic and

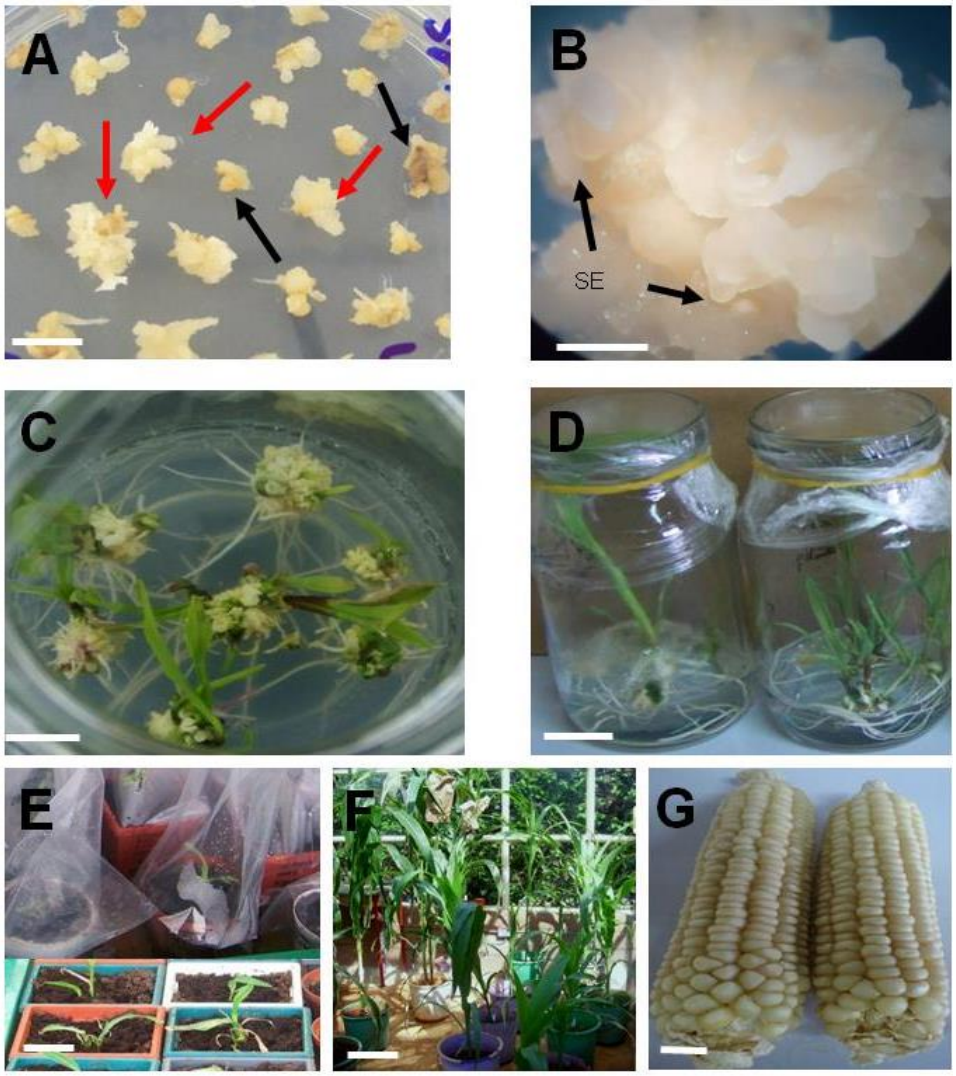

Figure 2. Transformation and regeneration profile of putative transgenic maize. A: Transformed embryos 6 weeks on SEM medium, (red arrows indicate transformed calli surviving on SEM and black arrows indicates untransformed calli dying on SEM), bar = 10 mm; B: Embryogenic callus with somatic embryos 7 days on REGI ready to be transferred to REGII, bar = 10 mm; C: Shooting transformed calli on REG II, bar = 10 mm; D: Putatively transformed T_0 plantlets with good root system ready for hardening, bar = 20 mm; E: Putative transformants undergoing hardening process, bar = 40 mm; H: Putative T_0 regenerants growing in the glass house, bar = 300 mm; G: Harvested T_0 cobs with seeds *bar* = 10 mm.

Table 2. Putative CML144 transformed with *XvPrx2* gene and recovered T_0 events

Exp	E.I	MRC	Event	T_0 Plants **	PCR+ plants **	No. Cobs (seeds)	TF (%)	RF (%)	TE (%)
1	224	22	22	5(3)	2(2)	3(~200)	9.8	22.7	0.89
2	295	45	45	16(8)	16(8)	22(~2000)	15.3	35.5	5.42
Total	519	67	67	21(11)	18(10)	25(~2200)	12.9	31.3	3.46

**In brackets represent number of events, Exp: Independent experiment; E. I: Embryos infected and co-cultivated; MRC: mannose resistant callus; T_0 PCR+: number of putative maize plants tested positive by PCR, TF (%): transformation frequency-obtained as a percentage of mannose resistant calli over the total number of immature zygotic embryos infected and co-cultivated, RF (%): Regeneration frequency-obtained as a percentage of number of T_0 plantlets per number of total number of calli events transferred to REGII medium, TE (%): Transformation efficiency-obtained as a percentage of PCR positive plants over the total number of immature zygotic embryos infected and co-cultivated.

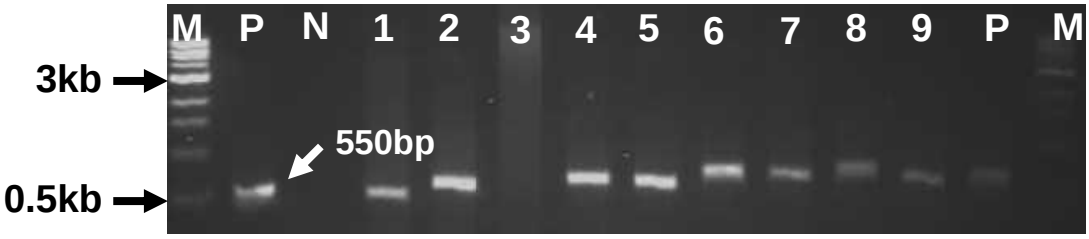

Figure 3. PCR detection of transgenic CML144 maize plants using *PMI* gene specific primers. M: 1 kb ladder, P: positive control (Plasmid construct used for transformation), N: negative control (DNA from conventional CML144 maize), Lanes 1-9 PCR product from DNA of putative transgenic CML144 maize. Expected band size for *manA* gene (positive control) was 0.550 kb.

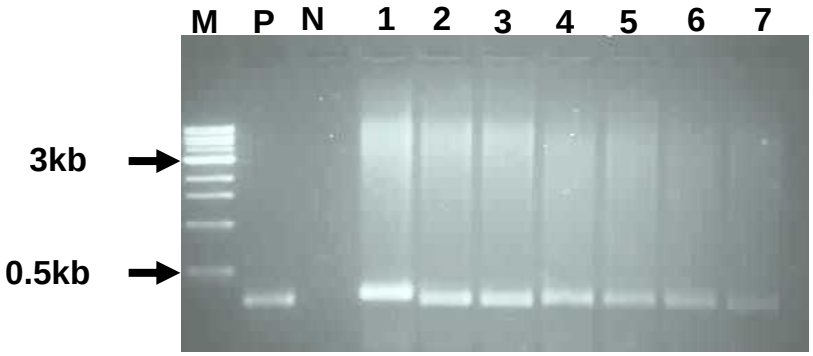

Figure 4. PCR detection of transgenic T_1 CML144 plants using XvPsap1 promoter specific primers. M: 1 kb ladder, P: Positive control (plasmid construct used for transformation, N: negative control (DNA of conventional CML144 maize), 1-7: PCR product from T_1 transgenic CML144 plants transformed with *XvPrx2* gene construct. Expected band size of 0.395 kb was observed (positive control).

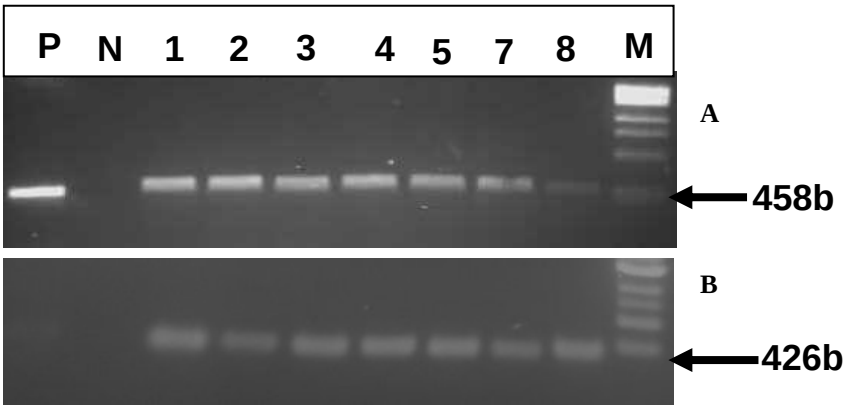

Figure 5. RT-PCR on transgenic and conventional CML144 maize. Panel A: Results obtained using *XvPrx2* specific primers, Panel B: The same substrates amplified with maize actin gene primers for loading control. M: 1 Kb ladder (New England Biolab, UK); P: Positive control (PCR on plasmid of the construct used for maize transformation, N: Negative control (RNA untreated with superscript reverse transcriptase), Lanes 1-7: RT-PCR product from transgenic maize plants cDNA under dehydration, 8: cDNA from conventional maize under dehydration. Expected band size for *XvPrx2* and *Zm-actin* gene was 458 and 426 bp, respectively.

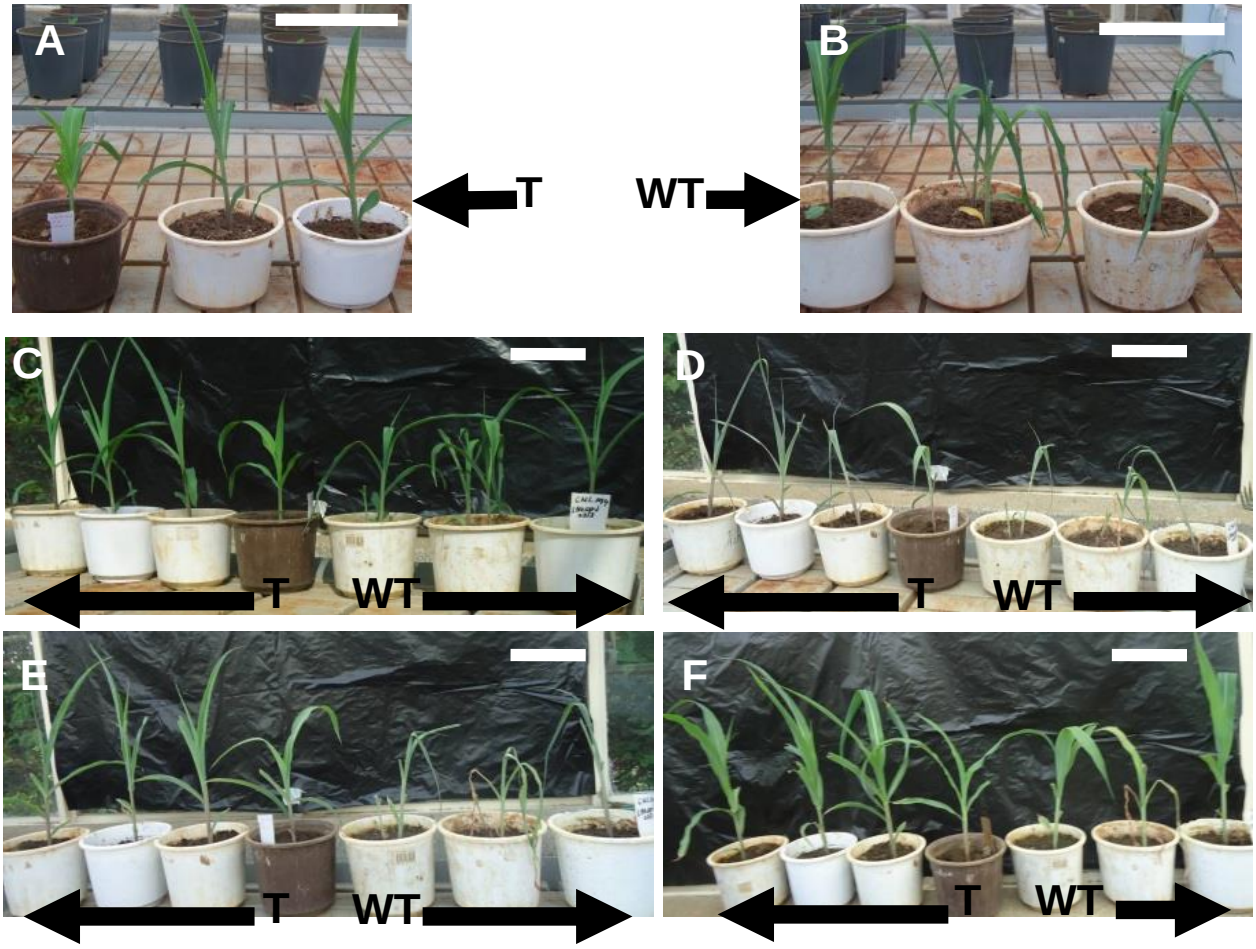

Figure 6. Six week old transgenic and conventional CML144 maize genotypes under different stages of drought stress. A: Transgenic maize after 7 days of drought stress, bar = 150 mm, B: Non transgenic maize after 7days of drought stress, bar = 150 mm, C: Transgenic (T) and non-transgenic (WT) 14 days after drought stress, bar = 150 mm, D: Transgenic and non-transgenic maize plants 21 days after stress just before re-watering, bar = 150 mm, E: Recovery irrigation of transgenic and non-transgenic plants 6 h after re-watering, bar = 150 mm, F: Recovery irrigation of transgenic and non-transgenic maize plants after 4 days of re-watering, bar = 150 mm. T: Transgenic (CML144-XvPrx2), WT: Conventional CML144.

conventional maize plants had wilted. However, conventional maize showed bleached leaf colour and almost collapsing (Figure 6D). Upon rewatering, recovery in transgenic CML144 maize genotype was noted within 6 to 24 h (Figure 6E) as compared to over 4 days in the conventional CML144 maize counterparts (Figure 6F).

Effect of drought stress on relative water content

RWC (%) of transgenic and conventional maize plants were found to be significantly different from each other at $p<0.05$ (Table 3). Pairwise comparison of RWC between transgenic and conventional maize also revealed significant difference according to LSD at $p<0.05$ in all the sampling points. Generally, there was a decrease in RWC in both conventional and transgenic maize plants upon exposure to drought stress. However, the effect was more pronounced in conventional maize plants than in the transgenic plants (Figure 7). RWC decreased from 85.2 (day 0) to 78.2 (day 7), 73.1 (day 14) and 62.1% (day 21) in conventional maize plants, whereas in transgenic maize the RWC decreased from 85.5% on day 0 to 82.3% on day 7, 78.7% on day 14 and 72.3% on day 21, respectively. Conventional maize plants lost a total of 23.2% of their water within three weeks of drought stress as compared to transgenic maize plants, which lost only 13.3% under similar conditions. RWC determined 24 h after rewatering increased from 62.06 to 74.9% in conventional plants whereas in transgenic plants, the increase ranged from 72.3 to 82.4%.

Effect of drought stress on total chlorophyll content

According to analysis of variance (Table 3), total

Table 3. Analysis of variance for chlorophyll *a*, *b*, TChl, carotenoids content, Chl*a*/*b* ratio and RWC of transgenic CML144 and non transgenic CML144 maize plants as affected by drought stress.

Source of variation	Df	Mean square					
		Chla	Chlb	TChl	Chl a/b ratio	Carotenoids content	RWC
Genotypes (G)	1	5.01982**	15.9036**	38.7933**	0.244247**	0.63704**	228.682**
Days on stress (D)	4	11.90134**	96.4891**	171.7673**	0.528486**	2.20529**	270.197**
GxD	4	2.76271**	19.6947**	31.6174**	0.135938**	0.73507**	20.766*
Residual (E)	20	0.07617	0.3923	0.5760	0.002689	0.01977	5.250
CV (%)		1.6	4.2	2.4	4.0	8.6	3.0

*****Significant differences and ******highly significant differences at $p<0.05$ and $p<0.01$, respectively. Df: Degrees of freedom, GxD: Interaction of genotype by days under stress, E: Residual (Error), CV (%): Coefficient of variation, Chla: Chlorophyll *a*, Chlb: Chlorophyll *b*, TChl: Total chlorophyll, Chl a/b: The ratio of chlorophyll *a* to that of *b*, RWC (%): Leaf relative water content.

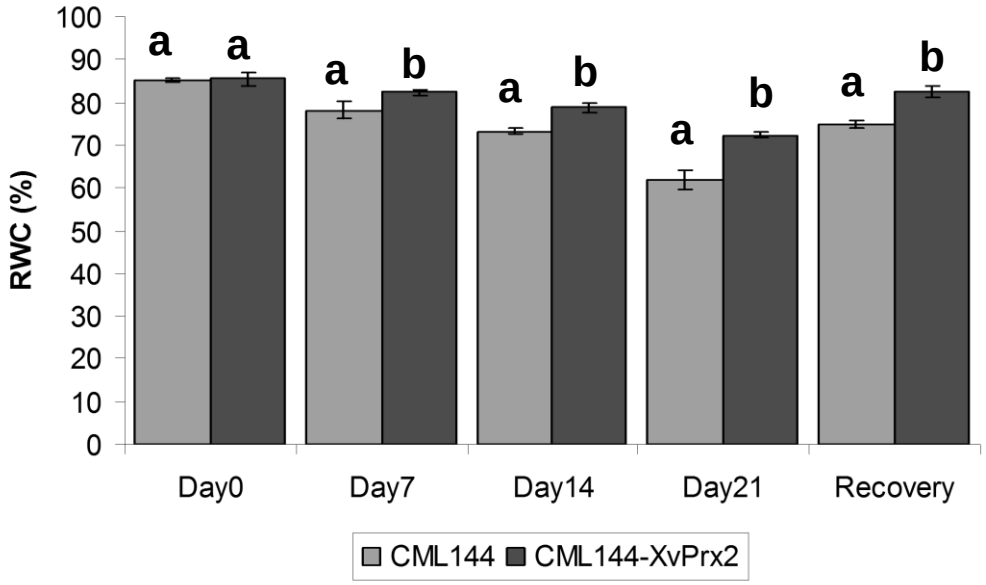

Figure 7. Leaf RWC as affected by drought stress and recovery after re-watering in transgenic maize and conventional CML144 maize plants. Bar graphs followed by different letters indicate that their means are statistically different from each other according to LSD at $p<0.05$.

chlorophyll (TChl) content was significantly different between the transgenic and conventional plants and between the days on which drought was imposed. Total chlorophyll was also significantly influenced by the interaction between the genotype and days of drought stress treatment ($p<0.05$). Pairwise comparison of TChl between transgenic and conventional maize was significantly different according to LSD at $p<0.05$ in all the sampling points. The TChl content in conventional plants decreased from 39.2 µg/gfw on day 0 to 35.8 µg/gfw on day 7, 28.9 µg/gfw on day 14 and 22.1 µg/gfw on day 21. In transgenics, the TChl content increased from 35.9 µg/gfw on day 0 to 37.9 µg/gfw on day 7 and thereafter it decreased to 35.7 µg/gfw and 28.9 µg/gfw on day 14 and day 21, respectively (Figure 8). Twenty four hours after rewatering, the total chlorophyll increased from 22.1 µg/ to 28.4 µg/gfw in conventional plants, whereas in transgenics, it decreased to 27.3 µg/gfw.

Effect of drought stress on chlorophyll *a* content

The Chl*a* contents were significantly different ($p<0.001$) among the genotypes, days of exposure to drought and the interaction between the genotypes by days of exposure to drought (Table 3). Pairwise comparison of Chl*a* between transgenic and conventional maize also revealed significant difference according to LSD at $p<0.05$ in all sampling points. Under drought stress, Chl*a* in conventional plants decreased from 18.5 to 18.3, 17.2 and 13.6 µg/gfw on day 0, day 7, day 14 and day 21, respectively. In transgenic plants, Chl*a* decreased from

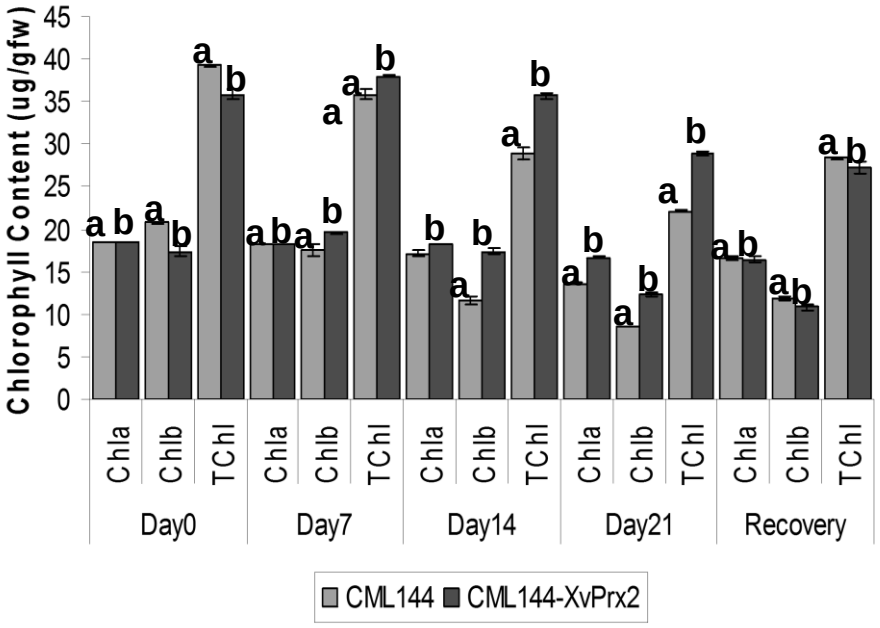

Figure 8. Effect of drought stress and recovery re-watering on chlorophyll content in transgenic and conventional CML144 maize plants. Bar graphs followed by different letters indicate that their means are statistically different from each other according to LSD at $p<0.05$.

18.5 µg/gfw on day 0 to 18.3 and 16.65 µg/gfw on day 7, and day 21, respectively (Figure 8). During recovery watering, Chl*a* increased from 13.6 to 16.6 µg/gfw after 24 h in conventional plants, whereas in transgenics, Chl*a* slightly decreased further from 16.7 to 16.5 µg/gfw.

Effect of drought stress on chlorophyll *b* content

Pairwise comparison of chlorophyll *b* (Chl*b*) between transgenic and conventional maize revealed significant difference according to LSD at $p<0.05$ in all the sampling points. The Chl*b* content in conventional CML144 maize plants decreased from 20.7 µg/gfw on day 0 to 17.5 µg/gfw on day 7, 11.7 µg/gfw on day 14 and 8.5 µg/gfw on day 21. For transformed (CML144-XvPrx2) plants, the content of Chl*b* increased from 17.4 µg/gfw on day 0 to 19.6 µg/gfw on day 7, followed by a steady decrease to 17.4 and 12.3 µg/gfw on D14 and day 21, respectively. After 24 h of rewatering, the content of Chl*b* in conventional plants increased from 8.5 to 11.8 µg/gfw, whereas in transgenic maize, Chl*b* content slightly decreased from 12.3 to 10.8 µg/gfw (Figure 8).

Effect of drought stress to Chlorophyll *a/b* ratio

Chlorophyll (Chl*a/b*) ratios were significantly different ($p<0.05$) between the transgenic and conventional maize genotypes and days under drought stress. There was also a significant interaction ($p<0.05$) between genotypes and days under exposure to drought stress (Table 3). Pairwise comparison of Chl*a/b* ratio between transgenic and conventional maize also revealed significant difference according to LSD at $p<0.05$ in all sampling points. The Chl*a/b* ratios increased with increase in severity of drought stress across the days for conventional plants. The transgenic plants also showed a relative increase in the Chl*a/b* ratios as compared to conventional plants (Figure 9). Upon rewatering, the chlorophyll ratio decreased in conventional plants whereas a slight increase was observed in the transgenic plants (Figure 9).

DISCUSSION

Genetic engineering has become an integral strategy in crop improvement particularly for polygenic traits like drought stress tolerance. In this study, the authors' successfully introgressed *XvPrx2* gene into CML144 maize, a tropical breeding line. Successful over expression of XvPrx2 gene transcripts was revealed by RT-PCR. However, conventional control also gave a faint signal revealing that Prx2 is conserved in maize. Hence, the reason to use PMI and XvPsap1 primers is to confirm the integration of the transgenes in the maize plant genome.

The transgenic plants showed higher RWC than the conventional plants. The amount of water lost by

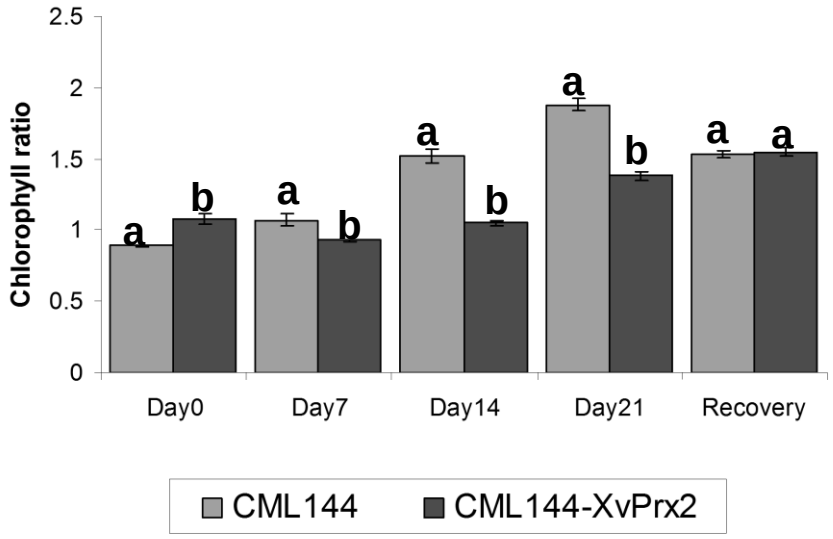

Figure 9. Effect of drought stress on chlorophyll *a/b* ratio in transgenic and conventional CML144 maize plants. Bar graphs followed by different letters indicate that their means are statistically different from each other according to LSD at $p<0.05$.

conventional plants after 21 days of stress was twice that lost by transgenic plants. The latter showed considerable ability to conserve cell water content which sustained the plants to conduct normal physiological activities efficiently under dehydration stress for long time.

The high RWC observed in transgenic maize plants under dehydration stress might have been due to a build up of peroxiredoxin2, the antioxidant product of the *XvPrx2* gene. Peroxiredoxin2 might be involved in ROS scavenging thereby maintaining integrity of the cell membrane by preventing shrinkage of the plasma membrane away from the cell wall and subsequence cytorhesis. Reduced RWC in drought stressed plants has also been reported by other researchers (Arjenaki et al., 2012; Najafinezhad et al., 2014). The more rapid recovery of transgenic plants than in their conventional counterparts in the present study might have been as a result of the higher RWC delaying the onset of wilting. Several authors have reported similar findings in which drought tolerant genotypes exhibit higher RWC in different species such as *Macrotyloma uniflorum* (Bhadwaj and Yadav, 2012), *Phaseolus vulgaris* (Turkan et al., 2005), barley (Kocheva and Georgiev, 2003), *triticum* (Sairam and Srivastava, 2001) and *Vicia faba* (El-Tyeb, 2006).

Peroxiredoxin2 is likely to be involved in the detoxification of free radicals. The excessive accumulation of ROS under drought stress might have caused injury to cells in drought sensitive conventional plants. Injuries caused by ROS include lipid peroxidation, degradation of membrane protein and inactivation of enzymes such as those responsible for photorespiration (example RUBiSco, PEP carboxylase) which are important components in the electron transport chain (Sairam et al., 2005; Zlatev and Lidon, 2012).

Under dehydration stress stomatal conductance is reduced to allow more water conservation which results in reduced CO_2 fixation thereby decreasing the rate of photosynthesis as noted by Flexas et al. (2004). Inflicting dehydration stress during vegetative growth stage, decreased substantially the content of TChl, Chl*a* and *b* in both the transgenic and conventional plants. This reduction in photosynthetic pigments might have resulted due to reduced RWC. These findings are similar to report by Terzi and Kadioglu (2006) who noted reduction in photosynthetic pigments while studying drought stress tolerance and the antioxidant enzyme system in *Ctenanthesetosa*. However, the reduction of TChl, Chl*a* and Chl*b* was less in transgenics than in conventional maize. Transgenic maize maintained a relatively higher content of chlorophylls than the conventional plants. These results complement the reports by Pastori and Trippi (1992) and Zaeifyzadeh and Goliov (2009) who revealed that resistant genotypes of wheat and corn had higher chlorophyll content than sensitive genotypes under the oxidative stress. Lamkemeyer et al. (2006) reported that the absence or presence of *Prx Q* gene (member of peroxiredoxin gene family) in transgenic *A. thaliana* affected chlorophyll *a* fluorescence parameters suggesting a role in maintaining photosynthesis.

Energy absorption in the photosynthetic apparatus acts as the main cause of excessive generation of ROS and plants tend to avoid this through degradation of photosynthetic pigments (Herbinger et al., 2002). The high resistance in degrading chlorophylls observed in transgenic plants in the present study might be attributed

to the presence of peroxiredoxin 2 which protects chlorophylls by quenching of ROS.

The increase in Chl*a/b* ratio in the dehydrated transgenic plants in this study suggests that Chl*a* was relatively stable. These results are in agreement with Ashraf et al. (1994) who reported that drought stress reduced the concentration of chlorophyll *b* more than chlorophyll *a*. This means that the Chl*a/b* ratio may be used as an indicator of plant response to environmental stress such as drought. Therefore, lower Chl*a/b* ratio observed in transgenic maize plants in the present study may indicate better adaptation to drought stress. However, different results were reported by Mafakheri et al. (2010) who worked on chickpea and observed that there was no effect on the chlorophyll *a/b* ratio.

Conclusion

The expression of the *XvPrx2* gene has revealed considerable tolerance to drought stress in transgenic maize as compared to conventional plants. In transgenic maize, RWC, Chl*a*, *b*, a/b ratios were higher than in conventional CML144 maize plants implying a functional role of *XvPrx2* gene. Thus, the *X. viscosa* peroxiredoxin2 gene could be used as a strategy for drought stress improvement of maize.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Allen GC, Vergara MAF, Krasnyanski S, Kumar S, Thompson WF (2006). A modified protocol for rapid DNA isolation from plant tissue using cetyltrimethylammonium bromide. Nat. Protoc. 1:2320-2325.

Arjenaki FG, Jabbari R, Morshedi A (2012). Evaluation of Drought Stress on Relative Water Content, Chlorophyll Content and Mineral Elements of Wheat (*Triticum aestivum* L.) Varieties Int. J. Agric. Crop Sci. 4(11):726-729.

Ashraf MY, Azmi AR, Khan AH Ala SA (1994). Effect of water stress on total phenols, Peroxidase activity and chlorophyll content in wheat. Acta Physiol. Plant. 16(3):185-191.

Baier M, Dietz KJ (1997). The plant 2-Cys peroxiredoxin BAS1 is a nuclear encoded chloroplast protein. Its expressional regulation, phylogenetic origin, and implications for its specific physiological function in plants. Plant J. 12:179-190.

Baier M, Dietz KJ (1999). Protective function of chloroplast 2-cysteine peroxiredoxin in photosynthesis. Evidence from transgenic Arabidopsis. Plant Physiol. 119(4):1407-1414.

Bhadwaj J, Yadav SK (2012). Comparative study on biochemical parameters and antioxidant enzymes in a drought tolerant and a sensitive variety of Horsegram (*Macrotyloma uniflorum*) under drought stress. Am. J. Plant Physiol. 7(1):17-29.

Blum A, Mayer J, Golan G, Sinmena B (1998). Drought resistance of a doubled-haploid line population of rice in the field. Workshop on Genetic Improvement of Rice for Water-Limited Environments. IRRI, Los Baños, Philippines, 1-3 December, 1998.

Cornic G (2000). Drought stress inhibits photosynthesis by decreasing stomatal aperture-not by affecting ATP synthesis. Trends Plant Sci. 5:187-188.

El-Tyeb MA (2006). Differential responses of pigments, lipid peroxidation, organic solutes, catalase and peroxidase activity in the leaves of two *Vici faba* L. cultivars to drought. Int. J. Agric. Biol. 1:116-122.

Flexas J, Bota J, Loreto F, Cornic G, Sharkey TD (2004). Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. Plant Biol. 6:1-11.

Govender K (2006). Characterisation of *XvPrx2*, a type II peroxiredoxin isolated from the resurrection plant *Xerophyta viscosa* (Baker). PhD Thesis, University of Cape Town, Cape Town, South Africa.

Govender K, Thomson JA, Mundree S, ElSayed AI, Rafudeen MS (2016). Molecular and biochemical characterisation of a novel type II peroxiredoxin (*XvPrx2*) from the resurrection plant *Xerophyta viscosa*. Functional Plant Biology. 2016 Jan 12.

Herbinger K, Tausz M, Wonisch A, Soja G, Sorger A, Grill D (2002). Complex interactive effects of drought and ozone stress on the antioxidant defense systems of two wheat cultivars. Plant Physiol. Biochem. 40:691-696.

Hood EE, Helmer GL, Fraley RT, Chilton MD (1986). The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J. Bacteriol. 168:1291-1301.

Kocheva K, Georgiev G (2003). Evaluation of the reaction of two contrasting barley (*Hordeum vulgare* L.) caltivars in response to osmotic stress with PEG 6000. Bulg. J. Plant Physiol. 49:290-294.

Lamkemeyer P, Laxa M, Collin V, Li W, Finkemeier I, Schöttler MA, Holtkamp V, Tognetti VB, Issakidis-Bourguet E, Kandlbinder A, Weis E (2006). Peroxiredoxin Q of *Arabdopsis thaliana* is attached to the thylakoids and functions in context of photosynthesis. Plant J. 45(6):968-981.

Lichtenthaler HK, Wellburn AR (1983). Determination of Total Carotenoids and Chlorophylls A and B of Leaf in Different Solvents. Biol. Soc. Trans. 11:591-592.

Linsmaier EM, Skoog F (1965). Organic growth factor requirements of tobacco tissue culture. Physiol. Plant. 18:100-127.

Lugojan C, Ciulca S (2011). Evaluation of relative water content in winter wheat. J. Hortic. Forest. Biotechnol. 15(2):173-177.

Mafakheri A, Siosemardeh A, bahrmnejad B, Struik PC, Sohrbi Y (2010). Effect of drought stress on yield, proline and chlorophyll contents in three chickpea cultivars. Aust. J. Crop Sci. 4(8):580-585.

Mundree SG, Baker B, Mowla S, Peters S, Marais S, Vander Willigen C, Govender K, Maredza A, Muyanga S, Farrant JM, Thomson JA (2002). Physiological and molecular insights into drought tolerance. Afr. J. Biotechnol. 1(2):28-38.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant 15:473-497.

Najafinezhad H, Sarvestani ZT, Sanavy SAMM, Naghavi H (2014). Effect of irrigation regimes and application of barley residue, zeolite and superabsorbent polymer on forage yield, cadmium, nitrogen and some physiological traits of corn and sorghum. Int. J. Biosci. 5(3):234-245.

Negrotto D, Jolley M, Beer S, Wenck AR, Hansen G (2000). The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via *Agrobacterium* transformation. Plant Cell Rep. 19:798-803.

Oduor RO, Iyer R, Machuka J, Thomson JA (2009). Plant promoters of the *XVSAP1* gene. World Intellectual Property Organization Patent no. Patent No. WO/2009/060402.

Pastori GM, Trippi VS (1992). Oxidative stress induces high rate of glutathione reductase synthesis in drought-resistant maize strain. Plant Cell Physiol. 33:957-961.

Rodriguez-Maribona B, Tenorio JL, Conde JR, Ayerbe L (1992). Correlation between yield and osmotic adjustment of peas (*Pisum sativum* L) under drought stress. Field Crops Res. 29:15-22.

Sairam R, Srivastava G, Agarwal S, Meena RC (2005). Differences in antioxidant activity in response to salinity stress in tolerant and susceptible wheat genotypes. Biol. Plant. 49:85-91.

Sairam RK, Srivastava GC (2001). Water stress tolerance of wheat (*Triticum aestivum* L.): Variations in hydrogen peroxide accumulation and antioxidant activity in tolerant and susceptable genotypes. J. Agron. Crop Sci. 186:63-70.

Smirnoff N (1995). Antioxidant system and plant responses, In: Smirnoff N. (ed) Environment and Plant metabolism: Flexibility and

Acclimation, Bios Scientific Publishers, Oxford. pp. 217-243.
Terzi R, Kadioglu A (2006). Drought stress tolerance and the antioxidant enzyme system in *ctenanthe setosa.* Acta Biol. Crac. Ser. Bot. 48(2):89-96.
Turkan I, Bor M, Ozdemir F, Koca H (2005). Differential response of lipid peroxidation and antioxidants in the leaves of drought–tolerant *P. acutifolius* Gray and drought-sensitive *P. vulgaris* L. subjected to polyethylene glycol mediated water stress. Plant Sci. 168:223-231.
Turner NC (1981). Techniques and experimental approaches for the measurement of plant water status. Plant Soil 58:339-366.
Zaeifyzadeh M, Goliov R (2009). The Effect of the Interaction between Genotypes and Drought Stress on the Superoxide dismutase and Chlorophyll Content in Durum Wheat Landraces. Turk. J. Biol. 33:1-7.
Zlatev Z, Lidon FC (2012). An overview on drought induced changes in plant growth, water relations and photosynthesis. Emir. J. Food Agric. 24(1):57-72.

academicJournals

Vol. 15(25), pp. 1363-1374, 22 June, 2016
DOI: 10.5897/AJB2015.15074
Article Number: FA5B4FA59057
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic diversity of stay-green sorghums and their derivatives revealed by microsatellites

Isaac K. A. Galyuon[1,2]*, R. Madhusudhana[3], Andrew K. Borrell[4], Tom C. Hash[5] and Catherine J. Howarth[2]

[1]Department of Molecular Biology and Biotechnology, University of Cape Coast, Cape Coast, Ghana.
[2]Institute of Biological, Environmental and Rural Sciences, Plas Gogerddan, Aberystwyth, SY23 3EB, Wales, UK.
[3]Directorate of Sorghum Research, Hyderabad, India.
[4]Department of Primary and Rural Industries, Queensland, Australia.
[5]International Crops Research Institute for the Semi-Arid Tropics, Pantacheru, India.

Received 31 October 2015; Accepted 23 May, 2016

The genetic variability of 28 sorghum genotypes of known senescence phenotype was investigated using 66 SSR markers well-distributed across the sorghum genome. The genotypes of a number of lines from breeding programmes for stay-green were also determined. This included lines selected phenotypically for stay-green and also RSG 03123, a marker-assisted backcross progeny of R16 (recurrent parent) and B35 (stay-green donor). A total of 419 alleles were detected with a mean of 6.2 per locus. The number of alleles ranged from one for Xtxp94 to 14 for Xtxp88. Chromosome SBI-10 had the highest mean number of alleles (8.33), while SBI-05 had the lowest (4.17). The PIC values obtained ranged from zero to 0.89 in Xtxp94 and Xtxp88, respectively, with a mean of 0.68. On a chromosome basis, mean PIC values were highest in SBI-10 (0.81) and lowest in SBI-05 (0.53). Most of the alleles from B35 in RSG 03123 were found on chromosomes SBI-01, SBI-02 and SBI-03, confirming the successful introgression of quantitative trait loci associated with stay-green from B35 into the senescent background R16. However, the alternative stay-green genetic sources were found to be distinct based on either all the SSRs employed or using only those associated with the stay-green trait in B35. Therefore, the physiological and biochemical basis of each stay-green source should be evaluated in order to enhance the understanding of the functioning of the trait in the various backgrounds. These genetic sources of stay-green could provide a valuable resource for improving this trait in sorghum breeding programmes.

Keywords: Simple sequence repeats, sorghum, stay-green, genetic diversity.

INTRODUCTION

The stay-green trait has been used for years by breeders as a measure of post-flowering drought tolerance (Rosenow and Clark, 1981; Borrell et al., 2001) and is a mechanism that prevents premature senescence under

*Corresponding author. E-mail: igalyuon@ucc.edu.gh. Tel: +233 (0) 242 337 612.

low soil moisture stress during grain-filling (Sanchez et al., 2002). The trait is characterised by green stems and green upper leaves when water is limiting during grain filling, which enables the stay-green genotypes to continue to fill their grain under drought conditions (Subudhi et al., 2000; Tao et al., 2000; Borrell et al., 2000 a, b, Borrell et al., 2001). Understanding the inheritance of traits, such as stay-green, is important for its successful application in plant breeding programmes. Markers associated with regions of the genome controlling stay-green can be used in the breeding programme to assist in the precise introgression of those regions into novel varieties. Alleles of simple sequence repeats (SSR) associated with stay-green quantitative trait loci (QTL) have been identified in a number of genetic sources (Borrell et al., 2000ab; Bhattramakki et al., 2000; Kebede et al., 2001; Kong et al., 2000; Haussmann et al., 2002), which might influence their tolerance on post-flowering drought stress. However, different genetic sources of stay-green may employ different genes and display different inheritance characteristics.

Analysis of genetic diversity is important for crop improvement and provides essential information to enable more efficient use of available genetic resources and a platform for stratified sampling of breeding populations (Mohammadi and Prasanna, 2003). Accurate assessment of the levels and patterns of diversity can be invaluable in the analysis of genetic variability in cultivars (Smith, 1984; Cox et al., 1986), identification of diverse parental combinations to create segregating progenies with maximum genetic variability for further selection (Barrett and Kidwell, 1998) and in introgressing desirable genes from diverse germplasm into the available genetic base (Thompson et al., 1998). Genetic relationships can be useful for the planning of crosses, assigning lines to specific heterotic groups and for precise identification with respect to plant varietal protection (Mohammadi and Prasanna, 2003).

Molecular markers have many advantages over phenotypic characters as they are unaffected by the environment. DNA-based markers, such as simple-sequence repeats (SSRs), have been or are being utilised in cultivar development, quality control of seed production, measurement of genetic diversity for conservation management, varietal identification and intellectual property protection (Smith et al., 2000; McIntyre et al., 2001) and are powerful tools in genetic similarity studies (Pejic et al., 1998). Furthermore, these markers can be used to obtain information on the genes that influence agriculturally important traits and to follow the introgression of these genes, thus facilitating the breeding process.

SSRs are highly useful as genetic markers as they are codominant, occur in high frequency and appear to be distributed well throughout the genomes of higher plants and animals (Bhattramakki et al., 2000) with simple Mendelian segregation (Brown et al., 1996). SSRs have higher information content, as measured by expected heterozygosity and number of alleles and polymorphism, as compared to amplified fragment length polymorphisms (AFLPs), random amplified polymorphic DNA (RAPDs) and restriction fragment length polymorphism (RFLPs) (Smith et al., 1997; Pejic et al., 1998; Uptmoor et al., 2003), indicating that SSRs are more discriminating. The high information content and favourable characteristics of SSRs make them excellent genetic markers for many types of investigations, including marker-assisted selection (MAS) and finger-printing of germplasm collections (Kong et al., 2000). Comparative studies in crop plants have shown that they provide a powerful tool for discriminating between genotypes and for MAS (Djè et al., 2000; Asare et al., 2010; Asare et al., 2011; Faustine et al., 2015).

In sorghum, SSRs are well distributed across the sorghum genome (Kong et al., 2000). It is thus possible to avoid overrepresentation of certain regions of the genetic map that can produce inaccurate estimates of genetic similarities among individuals. SSRs have revealed high levels of polymorphism to allow the vast majority of *Sorghum bicolor* accessions, including those within working groups, to be distinguished by using a relatively small number of SSR loci (Taramino et al., 1997; Kong et al., 2000 Smith et al., 2000; Xu et al., 2000; Uptmoor et al., 2003; Menz et al., 2004). Indeed, 10 SSRs were able to distinguish 324 individuals across 27 sorghum accessions (Ng'uni et al., 2011). In addition to their use in genetic diversity analysis, SSRs have been widely used in genetic mapping and QTL analysis in sorghum.

For the study of stay-green, the line B35 or its derivative QL41 has been employed as one of the parents in the development of mapping populations (Tuinstra et al., 1997; Crasta et al., 1999; Subudhi et al., 2000; Tao et al., 2000; Xu et al., 2000) and some of the QTL identified (stg1, stg2, stg3 and stg4) from B35 have been introgressed into the senescent R16 background using marker-assisted backcrossing (Hash et al., 2003, Harris et al., 2007; Kassahun et al., 2010; Vadez et al., 2011). Relatively, few studies have used different stay-green sources, such as SC56 (Kebede et al., 2001) and E36-1 (Haussmann et al., 2002). Although, the B35 and KS19 sources of stay-green have been employed in breeding programmes in the USA and Australia (Borrell et al., 2000a, b) little is known about the genetic basis of the stay-green trait in KS19. There may be other sources of stay-green genotypes yet untapped. It is important that breeding programmes should not rely on one or a few sources of any trait in order to reduce the risk of genetic vulnerability. Furthermore, the genetic and physiological determinants of the trait are varied (Thomas and Howarth, 2000). Therefore, it would be good to widen the base of the stay-green sources to avoid any future vulnerability yet unknown. In addition, as shown in studies by Borrell et al. (2000a, b) and Haussmann et al.

Table 1. Races, origins and senescence phenotypes of sorghum genotypes investigated.

Line	Race, country of origin, and breeding programmes	Senescence phenotype
2077-B	Durra, breeding line from India	Senescent
296B	Breeders' material from India	Stay-green
B35	Bred in Texas from a Durra line originally from Ethiopia. Employed in breeding programmes in Australia and the USA. Also referred to as BTx642	Stay-green
BTx623	Kafir x Zera-zera, Texas A & M, source 90C510	Senescent
DJ1195	Durra, breeding line from India	Senescent
E36-1	Widely-adapted zere-zera line from Ethiopia; high-yielding, Guinea-caudatum	Stay-green
#66	Guinea-caudatum, derived from a cross between E36-1 and R16 from ICRISAT	Stay-green
#68	Guinea-caudatum, derived from a cross between E36-1 and R16 from ICRISAT	Stay-green
ICSV112	Guinea-caudatum, bred at ICRISAT Centre and originally released as a variety in Zimbabwe	Stay-green
IS13441	Caudatum, landrace from Zimbabwe	Senescent
IS18530	Durra, breeding material from Egypt	Senescent
IS18551	Durra, breeding material from Ethiopia	Stay-green
IS2146	Durra, landrace from Nigeria	Senescent
IS22830	Caudatum, landrace from Sudan	Stay-green
IS3260C	Guinea/Margaritiferum, landrace from Nigeria	Stay-green
IS3762	Bicolor, landrace from China	Senescent
IS4845	Durra, landrace from India	Senescent
IS9302	Kafir, advanced cultivar from South Africa	Stay-green
IS9830	Caudatum, advanced cultivar from Sudan	Senescent
KS19	Derived from a cross between a Short Kaura (a landrace from Nigeria) and Combined Kafir 60. Used for breeding in Australia and the USA for the stay-green trait.	Stay-green
QL12	Dwarf genotype with a KS19 pedigree bred in Australia	Stay-green
QL39	Dwarf genotype with SC170C and KS4 pedigree bred in Australia	Senescent
QL41	A hybrid from a cross between B35 and QL33 bred in Australia	Stay-green
R16	High-yielding post-rainy season variety from Maharashta; a Guinea-caudatum of Ethiopian origin	Senescent
R9188	Texas A & M, Source 90C66	Stay-green
RSG 03123	A BC_1F_5 R16 introgression line with *Stg 1, 2, 3, and 4* QTL from B35 developed at ICRISAT	Stay-green
SC56	Caudatum-nigricans, breeding material from Sudan	Stay-green
SPV386	Caudatum from India	Senescent

(2002), the senescent parents of the mapping populations used (QL39 and N13) contributed major QTL for stay-green to the hybrids. Therefore, this study was designed to examine the genetic variability among sorghum germplasm normally grown in the semi-arid regions, determine the genetic variability using SSRs associated with the stay-green trait in B35 and to assess the genotypes of stay-green derivatives of B35, KS19 and E36-1 sources of stay-green which have been developed either using phenotypic or marker-assisted selection. The stay-green trait, as a post-flowering drought resistance mechanism, has great potential for use in breeding and selection of sorghum lines for drought-prone agro-ecological zones. Therefore, such studies should enhance our understanding of the genetic relatedness of sorghum germplasm and enhance breeding for elite cultivars of sorghum for the semi-arid and arid regions.

MATERIALS AND METHODS

Plant material and DNA extraction

The races, origins and senescence phenotypes of the sorghum genotypes employed in the study are described in Table 1. Leaves were harvested at the 3-4 leaf stage from 20 seedlings of each line and stored at -80°C prior to DNA extraction using the Nucleon PhytoPure Plant and Fungal DNA Extraction Kit RPN 8511 (Amersham Life Science, Little Chalfont, England) according to the manufacturer's instructions. DNA was quantified using a UV-VIS PU8720 spectrophotometer (Philips) and its integrity checked by running on 1% agarose gels stained with ethidium bromide. Each well contained a mixture of loading buffer (5 µl) and sample DNA

(10 µl). The gels were run with 1x TAE buffer from the cathode to the anode with a constant voltage of 70mV for 1.5 h. DNA samples were diluted with sterile nano-pure water to a final concentration of 10 ng/µl.

PCR amplification of SSRs

Seventy-four SSR primer pairs were chosen by initially selecting at least five SSR loci from each of the 10 sorghum genetic chromosomes to uniformly represent the entire mapped nuclear genome (Bhattramakki et al., 2000; Kong et al, 2000). Of these, only sixty-eight displayed polymorphism in a preliminary study and these were used with all genotypes tested (Table 2). PCR reactions were conducted in GeneAmp® PCR System 2700 (Applied BioSystems) in a 25 µl reaction mixture in 96-well plates. The 25-µl reaction mix consisted of 1× PCR buffer, 50 ng genomic DNA template, 4 µl of dNTP (5 mM equimolar solution of each dATP, dCTP, dGTP and dTTP), 1.25 µl each of forward and reverse primers (10 µM solution) and 0.65 U *Taq* DNA polymerase (Roche Applied Sciences). The PCR programme consisted of an initial denaturation for 2 min at 94°C and then 30 cycles of denaturation for 30 s at 94°C, primer specific annealing for 30 s (Table 2) and extension at 72°C for 90 s. After 30 cycles, there was a final extension step of 7 min at 72°C. Amplification products were separated on a 4.5% polyacrylamide, 8.5 M Urea denaturing gel (BIO-RAD Sequi-GenGT) and visualised by silver staining (Promega Silver Sequencing system) along with a 50 bp ladder (Gibco).

Analysis of data, genotyping and determination of genetic diversity

Bands for the same SSR locus with different molecular weights were scored as alleles. Presence or absence of each amplified band was scored as 1 and 0, respectively, for all markers to generate a binary data matrix. The genetic diversity for each microsatellite locus was calculated according to the following formula of Nei (1973):

Genetic diversity = $1- \Sigma P_{ij}^2$,

where P_{ij} is the frequency of *jth* allele for the *ith* locus summed across all the alleles of the locus. Calculated in this manner, the genetic diversity is synonymous with the term polymorphic information content (PIC) described by Anderson et al. (1993).
The binary data matrix generated from this scoring was used to calculate a similarity matrix using the Nei and Li (1979) coefficient. Cluster analysis was conducted using the unweighted paired group method using arithmetic averages (UPGMA) as defined by Sneath and Sokal (1973) to produce dendrograms of genetic similarities using the Numerical Taxonomy and Multivariate Analysis System software (NTSTSpc) version 2.1 (Exeter Software, New York). The correlation coefficient between the similarity matrix and the cophenetic values matrix was computed to test the goodness of fit of the cluster analysis. The binary data matrix was also analysed using only the data for 15 loci (as highlighted in Table 2) associated with the published B35 QTL for stay-green.

RESULTS

Size of SSRs, number of alleles and polymorphism information content (PIC)

The estimated sizes of SSR alleles, number of alleles produced and the PIC values of the 68 SSR loci examined are presented in Table 2. A total of 419 alleles were detected among the 28 genotypes assessed. The number of alleles per locus ranged from one (*Xtxp*94) to 14 (*Xtxp*88) with an overall mean of 6.2. Thus, only one locus (*Xtxp*94) was non-polymorphic. This marker was included in an attempt to increase genome coverage on chromosome SBI-05. SBI-02 and SBI-03 had the highest number of loci, while SBI-10 had the smallest number. The mean number of alleles was highest in SBI-10 (8.3) followed by SBI-09 and SBI-06 (7), SBI-01 and SBI-04 (6.8), SBI-02 (6.6) SBI-08 (6.4) SBI-07 (5.5), SBI-03 (5.3) and SBI-05 with the least (3.7). These markers had all been assessed previously in a set of 18 relatively diverse sorghum genotypes (Kong et al., 2000; Bhattramakki et al., 2000). In the set of 28 genotypes examined in this study, 49 (72%) loci had, at least, one allele more than previously reported, while 10 (15%) loci had at least one less allele and 9 (13%) loci had the same number. The mean number of alleles for SSR loci associated with the stay-green trait in the various published studies was 6.2, which was the same for all 68 SSR loci. Loci *Xtxp*88, *Xtxp*285, *Xtxp*56, *Xtxp*298, *Xtxp*207, *Xtxp*230, *Xtxp*217 and *Xtx*p67 associated with the stay-green trait were the most polymorphic with alleles varying from 14 in *Xtxp*88 to 7 in *Xtxp*67.

PIC values ranged from zero (*Xtxp*94) to 0.89 (*Xtxp*88 and *Xtxp*285) with a mean of 0.68. PIC values for these loci have not been reported before. *Xtxp*88 in chromosome SBI-01 had 14 alleles (highest), while *Xtxp*285 had 12 in chromosome SBI-03 (second highest). Chromosome SBI-10 again had the highest mean PIC value of 0.81. This was followed by chromosome SBI-06 (LG I) (0.78), SBI-04 (0.74), SBI-09 and SBI-08 (0.71), SBI-02 (0.70), SBI-01 (0.68), SBI-03 (0.64), SBI-07 (0.60) and SBI-05 again with the lowest PIC value of 0.53. The proportion of loci with PIC values equal to or more than 0.5 was 85%, while 82% had PIC values from 0.6 and PIC values in 69% were equal to or more than 0.7. The mean PIC value for SSR loci associated with the stay-green trait was 0.64, lower than the overall mean.

Genetic diversity among the 28 sorghum genotypes

PCR products from these 68 SSR primers were used to evaluate genetic diversity in 28 sorghum lines. Associations between the lines studied based on the cluster analysis of their genetic similarities are presented in a dendrogram (Figure 1). The set of markers used was able to uniquely classify the 28 lines included in this study and showed that considerable genetic diversity was present. Five distinct groups were identified in the resulting dendrogram. The most genetically distinct genotype of those examined was IS3620C, which did not cluster with any other line. This is the only Guinea-Margaritiferum line included in the study and was included (along with BTx623) as it is a parent in the

Table 2. Size, number of alleles and polymorphic information content (PIC) of SSR markers used in genetic diversity analyses.

Locus	[1]Chromosome no.	Annealing temperature (°C)	[2]No. of alleles	Size range in this study		No. of alleles in this study	PIC in this study
Xtxp58	SBI-01	55	7	145	161	6	0.79
Xtxp61	SBI-01	55	3	121	190	8	0.80
Xtxp88	SBI-01	53	6	106	150	14	0.89
Xtxp208	SBI-01	55	3	227	231	2	0.44
Xtxp229	SBI-01	55	2	153	157	3	0.43
Xtxp316	SBI-01	55	6	279	362	9	0.75
Xtxp335	SBI-01	55	5	133	220	9	0.80
Xtxp357	SBI-01	55	3	238	244	3	0.57
Xtxp8	SBI-02	60	6	115	155	9	0.84
Xtxp56	SBI-02	55	5	268	497	10	0.82
Xtxp96	SBI-02	52	5	140	174	7	0.77
Xtxp100	SBI-02	55	2	125	127	2	0.33
Xtxp207	SBI-02	55	4	149	172	5	0.79
Xtxp211	SBI-02	55	6	180	250	10	0.91
Xtxp283	SBI-02	55	6	203	205	2	0.32
Xtxp286	SBI-02	55	2	179	201	4	0.57
Xtxp296	SBI-02	55	5	165	177	5	0.70
Xtxp298	SBI-02	55	5	151	196	10	0.86
Xtxp348	SBI-02	55	4	226	350	9	0.80
Xtxp38	SBI-03	60	4	406	446	5	0.72
Xtxp69	SBI-03	50	4	186	270	9	0.84
Xtxp114	SBI-03	50	2	200	208	3	0.43
Xtxp183	SBI-03	55	3	165	170	3	0.65
Xtxp205	SBI-03	55	4	198	210	6	0.69
Xtxp215	SBI-03	50	3	172	182	4	0.66
Xtxp218	SBI-03	55	2	190	260	5	0.55
Xtxp228	SBI-03	55	4	205	228	4	0.66
Xtxp231	SBI-03	55	2	188	210	3	0.45
Xtxp285	SBI-03	55	5	215	258	12	0.89
Xtxp336	SBI-03	55	3	146	154	4	0.56
Xtxp12	SBI-04	55	4	164	210	6	0.77
Xtxp21	SBI-04	60	5	145	163	7	0.72
Xtxp177	SBI-04	55	4	150	160	4	0.61
Xtxp343	SBI-04	55	5	120	220	10	0.84
Xtxp94	SBI-05	50	2	220	220	1	0.00
Xtxp30	SBI-05	60	7	147	161	4	0.72

[1]Chromosome number based on Bhattramakki et al. (2000) as published by Kim et al. (2005); [2]Number of alleles detected among 18 diverse sorghum strains listed in Table 1 of Kong et al. (2000). SSRs markers associated with the stay-green QTL in B35 are highlighted (bold).

production of the most complete microsatellite map of sorghum (Bhattramakki et al., 2000) on which all the markers used here are mapped. This line also was the most genetically distinct in the study of Menz et al. (2004).

The largest group consisted of twelve genotypes with B35 at one end and IS9302 at the other. This group was subdivided into five, with the first subgroup containing B35 and QL41. QL41 is an inbred line derived from a cross in which B35 was a parent. The second subgroup contained KS19 and QL12, which had many alleles in common. Indeed these two lines were the most similar of

Table 2. Contd.

Locus	[1]Chromosome no.	Annealing temperature	[2]No. of Alleles	Size range in this study		No. of alleles in this study	PIC in this study
Xtxp115	SBI-05	60	2	201	207	3	0.56
Xtxp299	SBI-05	50	5	190	212	5	0.69
Xtxp123	SBI-05	55	ND	260	280	3	0.56
Xtxp136	SBI-05	55	ND	221	229	3	0.36
Xtxp225	SBI-05	55	4	159	178	7	0.80
Xtxp6	SBI-06	50	9	340	398	5	0.77
Xtxp17	SBI-06	55	3	148	177	7	0.81
Xtxp57	SBI-06	55	3	240	252	6	0.75
Xtxp145	SBI-06	55	5	184	210	8	0.77
Xtxp265	SBI-06	55	8	160	199	11	0.82
Xtxp274	SBI-06	55	6	305	350	5	0.77
Xtxp40	SBI-07	55	2	124	134	4	0.52
Xtxp92	SBI-07	50	2	148	162	2	0.32
Xtxp168	SBI-07	55	3	158	164	4	0.69
Xtxp278	SBI-07	50	3	246	254	3	0.43
Xtxp295	SBI-07	55	5	134	162	10	0.78
Xtxp312	SBI-07	55	9	130	194	10	0.85
Xtxp47	SBI-08	55	3	229	236	4	0.66
Xtxp105	SBI-08	55	3	265	274	4	0.60
Xtxp210	SBI-08	55	4	167	190	8	0.70
Xtxp273	SBI-08	55	5	180	207	7	0.76
Xtxp321	SBI-08	55	4	170	230	9	0.83
Xtxp67	SBI-09	55	8	145	172	7	0.83
Xtxp230	SBI-09	55	10	220	270	8	0.83
Xtxp258	SBI-09	55	5	162	199	8	0.77
Xtxp287	SBI-09	55	5	345	353	3	0.59
Xtxp289	SBI-09	55	5	227	282	10	0.84
Xtxp339	SBI-09	55	2	150	186	4	0.20
Xtxp358	SBI-09	55	4	220	299	9	0.88
Xtxp141	SBI-10	55	5	126	150	8	0.78
Xtxp217	SBI-10	55	5	152	168	8	0.82
Xtxp270	SBI-10	55	6	208	273	9	0.84

[1]Chromosome number based on Bhattramakki et al. (2000) as published by Kim et al. (2005); [2]Number of alleles detected among 18 diverse sorghum strains listed in Table 1 of Kong et al. (2000). SSRs markers associated with the stay-green QTL in B35 are highlighted (bold).

any of the genotypes examined. BTx623 also clustered with KS19 and QL12. The third sub-group included seven genotypes, but there was considerable diversity between these lines. R9188, which is a stay-green line from the Rio source of stay-green (Borrell, personal communication), was found in this sub-cluster as was QL39, the senescent parent used in the QTL analysis of Tao et al. (2000). This study found that unexpectedly, QL39 contributed a number of stay-green alleles. The next largest group comprised of nine genotypes with E36-1 and #68 at one end and IS18530 at the other. The group had four subgroups; the first included E36-1 and the 2 lines derived from it #66 and #68. The second subgroup consisted of IS22380, DJ1195 and IS13441. The third subgroup had ISCV112 and SPV386 with IS18530 alone in the fourth subgroup. E36-1, IS22380, IS13441, ICSV112 and SPV386 are all caudatum type sorghums. Clustering however did not always follow the sorghum race classification (or country of origin) particularly as a number of the advanced breeding lines

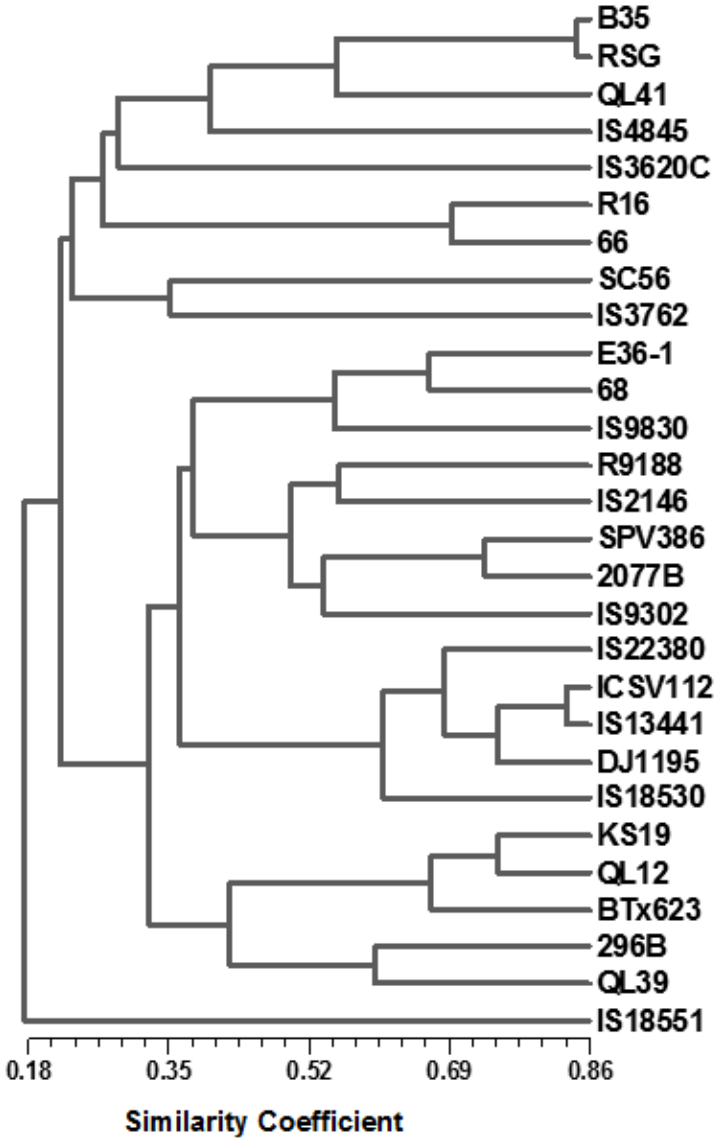

Figure 1. Dendrogram constructed with unweighted paired group method using the arithmetic average (UPGMA) clustering algorithm from pairwise matrix of genetic similarities of 28 sorghum lines using 68 microsatellite markers.

examined have more than one race in their genetic background. This has also been found in other studies of genetic diversity in sorghum (Menz et al., 2004).

The fourth major group consisted of four genotypes, with R16 and RSG 03123 in a subgroup, and IS18551 and IS4845 in another subgroup. R16 and RSG 03123 are guinea-caudatum race sorghums, but RSG 03123 has genes from the Durra sorghum B35; IS18551 and IS4845 are Durra race sorghums. The fifth major group had only two genotypes SC56 and IS9830, which both originate from Sudan and were about 40% similar.

Genetic similarities based on SSRs associated with the B35 stay-green QTL only

A dendrogram of genetic similarity based on 15 SSRs associated with the four major B35 stay-green QTL identified three major groups (Figure 2). The first distinct group had IS3762 and B35 at either end, with three subgroups. The largest subgroup comprised B35, RSG 03123, QL41, IS4845 and IS3620C. B35 and RSG 03123 were most related with a coefficient of more than 0.8 and were more similar to QL41 than the other genotypes. This

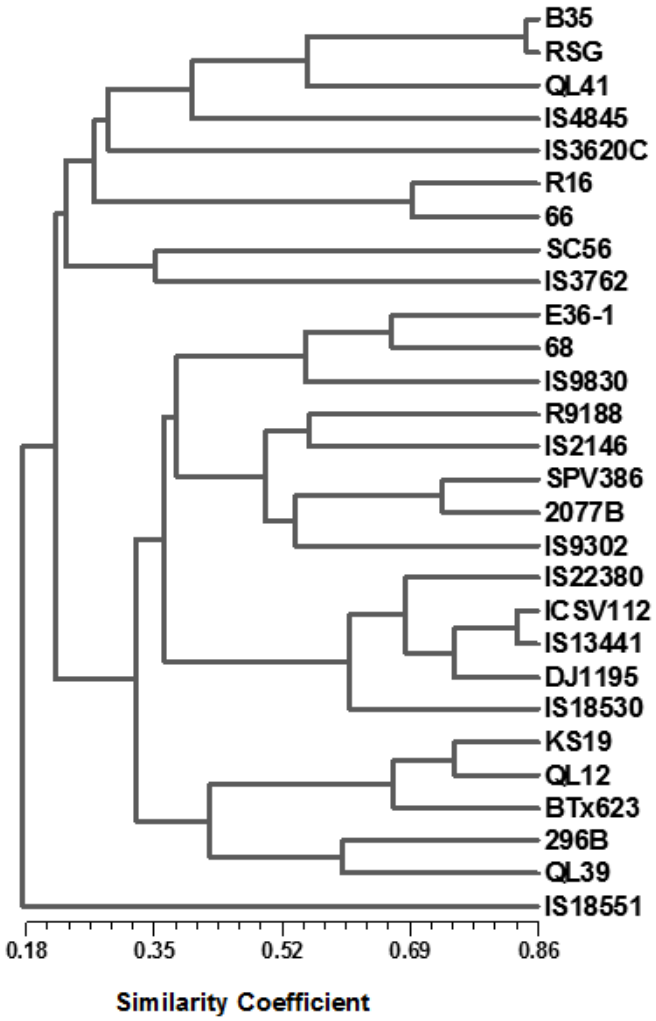

Figure 2. Genetic similarity among sorghum cultivars based on 15 SSRs associated with the B35 stay-green QTL only using UPGMA.

was not surprising as RSG 03123 was developed by marker-assisted backcrossing of B35 alleles associated with the stay-green QTL. The second subgroup had R16 and #66, while SC56 and IS3762 were in the third. The stay-green line SC56 was much more genetically similar to B35 when only regions of the genome comprising B35 stay-green QTL were considered. Kebede et al. (2001) found that a number of stay-green QTL were common between SC56 and B35, suggesting that common genes were involved in these otherwise genetically distinct lines. The second distinct group had 13 genotypes with IS18530 and E36-1 at each end. This indicates that, as with the overall genetic diversity, E36-1 shares little in common with B35 at the B35 stay-green regions. The stay-green line R9188 also clustered in this group as did IS22380. This latter line was found to have a high expression of stay-green in the study of Mahalakshmi and Bidinger (2002). The third major group included five genotypes, with KS19, QL12 and BTx623 in one subgroup, as well as 296B and QL39 in another. KS19 and QL12 were much more similar to B35 when their overall genetic similarity was examined. IS18551 which fell into the same group with IS4845 in Figure 1, this time stood alone with only 18% similarity with all other genotypes.

Genotypes of derivatives in relation to the parental lines

Five of the accessions studied (RSG 03123, #66, #68, QL12 and QL41) are known to be derived from accessions also included in this study. RSG 03123 is the product of marker-assisted backcrossing (MABC)

conducted at ICRISAT in which the target was to transfer genomic regions on chromosomes BSI-01, SBI-02, SBI-03 and SBI-05 from B35 where stay-green QTL have been identified in a wide range of studies (Kong et al., 2000; Bhattramakki et al., 2000; Huassmann et al., 2002).

For 31 loci, RSG 03123 was homozygous for the R16 parental allele whereas for 18 loci it was homozygous for the B35 allele. Ten SSR loci were non-polymorphic, one was heterozygous and 3 loci did not produce PCR products. Most of the alleles from B35 were found on chromosomes SBI-01, SBI-02 and SBI-03. Out of nine loci on chromosome SBI-01, alleles of four SSRs came from each parent while the remaining one was not polymorphic. The alleles from R16, however, were associated with the position of the B35 QTL stgA indicating that this QTL was not transferred from B35 into R16. In chromosome SBI-02 one locus was heterozygous for both parents; six of the rest were from B35 and three from R16. All the SSRs associated with *stg*3 from B35 were present in RSG 03123, while the SSRs associated with stgB originated from R16. In chromosome SBI-03, four SSRs were from B35; three of them are associated with *stg*1, while three SSRs were of R16 origin. In the genomic region where stg2 is located, there was no polymorphism detected in the markers used between B35 and R16. Chromosomes SBI-04, SBI-06, SBI-08 and SBI-09 were entirely made up of alleles from R16. In chromosomes SBI-05, SBI-07 and SBI-10, one allele from B35 was found in each of them. stg4 is in chromosome SBI05 and although for one marker associated with this QTL, the B35 allele was found in RSG 03123, again lack of polymorphism meant that it was not possible to determine whether this QTL had been transferred from B35 to RSG 03123 or not.

Two regions of the genome corresponding to QTL for stay-green (stg1 and stg3) were homozygous for B35 alleles confirming that stg1 and stg3 were successfully transferred from B35 to R16. The non-polymorphism between R16 and B35 in the other major regions containing stay-green QTL makes it impossible to confirm the transfer of *stg*2 and *stg*4 from B35 to R16. The minor QTL from B35 (stgA and stgB) would appear not to have been transferred to RSG 03123 as its genomic composition in these regions were homozygous for the R16 allele. As the majority of the genome of RSG 03123 is R16, these results indicate that this material is ideal for the study of the functioning of stay-green QTL in a novel or senescent background.

Genotypes #66 and #68 are phenotypic selections from a cross between E36-1 and R16. E36-1 is described as a stay-green line (van Oosterom et al., 1996) although its stay-green nature is dependent on the environment in which it is grown. In the phenotypic study of Mahalakshmi and Bidinger (2002), E36-1 was not described as stay-green, but it was used as the stay-green parent in both (2) populations used in the QTL analysis of stay-green of Haussmann et al. (2002). QTL in which E36-1 provided the positive alleles for stay-green were found on chromosomes SBI-01, SBI-03, SBI-07 SBI-08 and SBI-10. The hybrids #66 and #68 showed a different stay-green response in the field study of Mahalakshmi and Bidinger (2002) but no genetic analyses have been conducted previously with this material. #66 and #68 had the same alleles at 22 SSR loci. They both shared four alleles with R16 and 14 alleles with E36-1. However, #66 had 21 SSR alleles in which the parental allele came from E36-1 and 26 from R16, while in #68 35 SSR alleles originated from E36-1 and only 9 originated from R16. On chromosome SBI-10, #66 and #68 were 100% similar with all SSR alleles being of E36-1 origin. These alleles are also associated with the stay-green QTL from E36-1. Other SSR alleles associated with stay-green QTL from E36-1 on chromosomes SBI-01, SBI-07 and SBI-08 were found in #68 whereas #66 only contained stay-green alleles from E36-1 on SBI-03 in addition to those on SBI-10. QL12 is a sugarcane mosaic virus resistant BC_1 derivative of the stay-green line KS19 (Jordan et al., 2004). It was thus interesting to examine the genomic composition of QL12 as compared to KS19. Only six SSR alleles identified were unique to QL12; three of them occurred in chromosome SBI-03 with one each in SBI-02, SBI-07 and SBI-08. On chromosomes SBI-01, SBI-04, SBI-05, SBI-06, SBI-09 and SBI-10, all the SSR alleles on QL12 were common to KS19 and in chromosome SBI-02, nine out 10 SSRs were also observed in KS19. Neither KS19 nor QL12 have been included in any published mapping studies for stay-green, so it is not known, which regions of the genome control stay-green expression in these genotypes or whether there is any similarity to those found for other stay-green lines such as B35.

QL41 is a stay-green line derived from a cross between B35 and QL33 (Jordan et al., 2004) and has been used as a parent to identify QTL associated with stay-green in a cross with QL39 (Tao et al., 2000). Twelve SSR alleles were unique to QL41 and another 11 were common to B35 and QL41 as well as many other cultivars. SSR alleles from B35 were found in SBI-02 and these were associated with the stay-green QTL mapped in QL41 as well as stg3 and stgB in B35 suggesting that this stay-green QTL in QL41 originates from B35. QL41 did not have the same alleles as B35 at the position of the other B35 stay-green QTL. Furthermore, there were 13 SSR alleles found in QL41, which were common to KS19 but not B35.

DISCUSSION

Scoring of SSRs

The 68 SSR markers used in this study generated 416

alleles with 1 to 14 alleles per locus with an average of 6.2. Similarly, Shehzad et al. (2009) used 38 polymorphic SSR markers to generate 146 alleles which were able to uniquely classify 320 sorghum accessions of diverse origin and the number of alleles revealed per locus ranged from two to nine with average of 3.84. A study of 27 sorghum accessions from a gene bank in Zambia using only 10 SSR loci found a total of 44 alleles from 324 individuals across accessions, a range of 2 to 9 alleles per locus and an average of 4.4 alleles per locus. In the current study, average number of alleles per locus was higher than that in the studies of Shehzad et al. (2009) and Ng'uni et al. (2011).

SSRs are highly polymorphic and useful genetic markers that have been used in genetic similarities studies in wheat (Plaschke et al., 1995; Röder et al., 1995), maize (Smith et al., 1997) as well as sorghum (Taramino et al., 1997; Uptmoor et al., 2003; Menz et al., 2004). The results of Taramino et al. (1997) showed that SSRs have great potential for discriminating among sorghum inbred lines and one SSR locus alone could allow all nine inbred lines to be identified. Polymorphic information content (PIC), a measure of the discrimination ability of a locus, has been found to be comparable between SSRs and RFLPs (Smith et al., 1997) or AFLPs (Menz et al., 2004) or even higher for SSRs (Pejic et al., 1998). The mean PIC and the mean number of alleles per locus for this study were higher than those found by Smith et al. (1997) among 58 maize inbred lines using 131 SSRs and Smith et al. (2000) among 50 elite sorghum lines and similar to that found of Menz et al. (2004). Eight SSR loci with number of alleles from 7 to 14 also had high PIC values (0.79 to 0.89). SSR allele sizes were also similar to those previously reported (Kong et al., 2000; Bhattramakki et al., 2000).

Genetic similarities based on 68 SSRs

All 28 sorghum lines were distinguished and the clusters obtained consisted of the derivatives in close association with their parental lines (Figures 1 and 2). QL41 was in the same cluster with B35, #66 and #68 with E36-1, and RSG 03123 was closely associated with R16. KS19 and QL12 were highly related with a coefficient of more than 0.8, while all other similarities were below this value. In previous studies (Taramino et al., 1997; Smith et al., 2000; Uptmoor et al., 2003; Menz et al., 2004), comparisons were made between R-lines and B-lines or were based on region of origin. The current study was not based on any of these classifications; however, clusters were not always made up of only lines from the same region or race. For example, IS22830 (Sudan, caudatum), DJ1195 (India, Durra), IS13441 (Zimbabwe, caudatum) from different geographical regions or races belonged to the same cluster. Similarly, ICSV112 (India, guinea-caudatum) and SPV386 (India, caudatum) also belonged to same cluster (Figure 1), while IS3620C and KS19 did not cluster together even though both originated from Nigeria. The advantage of using markers with known map positions instead of a random sample is that there is control over the coverage of the genome and although there were a few gaps, the markers used in his study provided extensive marker coverage of the sorghum genome.

Genetic diversity based on SSRs associated with the B35 stay-green QTL

Genetic diversity is vital in the success of any breeding programme (Ali et al., 2007) and molecular markers are an excellent tool for assessment of genetic relationships (Ritter et al., 2007). Initial inheritance studies of the stay-green trait in B35 suggested that it is influenced by a major gene that exhibits varied levels of dominant gene action depending on the environment in which evaluations are made (Tenkouano et al., 1993; Walulu et al., 1994). Subsequent QTL analysis studies indicated at least 4 regions of the genome controlling the trait. A number of clusters were obtained based on differences in allelic sizes of the SSRs associated with the stay-green trait (Figure 2). Derivatives of B35 and KS19 were always in the same clusters with the parental lines, #68 was grouped with E36-1, while #66 was grouped with R16. These associations were consistent with the data information on the data matrix (Table 2). In RSG 03123 stg1 and stg3 were polymorphic between R16 and B35 and RSG 03123 had the same alleles as B35, while stg2 and stg4 were non-polymorphic, hence the close association of RSG 03123 with B35. Similarly, for QL12, apart from a few loci, the SSR alleles were same as in KS19, thus their close relationship. The clustering of #66 with R16 is not surprising since #66 shared 26 SSR alleles of the same size with R16, and 21 with E36-1. #68, which had 35 SSRs of the same size as in E36-1 and only 7 of the same size in R16, was clustered with E36-1, as expected. Panicle shape and size were also similar between E36-1 and #68, and also for #66 and R16 (data not shown). Since R16 has not been involved in any mapping, it is impossible to tell whether it has genomic regions associated with stay-green or not, even though some alleles of SSRs associated with stay-green in B35 were revealed in it.

A mapping population of a cross between B35 and R16 could enhance the understanding of the genetic basis of the trait. KS19 has not been reported as a parent in any mapping population even though it is being used in breeding programmes in the USA and Australia. QTL determined with KS19 as a parent would reveal those contributed by this line and what phenotypic attributes they are associated with.

Conclusions

The 28 sorghum lines were highly variable genetically and clustered into groups not necessarily based on country of origin or race. Derivatives of B35, KS19 and E36-1 clustered together with their parental lines based on all 68 SSRs, while IS3620C was the least related with any other genotype.

Clusters based on the SSRs associated with QTL from B35 alone were similar to those observed with all 68 SSRs for B35, KS19 and E36-1 and their derivatives, except #66 which was in the same cluster with R16 and RSG 03123 clustered with B35. This confirms the introgression of the QTL from B35 into R16. The stay-green line SC56 was much more genetically similar to B35 when only regions of the genome comprising B35 stay-green QTL were considered, suggesting that common genes were involved in these otherwise genetically distinct lines. The stay-green lines KS19, E36-1, R9188 and IS22380 clustered distinctly from B35 indicating that these lines share little in common with B35 at the B35 stay-green regions. This might be so with other sources of stay-green. Hence, there is the need to investigate the physiological and biochemical basis of the trait in these lines and other sources of stay-green as well. These stay-green lines have considerable potential to increase the genetic diversity of the stay-green trait within sorghum breeding programmes. This study also shows how it is possible to follow, through a breeding programme, alleles associated with key agronomic QTL of interest.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Ali ML, Rajewski JF, Baenziger PS, Gill KS, Eskridge KM, Dweikat L (2007). Assessment of genetic diversity and relationship among a collection of US sweet sorghum germplasm by SSR markers. Mol. Breed. 21(4):497-509.

Anderson JA, Churchill GA, Sutrique JE, Tanksley SD, Sorrells ME (1993). Optimizing parental selection for genetic linkage maps. Genome 36:181-186.

Asare AT, Gowda BS, Galyuon IKA, Aboagye, LL, Takrama JF, Timko MP (2010). Assessment of the genetic diversity in cowpea (*Vigna unguiculata* L. Walp.) germplasm from Ghana using simple sequence repeat markers. Plant Gen. Res. Charact. Util. 8:142-150.

Asare PA, Galyuon IKA, Tetteh JP Sarfo JK (2011). Morphological and molecular based diversity studies of some cassava (*Manihot esculenta C*rantz) germplasm in Ghana. Afr. J. Biotechnol. 10(63):13900-13908.

Barrett BA, Kidwell KK (1998). AFLP-based genetic diversity assessment among wheat cultivars from the Pacific Northwest. Crop Sci. 38:1261-1271.

Bhattramakki D, Dong J, Chhabra AK, Hart GE (2000). An integrated SSR and RFLP linkage map of *Sorghum bicolor* (L.) Moench. Genome 43:988-1002.

Borrell AK, Bidinger FR, Tao YZ, McIntyre CL, Douglas AC L (2001). Physiological and molecular aspects of stay-green in sorghum. In, Borrell AK, Henzell RG (eds). Proceedings from the Fourth Australian Sorghum Conference, Kooralbyn, 5-8 February 2001.

Borrell AK, Hammer GL, Douglas ACL (2000a). Does maintaining green leaf area in sorghum improve yield under drought? I. Leaf growth and senescence. Crop Sci. 40:1026-1037.

Borrell AK, Hammer GL, Douglas ACL (2000b). Does maintaining green leaf area in sorghum improve yield under drought? II. Dry matter production and yield. Crop Sci. 40:1037-1048.

Brown SM, Hopkins MS, Mitchell SE, Senior ML, Wang TY, Duncan RR, Gonzales-Candelas F, Kresovich S (1996). Multiple methods for the identification of polymorphic simple sequence repeats (SSRs) in sorghum [*Sorghum bicolor* (L.) Moench]. Theor. Appl. Genet. 93:190-198.

Cox TS, Murphy JP, Rodgers DM (1986). Changes in genetic diversity in the red winter wheat regions of the United States. Proc. Natl. Acad. Sci. USA. 83:5583-5586.

Crasta OR, Xu WW, Rosenow DT, Mullet JE, Nguyen HT (1999). Mapping of post-flowering drought resistance traits in sorghum: association between QTLs influencing premature senescence and maturity. Mol. Gen. Genet. 262:579-588.

Djè Y, Heuertz M, Lefèbvre C, Vekemans X (2000). Assessment of genetic diversity within and among germplasm accessions in cultivated sorghum using microsatellite markers. Theor. Appl. Genet. 100:918-925.

Faustine C, Hoffmann LV, Tibazawa FI, Lukonge E (2015). Use of simple sequence repeats (SSR) markers for screening blue disease resistance in cotton germplasm. Afr. J. Biotechnol. 14(14):2871-2875.

Harris K, Subudhi PK, Borrell A, Jordan D, Rosenow D, Nguyen H, Klein P, Klein R, Mullet J (2007). Sorghum stay-green QTL individually reduce post-flowering drought-induced leaf senescence. J. Exp. Bot. 58: 327-338.

Hash CT, Bhasker Raj AG, Lindup S, Sharma A, Beniwal CR, Folkertsma RT, Mahalakshmi V, Zerbini E, Blümmel M (2003). Opportunities for marker-assisted selection (MAS) to improve the feed quality of crop residues in pearl millet and sorghum. Field Crops Res. 84:79-88.

Haussmann BIG, Mahalakshmi V, Reddy BVS, Seetharama N, Hash CT, Geiger HH (2002). QTL mapping of stay-green in two sorghum recombinant inbred populations. Theor. Appl. Genet. 106:133-142

Jordan DR, Tao YZ, Godwin ID, Henzell RG, Cooper M, McIntyre CL (2004). Comparison of identity by descent and identity by state for detecting genetic regions under selection in a sorghum pedigree breeding program. Mol. Breed. 14:441-454.

Kassahun B, Bidinger F, Hash C, Kuruvinashetti M (2010). Stay-green expression in early generation sorghum [*Sorghum bicolor* (L.) Moench] QTL introgression lines. Euphytica 172:351-362.

Kebede H, Subudhi PK, Rosenow DT, Nguyen, HT (2001). Quantitative trait loci influencing drought tolerance in grain sorghum (*Sorghum bicolor* L. Moench**).** Theor. Appl. Genet. 103:266-276.

Kong L, Dong J, Hart GE (2000). Characteristics, linkage-map positions, and allelic differentiation of *Sorghum bicolor* (L.) Moench DNA simple-sequence repeats (SSRs). Theor. Appl. Genet. 101:438-448.

Mahalakshmi V, Bidinger F (2002). Evaluation of stay-green sorghum germplasm lines at ICRISAT. Crop Sci. 42:965-974.

McIntyre CL, Tao Y, Jordan DR, Drenth J, Henzell RG (2001). In: AK Borrell and RG Henzell (eds). Proceedings from the Fourth Australian Sorghum Conference, Kooralbyn, 5-8 February 2001.

Menz MA, Klein, RR, Mullet JE, Obert JA, Unruh NC, Klein PE (2002) A high-density genetic map of *Sorghum bicolor* (L) Moench based on 2926 AFLP, RFLP and SSR markers. Plant. Mol. Biol.48:483-499.

Menz MA, Klein RR, Unruh NC, Rooney WL, Klein PE, Mullet JE (2004). Genetic diversity of public inbreds of sorghum determined by mapped AFLP and SSR markers. Crop Sci. 44:1236-1244.

Mohammadi SA, Prasanna BM (2003). Analysis of genetic diversity in crop plants - Salient statistical tools considerations. Crop Sci. 43:1235-1248

Nei M (1973). Analysis of gene diversity in subdivided populations.

Proc. Natl. Acad. Sci. USA 70:3321-3323.
Nei M, Li WH (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA. 76:5269-5273.
Ng'uni D, Geleta M, Bryngelsson T (2011). Genetic diversity in sorghum (*Sorghum bicolor* (L.) Moench) accessions of Zambia as revealed by simple sequence repeats (SSR). Hereditas 148:52-62.
Pejic I, Ajmone-Marsan P, Morgante M, Kozumplick V, Castiglioni P, Taramino G, Motto M (1998). Comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, RAPDs, SSRs, and RFLPs. Theor. Appl. Genet. 97:1248-1255.
Plaschke J, Ganal MW, Röder MS (1995) Detection of genetic diversity in closely related bread wheat using microsatellite marker. Theo. Appl. Genet. 91:1001-1007.
Ritter KB, McIntyre CL, Dodwin ID, Jordan DR, Chapman, SC (2007). An assessment of the genetic relationship between sweet and grain sorghum, within *Sorghum bicolor* ssp. *Bicolor* (L.) Moench, using AFLP markers. Euphytica 157:161-176.
Röder MS, Plaschke J, Konig SU, Borner A, Sorrels ME, Tanksley SD, Ganal MW (1995). Abundance, variability and chromosomal location of microsatellites in wheat. Mol. Gen. Genet. 246:327-333.
Rosenow DT, Clark LE (1981) Drought tolerance in sorghum. In: Loden, H. D. and Wilkinson, D. (eds) *Proc. 36th Annu. Corn and Sorghum Industry Res. Conf.* Chicago, IL., 9-11 Dec., 1981. Am. Seed Trade Assoc., Washington, DC. pp. 18-31.
Shehzad T, Okuizumi H, Makoto KM, Okuno, K (2009). Development of SSR-based sorghum (*Sorghum bicolor* (L.) Moench) diversity research set of germplasm and its evaluation by morphological traits. Genet. Resour. Crop Evol. 56:809-827
Smith JSC (1984). Genetic variability within U.S. hybrid maize: Multivariate analysis of isozyme data. Crop Sci. 24:1041-1046.
Smith JSC, Chin ECL, Shu H, Smith OS, Wall SJ, Senior ML, Mitchell SE, Kresovich S, Ziegle J (1997). An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): comparisons with data from RFLPs and pedigree. Theor. Appl. Genet. 95:163-173.
Smith JSC, Kresovich S, Hopkins MS, Mitchell SE, Dean RE, Woodman WL, Lee M, Porter K (2000). Genetic diversity among elite sorghum inbred lines assessed with simple sequence repeats. Crop Sci. 40:226-232.
Smith OS, Smith JSC (1992). Measurement of genetic diversity among maize hybrids: A comparison of isozymic, RFLP, pedigree, and heterosis data. Maydica 37:53-60.
Sneath PHA, Sokal RR (1973). Numerical Taxonomy. Freeman, San Francisco.
Subudhi PK, Nguyen HT (2000). Linkage group alignment of sorghum RFLP maps using RIL mapping population. Genome 43:240-249.
Subudhi PK, Rosenow DT, Nguyen HT (2000). Quantitative trait loci for the stay-green trait in sorghum [*Sorghum bicolor* (L.) Moench]: consistency across genetic backgrounds and environments. Theor. Appl. Genet. 101:733-741,
Tao Y, Henzell RG, Jordan DR, Butler DG, Kelly AM, McIntyre CL (2000). Identification of genomic regions associated with stay-green in sorghum by testing RILs in multiple environments. Theor. Appl. Genet. 100:1225-1232.
Tao Y, Manners JM, Ludlow MM, Henzell RA (1993). DNA polymorphisms in grain sorghum [*Sorghum bicolor* (L.) Moench]. Theor. Appl. Genet. 86:678-688.
Taramino G, Tarchini R, Ferrario S, Lee M, Pè ME (1997). Characterization and mapping of simple sequence repeats (SSRs) in *Sorghum bicolor*. Theor. App. Genet. 95:66-72.
Tenkouano A, Miller FR, Frederiksen RA, Rosenow DT (1993). Genetics of nonsenescence and charcoal rot resistance in sorghum. Theor. Appl. Genet. 85:644-648.
Thomas H (1982). Leaf senescence in a non-yellowing mutant of *Festuca pratensis.* I. Chloroplast membrane polypeptides. Planta 154:212-218.
Thomas H, Howarth CJ (2000). Five ways to stay green. J. Exp. Bot. 51:329-337.
Thompson JA, Nelson RL, Vodkin LO (1998). Identification of diverse soybean germplasm using RAPD markers. Crop Sci. 38:1348-1355.
Tuinstra MR, Grote EM, Goldsborough PB, Ejeta G (1997). Genetic analysis o post-flowering drought tolerance and components of grain development in *Sorghum bicolor* (L.) Moench. Mol. Breed. 3:439-448.
Uptmoor R, Wenzel W, Friedt W, Donaldson G, Ayisi K, Ordon F (2003). Comparative analysis on the genetic relatedness of *Sorghum bicolor* accessions from Southern Africa by RAPDs, AFLPs and SSRs. Theor. Appl. Genet. 106:1316-1325.
van Oosterom EJ, Jayachandran R, Bidinger F (1996). Diallel analysis of stay-green trait and its components in sorghum. Crop Sci. 36:540-555.
Vadez V, Deshpande SP, Kholova J, Hammer GL, Borrell AK, Talwar HS, Hash CT (2011). Stay-green quantitative trait loci's effects on water extraction, transpiration efficiency and seed yield depend on recipient parent background. Funct. Plant Biol. 38:553-566.
Walulu RS, Rosenow DT, Wester DB, Nguyen HT (1994). Inheritance of stay-green trait in sorghum. Crop Sci. 34:970-972.
Xu W, Subudhi PK, Crasta OR, Rosenow DT Mullet JE, Nguyen HT (2000). Molecular mapping of QTLs conferring stay-green in grain sorghum (*Sorghum bicolor* L. Moench). Gen. 43:461-469.

academicJournals

Vol. 15(25), pp. 1375-1382, 22 June, 2016
DOI: 10.5897/AJB2016.15377
Article Number: A72D7DA59059
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Sapucaia nuts (*Lecythis pisonis*) modulate the hepatic inflammatory and antioxidant metabolism activity in rats fed high-fat diets

Marcos Vidal Martins[1]*, Izabela Maria Montezano de Carvalho[2], Mônica Maria Magalhães Caetano[1], Renata Celi Lopes Toledo[1], Antônio Avelar Xavier[1] and José Humberto de Queiroz[1]

[1]Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa, Brazil.
[2]Departamento de Nutrição, Universidade Federal de Sergipe, Brazil.

Received 1 April 2016, Accepted 18 May, 2016.

Lecythis pisonis **Cambess is commonly known as "sapucaia" nut. Previous studies show that it is rich in unsaturated fatty acids and in antioxidant minerals. The aim of the present study was to assess the antioxidant and anti-inflamatory effects of this nut after its introduction into a control (AIN-93G) or high-fat diet in Wistar rats. The animals were divided into four groups: a control diet, the same control diet supplemented with sapucaia nuts, a high-fat diet or the high-fat diet supplemented with sapucaia nuts and were fed with these diets for 14 or 28 days. The gene expression of the markers tumor necrosis factor (TNF)-α NFκB (p65) zinc superoxide dismutase (ZnSOD) and heat shock protein 72 (HSP72) was determined by the chain reaction to the quantitative reverse transcription-polymerase chain reaction (q-PCR). The antioxidant activity was also measured as thiobarbituric acid reactive substances (TBARS) through the activity of the SOD enzyme. The groups treated with "sapucaia" nuts presented reduced lipid peroxidation values and increased ZnSOD and HSP72 gene expression, as well as decreased TNF-α and NFκB (p65) gene expression levels. The significant results showed that "sapucaia" could serve as a potential source of antioxidants and as a protector agent for the examined animals.**

Key words: Sapucaia nuts, inflammation, oxidative stress, gene expression.

INTRODUCTION

Excessive consumption of food associated or not with sedentary lifestyle contributes to the onset of metabolic disorders linked to increased body weight and systemic insulin resistance due to chronic inflammatory condition (Carobbio et al., 2011). Experimental data demonstrates that Wistar rats can develop obesity when they are fed with high-fat diets (Burneiko et al., 2006; Estadella et al., 2004; Kretschmer et al., 2005). The intake of these high-fat diets increases the total amount of body lipids, and thus, raises the oxidative stress, which is strongly related with inflammation (Brunetti et al., 2009; Pérez-Echarri et al., 2009). The increase of body fat reserves stimulates the macrophage infiltration into adipose tissue that could activate a chronic inflammation cascade, which may

*Corresponding author. E-mail: marcosvidalmartins@yahoo.com.br.

expand to other tissues and cause several health disorders (Alemany, 2013).

High-fat diets can affect the redox balance in the body Fispecies (ROS) (Vial et al., 2011). On the other hand, human and animal organisms have developed a very effective antioxidant defense system, which mainly consist of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione-S-transferase (GST) enzymes. These enzymes co-operate to protect cells from free radicals (Domínguez-Avila et al., 2015).

In addition to the enzymatic antioxidant defense, there are also highly conserved proteins that help in protecting the body, such as heat shock proteins (HSP). Different stimuli can interfere with the gene expression of these proteins and could result in the intracellular accumulation of denatured proteins. An increased gene expression of the HSP 70 protein family generates an anti-inflammatory effect, since these proteins down-regulate the Kappa Beta Transcription Factor (NFκB). Consequently, some inflammatory expression markers such as the tumor necrosis factor alpha (TNF-α) and the interleukin 1 beta (IL 1β) are inhibited (De la Fuente et al., 2015; Kim et al., 2015).

Literature shows that the consumption of nuts has beneficial effects on human health due to their nutritional components. Nuts are rich in unsaturated fatty acids, sulfur amino acids and minerals, such as selenium, magnesium, manganese, zinc, iron and copper. At the same time, they contain satisfactory levels of vitamin C and E (Dominguez-Avila et al., 2015; Sabaté et al., 2010; Serrano et al., 2007). The combined effects of these acids, minerals and vitamins may influence specific processes related to the regulation of cell differentiation, DNA protection and inflammatory responses due to their protective role in oxidative stress (Casas-Agustench et al., 2009; Cassidy et al., 2014; Domínguez-Avila et al., 2015; González and Salas-Salvadó, 2006; Ross, 2010; Sabaté et al., 2010).

The vast Brazilian biome remains almost untouched when it comes to nut extraction. Only the cashew and the Pará nuts are commercially available. The “sapucaia” nut (*Lecythis pisonis* Cambess) for human consumption remains commercially unexplored, although some local citizens consider it as the best Amazon nut (Clay et al., 2000). revious studies using “sapucaia” nuts conducted in our laboratory have shown that their use in diets can favor health. The composition of this nut makes it a significant source of unsaturated fatty acids, proteins and minerals such as manganese, magnesium, iron and calcium. Besides, “sapucaia” serves as a potential protective agent against different metabolic disorders (Carvalho et al., 2012). Thus, the aim of the current study is to verify the sapucaia nut ability to reduce oxidizing agent production and to modulate the gene expression of pro and anti-inflammatory molecules in the liver tissue of Wistar rats fed with a high-fat diet.

MATERIALS AND METHODS

Plant

The “sapucaia” nuts used in the present study were collected from five trees at the Federal University of Viçosa campus, which is located in the Zona da Mata Mineira region (20°76’S and -42°86’W), Southeastern of Minas Gerais State. Five fruits were collected from each tree and ten nuts were taken from each of them. The current study uses information about the chemical composition of these nuts indicated by Carvalho et al. (2012).

Animals and diets

The experiment followed the standards established by Law 11.794 and the CONCEA/MCTI Regulatory Resolutions, 2008. The study was approved by the Ethics Committee for Animal Experimentation of Federal University of Viçosa (CEUA-UFV) (Protocol 77/2014). Forty eight male albino rats (Rattus norvegicus, Wistar) were used in the present experiment. The newly weaned animals were obtained from the Central Animal Laboratory of the Biological Sciences Center (CCB) at Federal University of Viçosa, Minas Gerais State - Brazil. The weight of the animals was recorded and they were, then, randomly allocated to eight groups, each with six animals, and housed in individual cages with a controlled environment under temperatures between 22 and 25°C and light-dark cycle of 12 h per day. Experimental diets and water were offered *ad libitum* either for 14 or 28 days. Calculations were performed to simulate the human standard consumption of three nuts a day. The nutritional information of the “Sapucaia” nut (SAP) was obtained by the study of Carvalho et al. (2012). A reference intake of 2000 kcal per day, which corresponds to 4.65% of the total calories of three Sapucaia nuts (93.08 kcal for three nuts), was adopted; thus, the diets contained 4.65% of sapucaia nut.

The animals were fed with four different diets for 14 or 28 days, as follows: Group 1, was fed with a standard diet (AIN-93G) (Reeves et al., 1993); Group 2, was fed with the standard diet supplemented with sapucaia nuts (24 g/kg; 4.65% of total calories) (AIN-93G + SAP), although caloric density (3.95 kcal/g) remained the same (Table 1); Group 3, was fed with a high-fat diet (HFD); and Group 4, was fed with the high-fat diet supplemented with sapucaia nuts (HFD + SAP) (52.2 g/kg; 4.65% of total calories), although again the caloric density remained unaffected (6.92 kcal/g) (Table 2). The rats had *ad libitum* access to the diets and water. The diets were prepared weekly and stored at 4°C to prevent oxidation. The animals were euthanized with ketamine (25 mg/kg IM) and xylazine (2 mg/kg IM) at the end of each study period (14 or 28 days), after fasting for 12 h. The liver tissue of the euthanized animals was collected, frozen in liquid nitrogen and stored in an Ultrafreezer (-80°C).

Gene expression

The total RNA extraction was performed in Trizol reagent (Invitrogen, CA, USA) using 100 mg of liver tissue, according to the recommendation of the manufacturer. The concentration and purity were assessed in a spectrophotometer (Multiskan Go, Thermo Scientific DE, USA) and the integrity of the mRNAs was checked through agarose gel electrophoresis. The recovered mRNA was treated with RNase-free DNase (Promega). The cDNA synthesis was performed using the M-MLV Reverse Transcriptase kit (Invitrogen, CA, USA), according to the protocol of the manufacturer. cDNA was used to determine the expression of mRNAs for the markers TNF-α, NFκB, ZnSOD, HSP72 and the reference house keeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Table 1. Diet compositions (g/1000 g of diet).

Ingredient	AIN93G (g)	AIN93G+Sapucaia
Caseine (81.50%)	200	192.2
Dextrinized starch	132	123
Saccharose	100	100
Soy oil	70	54
Microcrystalline cellulose	50	50
Mineral mixture	35	35
Vitamin mix	10	10
L-cystine	3	3
Coline	2.5	2.5
Sapucaia nuts	-	29.4
Corn starch	397.5	400.9
Caloric density (kcal/g)	3.95	3.96

Table 2. High hat diet (HFD) compositions with sapucaia nuts (g/1000 g of diet).

Ingredient	AIN93G (g) [HFD]	AIN93G+Sapucaia (g) [HFD+SAP]
Ham pâté	222.22	215.7
Potato sticks	111.11	104.61
Bacon	111.11	104.61
Bologna	111.11	104.61
Sweet biscuit cornstarch	111.11	104.61
Chocolate powder	111.11	104.61
whole milk powder	111.11	104.61
Commercial Diet	111.11	104.61
Sapucaia nuts	-	52.2
Caloric density (kcal/g)	6.94	6.92

gene.

The relative quantification of the gene expression was performed through real-time polymerase chain reaction (qPCR) using the Sybr Green Reagent 2X Master Mix (Applied Biosystems, CA, USA). The final volume of each reaction was 10 µl: 2 µl of the cDNA, 0.8 µl of the primers mixture (2.5 µM) (sense and antisense), 5.0 µl of 2X Master Mix Sybr Green reagent and 2.2 µl of ultrapure water in each tested gene. The used qPCR reaction protocol was: 15 min at 95°C, then 40 cycles at 95°C (15 s), 60°C (30 s) and 72°C (30 s) followed by melting curve analysis. Samples were analyzed in four biological repetitions and two technical repetitions and quantified in independent runs.

The negative controls (NTC) were made in two technical repetitions by replacing the cDNA samples by the same water volume in the reaction. "AB Step One Real Time PCR System" (Applied Biosystems) equipment was used to run the experiment. The relative quantification of the gene expression was analyzed through the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

The pairs of oligonucleotides used to amplify the genes of interest were: NFκB (p65) (Fw 5'-CTTCTGGGCCATATGTGGAGA-3') and (Rw 5'-TCGCACTTGTAACGGAAACG-3'), TNF-α (Fw5′-GCCGATTTGCCATTTCATACC-3′) and (Rw 5′-GGACTCCGTGATGTCTAAGTAG-3′), HSP 72 (Fw 5'-AGGCCAACAAGATCACCATC-3') and (Rw 5'-TAGGACTCGAGCGCATTCTT-3'), ZnSOD (Fw 5'-GAGCAGAAGGCAAGCGGTGAA-3') and (Rw 5'-CCACATTGCCCAGGTCTC-3'), GAPDH (Fw 5'-GGTTGTCTCCTGTCACTTC-3') and (Rw 5'-CTGTTGCTGTAGCCATATTC-3'). The oligonucleotides were designed based on the gene sequences of the Wistar *Rattus norvegicus* found in the GenBank, using Primer 3 plus software. The experiment followed the MIQE guidelines established for studies that use the qPCR technique in real time (Bustin et al., 2009).

Preparation of liver homogenate

Liver samples (200 mg) were collected from each rat and after their thawing, were homogenized in Tris-HCl 0.01 M buffer, pH 7.4 at the ratio of 5 ml buffer per 500 mg of tissue and finally, centrifuged at 10.000 g for 15 min at 4°C. The supernatants were used to determine the total protein content, the superoxide anion dismutase antioxidant activity, and hepatic lipid peroxidation.

Enzymatic activity of the hepatic SOD

Superoxide dismutase (SOD) activity was determined in relative units. Each SOD unit was defined by the amount of enzyme able to

Table 3. Mean body weight gain, daily diet consumption and hepatosomatic index in the initial and final stages of the treatment.

Group/Treatment	Weight gain (g)	Daily diet consumption (g)	Hepatossomatic Index
14 days			
AIN-93G	95.10±19.89[a]	26.60±7.02[b]	4.9±0.44[a]
AIN-93G+SAP	96.85±16.46[a]	29.54±4.36[b]	4.7±0.51[a]
HFD	97.82±14.46[a]	32.73±6.05[a]	4.7±0.62[a]
HFD+SAP	94.32±11.86[a]	25.40±5.12 [b]	4.6±0.71[a]
28 days			
AIN-93G	152.27±12.49[b]	26.56± 6.24[b]	4.8±0.63[a]
AIN-93G+SAP	160.89±8.14[b]	30.21± 7.23[a]	4.6±0.48[a]
HFD	186.67±26.88[a]	32.62±9.39[a]	4.6±0.52[a]
HFD+SAP	187.38±11.54[a]	32.21±5.06[a]	4.7±0.73[a]

Means (n = 6) followed by different letters in the same column are statistically different (Tukey test, p <0.05). AIN-93G, AIN-93G diet; AIN-93G + SAP, AIN-93G + sapucaia nuts diet; HFD, high-fat diet; HFD + SAP, high-fat diet + sapucaia nuts diet, liver somatic index (liver's relative weight = liver weight / body weight).

inhibit 50% of the pyrogallol oxidation rate. The used reaction medium contained 30 µl of liver homogenate and 15 µl of pyrogallol 24 mmol/L and 15 µl of catalase prepared with 2.4 mg in 2 ml of distilled water. The volume was completed with Tris-HCl buffer 50 mM 270 µl up to 300 µl, pH 8.2 and 1 mM of EDTA. A standard 100% pyrogallol oxidation was conducted in 15 µl of catalase and 15 µl of pyrogallol 24 mmol/L. The blank solution contained 15 µl of catalase and 285 µl of the buffer. Initially, the readings were performed at 420 nm absorbance. The reaction was then incubated at 37°C for 5 min and the readings were performed again at 420 nm. The absorbance found before and after the 5 min incubation was subtracted to minimize the presence of interferents. The results were expressed as U SOD/mg of protein (Marklund, 1985). Calculations were made based on the standard absorbance value, by considering that such value had zero SOD units. Protein quantification was performed according to Bradford (1976) using a standard curve, which was based on bovine serum albumin callibration.

Hepatic lipid peroxidation analysis

This was conducted through the measurement of malondialdehyde (MDA), which is produced by the oxidation of fatty acids through the reaction of thiobarbituric acid reactive substances (TBARS) (Kohn and Liversedge, 1944). The lipid peroxidation was measured by means of a reaction containing 400 µl liver homogenate, 1 ml trichloroacetic acid (20%) and 400 µl thiobarbituric acid (1.6%). The mixture was incubated at 95°C for 1 h, 1.6 ml n-butanol was added and it was then centrifuged at 3000 rpm/10 min. The reading was performed at 510, 532, and 560 nm. Equation proposed by Pyles et al. (1993) was used to calculate the final values in order to minimize interferences from pigments of heme in the hemoglobin in the MDA dosage:

$$MDA_{532}=1.22[(A_{532}) – (0.56)\ (A_{510}) + (0.44)\ (A_{560)}]$$

Malondialdehyde concentration was determined through the coefficient of molar absorptivity E 0 = 1.56 × 105 L mol^{-1} cm^{-1} (Buege and Aust, 1978) and the results were expressed in nmoles of MDA per mg of protein.

Statistical analysis

ANOVA analysis was performed to assess the distribution of variables in the experiments. It was followed by the Tukey test (Graphpad Prism 5) in all treatments. The significance level was 95%, p <0.05. The results were expressed as mean ± standard deviation. The comparisons were conducted between the AIN-93G and AIN-93G groups + SAP and between HFD and HFD + SAP groups by considering the caloric density difference between the administered diets.

RESULTS

Weight gain and diet consumption

Weight assessments comparing animals subjected to HFD/HFD + SAP and those fed with AIN-93G/AIN-93G + SAP showed that the weight gain of animals was significantly different after 28 days of treatment. Daily feed intake per animal was higher in the HFD group at day 14 and lower in the AIN-93G group at day 28 of the experiment compared to the other groups (Table 3).

Lipid peroxidation values and antioxidant activity

Lipid peroxidation (MDA) values and the antioxidant activity expressed in SOD units (Table 4) were determined with the intention to assess the impact of the high-fat diet on the oxidative stress in the liver of rats and to discover the possible protective effect of sapucaia nut supplementation. All groups treated with “sapucaia” nut-enriched diets showed lower MDA concentration compared to the other groups in both tested diets (HFD and AIN-93G).MDA level measurements showed that the AIN-93G diet had similar values at days 14 and 28 of the

Table 4. Means of the hepatic malondialdehyde (MDA) concentrations (nmol MDA / mg STP) and superoxide dismutase units (SOD) (U SOD/mg LWA) at the 14th and 28th day in each diet (AIN-93G, AIN-93G + SAP, HFD, HFD + SAP) administered to the animals.

Group	MDA		SOD	
	14 days	28 days	14 days	28 days
AIN-93G	1.10 ± 0.04^{aA}	1.16 ± 0.02^{aA}	8.10 ± 0.44^{bA}	8.45 ± 0.72^{bA}
AIN-93G+SAP	0.62 ± 0.05^{bB}	1.03 ± 0.03^{cB}	11.78 ± 1.14^{aB}	13.65 ± 0.50^{aB}
HFD	1.39 ± 0.01^{aC}	1.72 ± 0.05^{bC}	6.30 ± 0.48^{aA}	10.35 ± 0.47^{bC}
HFD+SAP	0.65 ± 0.05^{bB}	0.96 ± 0.02^{aB}	14.28 ± 0.81^{bB}	14.86 ± 0.62^{bB}

Means (n=6) followed by different letters in the same column are statistically different (Tukey test, $p < 0.05$). AIN-93G, AIN-93G diet; AIN-93G + SAP, AIN-93G + sapucaia nuts diet; HFD, high-fat Diet; HFD + SAP, HFD + sapucaia nuts diet, PTN - Proteins.

experiment, although a significant increase in the MDA levels was observed in the other groups. The antioxidant enzyme activity expressed in SOD units was significantly higher in the sapucaia supplemented groups both at days 14 and 28.

Antiinflamatory and antioxidant hepatic gene expressions

Relative mRNAs gene expression was determined to help understand the effects caused by the inclusion of the “sapucaia” nuts in the diets. The experiment compared the diets with or without sapucaia nuts to find the genes involved in the inflammatory process and the hepatic antioxidant activity by real time RT qPCR (Figure 1). The gene expression of TNF-α was lower in animals fed with the sapucaia nuts than in those of the controls. There were no significant differences in both AIN-93G + SAP and HFD + SAP in the TNF-α gene expression in all treatment periods.

It was also observed that the gene expression of the NFkB (p65) was significantly lower in the group of rats that consumed the diets supplemented with “sapucaia” nuts. The gene expression of ZnSOD, in turn, was relatively higher in sapucaia supplemented groups than in the controls.

There was also an increased gene expression in the hepatic HSP72 gene in both diets. However, the HFD + SAP showed more significant amounts.

DISCUSSION

High-fat diets administered to rats can induce inflammation and oxidative stress (Burneiko et al., 2006; Brunetti et al., 2009; Estadella et al., 2004; Kretschmer et al., 2005; Pérez-Echarri et al., 2009; Scoaris et al., 2010). Rats fed with the hyperlipidic diet (HFD) did not show a significantly higher hepatosomatic index (HIS; relative liver weight) compared to the other groups. Sapucaia dietary supplementation and the duration of the experiment appeared not to affect HIS value. Although, diets enriched with “sapucaia” nuts did not have any effect on weight gain and on HSI, our findings showed that sapucaia enrichment contributed in the prevention of the redox imbalance caused by high-fat diets due to its interference in both the MDA concentrations and in the SOD enzyme activity. As it was expected, more oxidizing agents in animals fed with high-fat diet were observed. However, this negative effect was diminished after sapucaia supplementation as shown by the significant increase in the SOD activity and of the decrease in MDA values.

Carvalho et al. (2012) showed that 100 g of sapucaia nuts cover the daily needs for manganese and magnesium and the half of them for iron, according to the reference values. Other studies using “sapucaia” nuts found high magnesium, iron, selenium and phosphorus concentration levels (Kerdell-Vargas, 1966; Souza et al., 1996; Vallilo et al., 1999), as well as a desired protein digestibility (Denadai et al., 2007). Such findings about the nutritional value of the the sapucaia nut (*L. pisonis*), reinforce the hypothesis that are several benefits in the metabolism of animals fed with the sapucaia enriched diets.

The enzyme system is the first line of antioxidant defense of the organism. The constituents of the nuts associated with the gene stimulation of the enzymes can play an important role as exogenous antioxidants, such as tocopherol, ascorbate, carotenoids, and phenolic compounds, due to their mineral components. This set of nutrients can cause many benefitial health effects against metabolic disorders such as obesity and diabetes (Borges and Lessa, 2015; Casas-Agustench et al., 2009; Cassidy et al., 2014; Dominguez-Avila et al., 2015; Fernandes et al., 2015; Shahidi and Ambigaipalan, 2015).

The liver has multiple functions and is directly involved in the oxidative processes (Rodríguez-Hernández et al., 2013; Stadler et al., 2004). Changes in the hepatic redox balance can interfere in the translocation of proteins

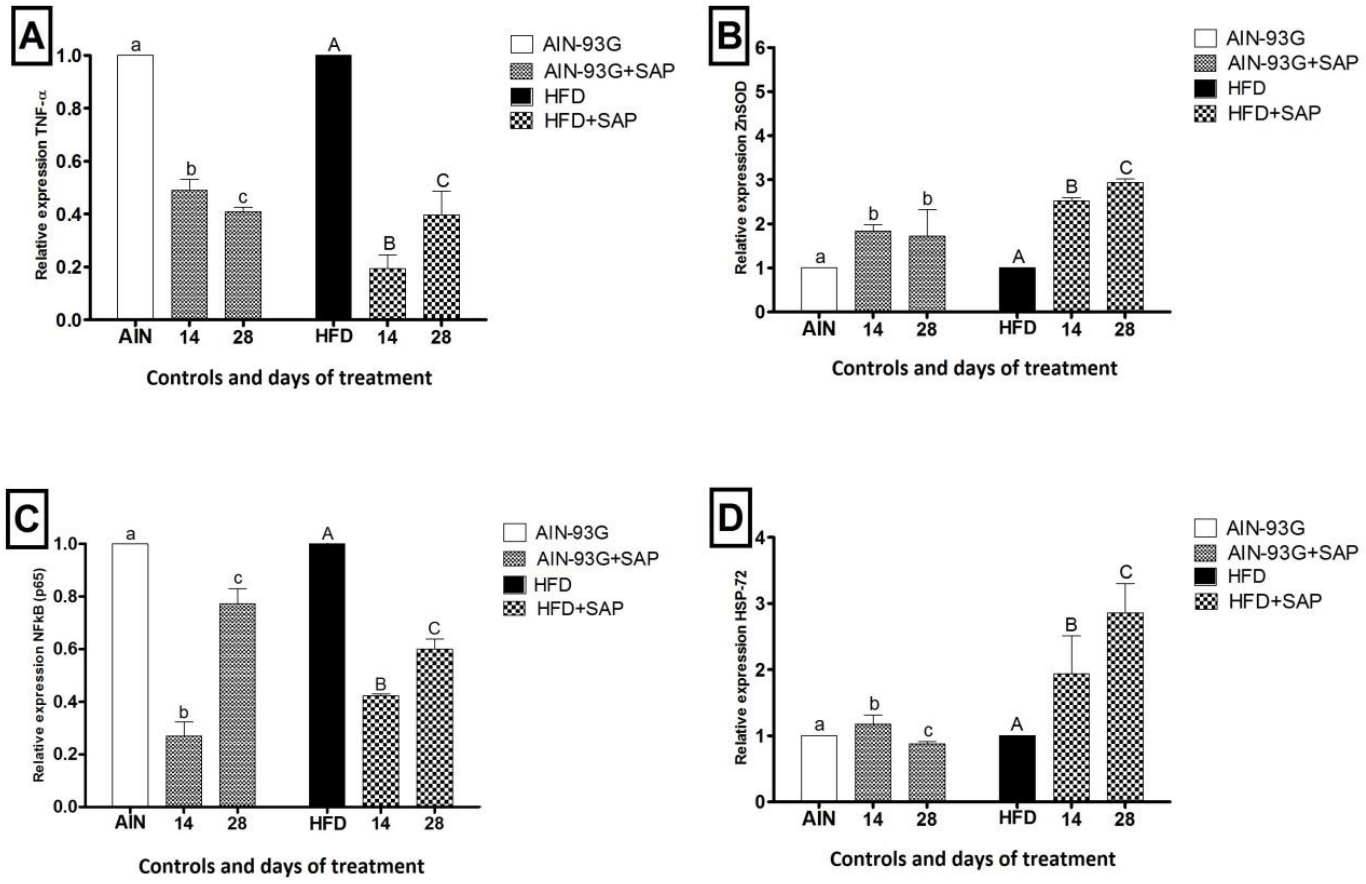

Figure 1. Averages of the mRNA relative gene expression (calibrator sample, y=1) of TNF-α (A), ZnSOD (B), NFkB (p65) (C) and HSP-72 (D) byqPCR for the animals fed with AIN-93G + SAP and HFD + SAP for 14 and 28 days. Means followed by different letters (lowercase for AIN-93G and capital to HFD) are significantly different according to the Tukey's test ($p<0.05$).

sensitive to oxidative stress from the cytoplasm to the cell nucleus (Cassidy et al., 2014; Maritim et al., 2003; Sadi et al., 2008). Thus, the oxidative stress induced by high-fat diets tends to increase the gene expression of TNF-α and NFkB (p65), as a response to the process triggered by the oxidative imbalance associated to the high content of saturated fatty acids. In addition, the HSP70 HSP family is also affected by the oxidative stress caused by dietary fats (Jangale et al., 2013; Lightfoot et al., 2015; Yaglom et al., 2003; Gabai and Sherman, 2002).

There has been a significant difference in the HSP-72 gene expression between rats treated with both HFD and HFD + SAP and those fed with AIN-93G and AIN-93G + SAP. Rats treated with the sapucaia nut-enriched diets showed significantly higher HSP-72 gene expression than those fed just with the control diets (HFD and AIN-93G), except AIN-93G at day 28. As it is concluded, "sapucaia" nut could play an important role in the modulation of this gene expression. The HSP-72 is part of the HSP-70 proteins family, which inhibits NFkB (p65) activation by reducing the production of pro-inflammatory cytokines (Dokladny et al., 2010; Shi et al., 2006; Tanaka et al., 2014). Therefore, increased HSP-72 expression seen in groups fed with the sapucaia nuts-enriched diets (HFD + SAP and AIN-93G + SAP) could be correlated with the decrease in the NFkB activation cascade activity (p65).

There is an interaction of NFkB signaling cascade (p65) with the TNF-α gene expression, since TNF-α is a pro inflammatory agent and can be activated by the NFkB (p65) protein (Dokladny et al., 2010; Tanaka et al., 2014). As it was shown in the present study, there was a decrease in the TNF-α gene in animals fed with HFD + SAP compared to the HFD group. Higher ZnSOD gene expression was observed in all groups treated with sapucaia nuts-enriched diets, in addition to all the aforementioned findings. It allows inferring that the presence of these nuts in the diets played an important role, since the gene expression of this enzyme was higher in the HFD + SAP than in the HFD.

The correlation between saturated fat acids consumption and oxidative stress is not simple, because there are many biochemical mechanisms involved. Thus, saturated fat acids consumption can increase oxygen

consumption and generate other oxidizing molecules (Dandekar et al., 2015; Hybertson et al., 2011; Seifert et al., 2009). However, our results showed increased antioxidant and anti-inflammatory capacity due to the lower gene expressions of TNF-α and NFκB. These findings reinforce the hypothesis that dietary antioxidants are particularly important for protection against chronic diseases. Finally, the sapucaia nuts could serve as a potential antioxidant source and their protection is profound even in animals fed with high-fat diets.

Conclusion

The sapucaia nut dietary supplementation stimulated the enzymatic activity of SOD and reduced lipid peroxidation values in the hepatic tissue of rats fed with high-fat diets. These results were confirmed by the observed differencies in the expression of genes directly involved in the metabolic processes. ZnSOD enzyme and HSP72 protein expressions were significantly higher in rats fed with the sapucaia nuts-enriched diets. Diet enriched with sapucaia nuts was found to influence the gene expression of the inflammation markers TNF-α and NFκB; reduction in their expression levels can be linked with the increased antioxidant activity, since these parameters are related. Further studies on sapucaia must be conducted in order to assess the impacts of its consecutive use as food, since is rich in nutrients and could have beneficial effects on health status.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

We are grateful to Fundação de Amparo a Pesquisa de Minas Gerais (FAPEMIG) for the finantial support (APQ 00832 12).

REFERENCES

Alemany M (2013). Relationship between energy dense diets and white adipose tissue inflammation in metabolic syndrome. Nutr. Res. 33(1):1-11.

Borges J P and Lessa M A (2015). Mechanisms Involved in Exercise-Induced Cardioprotection: A Systematic Review. Arq. Bras. Cardiol. 105(1):71-81.

Bradford M (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 72(1-2):248-254.

Brunetti L, Leone S, Orlando G, Recinella L, Ferrante C, Chiavaroli A, Vacca M (2009). Effects of obestatin on feeding and body weight after standard or cafeteria diet in the rat. Peptides. 30(7):1323-1327.

Buege JA and Aust SD (1978). Microsomal lipid peroxidation. Biomembranes- Part C: Biological Oxidations. pp. 302-310.

Burneiko RCM, Diniz YS, Galhardi CM, Rodrigues HG, Ebaid GMX, Faine LA, Novelli ELB (2006). Interaction of hypercaloric diet and physical exercise on lipid profile, oxidative stress and antioxidant defenses. Food Chem. Toxicol. 44(7):1167-1172.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Wittwer C T (2009). "The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments." Clin. Chem. 55(4):611-622.

Carobbio S, Rodriguez-Cuenca S, Vidal-Puig A (2011). Origins of metabolic complications in obesity. Curr. Opin. Clin. Nutr. Metab. Care. 14(6):520-526.

Casas-Agustench P, López-Uriarte P, Ros E, Bulló M, Salas-Salvadó J (2011). Nuts, hypertension and endothelial function. Nutr. Metab. Cardiovas. 21:S21-S33.

Clay JW, Paulo de Tarso BS, Clement CR (2000). Biodiversidade amazônica: exemplos e estratégias de utilização. (1th ed.) Brasilia: Ministerio de Ciência e Tecnologia.

Dandekar A, Mendez R, Zhang K (2015). Cross talk between er stress, oxidative stress, and Inflammation in Health and Disease. Methods Mol. Biol. pp. 205-214.

Carvalho IM, Queiros LD, Brito LF, Santos FA, Moreira Bandeira AV, de Souza, AL, Queiroz JH (2012). Chemical characterization of sapucaia nuts (LecythispisonisCambess.) from zona da matamineira region. Biosci. J. 28(6):971-977.

De la Fuente V, Federman N, Zalcman G, Salles A, Freudenthal R, Romano A (2015). NF-κB transcription factor role in consolidation and reconsolidation of persistent memories. Front. Mol. Neurosci. 8: 1-9.

Denadai SMS, Hiane PA, Marangoni S, Baldasso PA, Miguel AMRO, Macedo MLR (2007). In vitro digestibility of globulins from sapucaia (LecythispisonisCamb.) nuts by mammalian digestive proteinases. Ciênc. Tecnol. Aliment. 27(3):535-543.

Dokladny K, Lobb R, Wharton W, Ma TY, Moseley PL (2009). LPS-induced cytokine levels are repressed by elevated expression of HSP70 in rats: possible role of NF-κB. Cell Stress and Chaperones. 15(2):153-163.

Domínguez-Avila JA, Alvarez-Parrilla E, López-Díaz JA, Maldonado-Mendoza, I E, Gómez-García MC, De la Rosa LA (2015). The pecan nut (Caryaillinoinensis) and its oil and polyphenolic fractions differentially modulate lipid metabolism and the antioxidant enzyme activities in rats fed high-fat diets. Food Chem. 168:529-537.

Estadella D (2004). Effect of Palatable Hyperlipidic Diet on Lipid Metabolism of Sedentary and Exercised Rats. Nutrition 2(2):218-224.

Fernandes DC, Alves AM, Castro GSF, Junior AAJ, Naves MMV (2015). Effects of Baru Almond and Brazil Nut Against Hyperlipidemia and Oxidative Stress In Vivo. J. Food Res. 4(4):38.

Gabai VL, Sherman MY (2002). Invited Review: Interplay between molecular chaperones and signaling pathways in survival of heat shock. J. Appl Physiol. 92(4):1743-1748.

González CA, Salas-Salvadó J (2006). The potential of nuts in the prevention of cancer. Br. J. Nutr. 96(S2): S87-S94.

Hybertson BM, Gao B, Bose SK, McCord JM (2011). Oxidative stress in health and disease: The therapeutic potential of Nrf2 activation. Mol. Aspects Med. 32(4-6):234-246.

Jangale NM, Devarshi PP, Dubal AA, Ghule AE, Koppikar SJ, Bodhankar SL, Harsulkar AM (2013). Dietary flaxseed oil and fish oil modulates expression of antioxidant and inflammatory genes with alleviation of protein glycation status and inflammation in liver of streptozotocin–nicotinamide induced diabetic rats. Food Chem. 141(1):187-195.

Kerdell-Vargas F (1966). The depilatory and cytotoxic action of coco de mono (*Lecythis ollaria*) and its relationship to chronic seleniosis. Econ. Bot. 20:187-105.

Kim JY, Yenari MA, Lee, JE (2015). Regulation of inflammatory transcription factors by heat shock protein 70 in primary cultured astrocytes exposed to oxygen–glucose deprivation. Neuroscience 286:272-280.

Kohn HI, Liversedge M (1944). On a new aerobic metabolite whose production by brain is inhibited by apomorphine, emetine, ergotamine, epinephrine, and menadione. J. Pharmacol. Exp. Ther. 82(3):292-300.

Kretschmer BD, Schelling P, Beier N, Liebscher C, Treutel S, Krüger N, Haus A (2005). Modulatory role of food, feeding regime and physical exercise on body weight and insulin resistance. Life Sci. 76(14): 1553-1573.

Lightfoot AP, Jackson MJ, McArdle A, Cooper RG (2015). Heat Shock Protein 70 Protects Against ER Stress-Induced Mitochondrial Dysfunction; A Potential Therapeutic Target to Reduce Muscle Weakness in IIM. Rheumatology, 54(1):i31-i32.

Livak KJ, Schmittgen TD (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods 25(4):402-408.

Maritim AC, Sanders RA, Watkins J (2003). Diabetes, oxidative stress, and antioxidants: a review. J. Biochem. Mol. Toxicol. 17(1):24-38.

Marklund SL (1985). Pyrogallol autoxidation. Handbook of methods for oxygen radical research.CRC Press. Boca Raton. pp. 243-247.

Pérez-Echarri N, Pérez-Matute P, Marcos-Gómez B, Marti A, Martínez JA, Moreno-Aliaga MJ (2009). Down-regulation in muscle and liver lipogenic genes: EPA ethyl ester treatment in lean and overweight (high-fat-fed) rats. J. Nutr. Biochem. 20(9):705-714.

Pyles LA, Stejskal EJ, Einzig S (1993). Spectrophotometric Measurement of Plasma 2-Thiobarbituric Acid-Reactive Substances in the Presence of Hemoglobin and Bilirubin Interference. Exp. Biol. Med. 202(4):407-419.

Reeves PG, Nielsen FH, Fahey GC (1993). AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr. 123(11):1939-1951.

Rodríguez-Hernández H, Simental-Mendía LE, Rodríguez-Ramírez G, Reyes-Romero MA (2013). Obesity and inflammation: epidemiology, risk factors, and markers of inflammation. Int. J. Endocrinol. Metab. pp. 1-11.

Ross E (2010). Health Benefits of Nut Consumption. Nutrients. 2(7):652-682.

Sabate J, Ang Y (2010a). Nuts and health outcomes: new epidemiologic evidence. Am. J. Clin. Nutr. 89(5):1643S-1648S.

Sabaté J, Oda, K, Ros, E (2010b). Nut consumption and blood lipid levels: a pooled analysis of 25 intervention trials. Arch. Intern. Med. 170(9):821-827.

Sadi G, Yılmaz Ö, Güray T (2008). Effect of vitamin C and lipoic acid on streptozotocin-induced diabetes gene expression: mRNA and protein expressions of Cu–Zn SOD and catalase. Mol. Cell. Biochem. 309(2):109-116.

Serrano J, Goñi I, Saura-Calixto F (2007). Food antioxidant capacity determined by chemical methods may underestimate the physiological antioxidant capacity. Food Res. Int. 40(1):15-21.

Shahidi F and Ambigaipalan P (2015). Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects–A review. J. Funct. Foods 18:820-897.

Scoaris CR, Rizo GV, Roldi LP, Moraes SMF, Proença ARG, Peralta RM, Natali MRM (2010). Effects of cafeteria diet on the jejunum in sedentary and physically trained rats. Nutrition 26(3):312-320.

Seifert EL, Estey C, Xuan JY, Harper ME (2009). Electron Transport Chain-dependent and -independent Mechanisms of Mitochondrial H2O2 Emission during Long-chain Fatty Acid Oxidation. J. Biol. Chem. 285(8):5748-5758.

Shi Y, Tu Z, Tang D, Zhang H, Liu M, Wang K, Xiao X. (2006). The inhibition of LPS-induced production of inflammatory cytokines by HSP70 involves inactivation of the NF-κB pathway but not the MAPK pathways. Shock. 26(3):277-284.

Souza ADGD, Sousa NR, Silva SD, Nunes CDM, Canto ADC, Cruz LDA (1996). Fruteiras da Amazônia. (1th ed.) Brasília: Embrapa-SPI.

Stadler K, Jenei, V, Von Bölcsházy G, Somogyi A, Jakus J (2004). Role of free radicals and reactive nitrogen species in the late complications of diabetes mellitus in rats. Orvosihetilap. 145(21):1135-1140.

Tanaka T, Shibazaki A, Ono R, Kaisho T (2014). HSP70 mediates degradation of the p65 subunit of nuclear factor κB to inhibit inflammatory signaling. Sci. Signal. 7(356):ra119-ra119.

Vial G, Dubouchaud H, Couturier K, Cottet-Rousselle C, Taleux N, Athias A, Leverve XM (2011). Effects of a high-fat diet on energy metabolism and ROS production in rat liver. J. Hepatol. 54(2):348-356.

Vallilo M (1999). Lecythis pisonis Camb. nuts: oil characterization, fatty acids and minerals. Food Chem. 66(2):197-200.

Yaglom J, O'Callaghan-Sunol C, Gabai V, Sherman, MY (2003). Inactivation of dual-specificity phosphatases is involved in the regulation of extracellular signal-regulated kinases by heat shock and hsp72. Mol. Cell. Biol. 23(11):3813-3824.

African Journal of Biotechnology

Related Journals Published by Academic Journals

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

academicJournals