SUSTAINABLE AND INTEGRAL EXPLOITATION OF AGAVE

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ESTRATEGIA NACIONAL PARA LA CONSERVACIÓN Y USO SOSTENIBLE DE LOS RECURSOS FITOGENÉTICOS DEL PAÍS

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RESUMEN

México es considerado un país megadiverso por la gran diversidad de especies albergadas en su territorio (Villasenor 2004), ha contribuido con el enriquecimiento de los Recursos Fitogenéticos para la Alimentación y la Agricultura (RFAA) del mundo. Es centro de origen y/o diversidad, ha contribuido a la domesticación de cultivos con importancia mundial (maíz, frijol, chile, cacao, algodón, aguacate, agaves, etc), para la Alimentación y la Agricultura del mundo (Molina 2006). Es por ello que, la Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA) a través del Servicio Nacional de Inspección y Certificación de Semillas (SNICS) implementaron desde el 2002 el Sistema Nacional de Recursos Fitogenéticos para la Alimentación y la Agricultura (SINAREFI), con el objetivo de vincular a los actores relacionados con los RFAA para promover el trabajo colaborativo mediante redes de cooperación por cultivo. El SINAREFI ha funcionado como un modelo de asociatividad y agente catalizador en el trabajo colaborativo con más de 60 instituciones, 400 investigadores y más de 500 productores de todo el país, agrupados en 45 Redes de investigación y desarrollo tecnológico de 44 cultivos nativos, aglomerados en 5 grandes Macro-Redes y una Red temática: Centros de Conservación. Lo anterior ha permitido conocer el estado que guardan cada uno de los cultivos atendidos, definiendo un plan estratégico con acciones a corto, mediano y largo plazo, así como el resguardo de 66,000 accesiones en los diferentes Centros de Conservación distribuidos estratégicamente en el territorio nacional, evitando de esta forma la duplicidad de actividades, realizando un uso eficiente de recurso económico, material y humano; y fomentando el desarrollo sustentable e integral del sector agroalimentario del país. Es así como el modelo del SINAREFI ha contribuido a la seguridad alimentaria del país, a la conservación de especies nativas y al fortalecimiento de las capacidades interinstitucionales

Palabras clave: Conservación, Fitogenéticos, Sostenible, Redes, Aprovechamiento.

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INTRODUCCIÓN

La República Mexicana presenta variadas condiciones climáticas, estas condiciones naturales ofrecen numerosos ambientes para el florecimiento de un gran número de especies de plantas, razón por la cual el país posee una de las biotas más diversas y es considerado uno de los 17 países megadiversos y el cuarto en recursos fitogenéticos del mundo. La existencia de una gran diversidad vegetal y de numerosos grupos humanos, permitieron el uso y domesticación de un amplio número de especies, como lo son el maíz, frijol, chile, cacao, algodón, aguacate, etc., cultivos que han enriquecido los recursos fitogenéticos disponibles para la alimentación y la agricultura en el mundo.

Los RFAA se refiere a cualquier material de origen vegetal, incluido el material reproductivo y de propagación vegetativa que contiene unidades funcionales de herencia, y que tiene valor real o potencial para la alimentación y la agricultura (Hammer et al., 2003), incluye formas primitivas de las especies cultivadas, variedades locales, cultivares modernos, cultivares obsoletos, líneas de cruzas, malezas y los parientes silvestres (Upadhyaya et al., 2008); los cuales proporcionan alimento, medicinas, fibras, energía y otros usos (Hammer et al., 2003, Lobo y Medina 2009).

En este sentido la SAGARPA creo en 2002 al SINAREFI coordinado por el SNICS como una estrategia para salvaguardar la diversidad fitogenética de nuestro país. En el 2006 se logró obtener el segundo informe en materia de recursos fitogenéticos.

Las principales premisas de acción arrojadas en dichos documentos fueron:
- ✓ Fortalecer las capacidades nacionales de manera coordinada.
- ✓ Protección al patrimonio genético (Técnico / Legal).
- ✓ Atención prioritaria a especies originarias de México.
- ✓ Prevenir la biopiratería / saqueo.

Derivado de éste análisis y con el propósito de resolver estas premisas, la SAGARPA adoptó a través del SNICS el Plan de Acción Mundial para la Conservación y Utilización Sostenible de los Recursos Fitogenéticos para la Alimentación y la Agricultura implementado por la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO por sus siglas en Inglés), de este modo se creó el Plan Nacional de Acción para los RFAA en México integrado por cuatro áreas estratégicas y 20 líneas de acción. Cuadro 1.

Tabla 1. Áreas estratégicas y líneas de acción del SINAREFI

I. Conservación *in situ*	**II. Conservación *ex situ***	**III. Uso y potenciación**	**IV. Creación de capacidades**
1. Inventario	5. Mantenimiento de colecciones	9. Caracterización	15. Coordinación de Redes
2. Mejoramiento participativo	6. Regeneración	10. Mejoramiento Genético	16. Promoción Redes
3. Asistencia en caso de catástrofes	7. Recolección	11. Promover diversificación	17. Sistemas de Información
4. Promoción especies subutilizadas	8. Ampliar actividades de conservación	12. Desarrollo de especies subutilizadas	18. Sistemas vigilancia y alerta
		13. Producción de semillas	19. Enseñanza y capacitación
		14. Nuevos mercados	20. Sensibilización a la opinión pública

La Ley de Desarrollo Rural Sustentable señala en su artículo 102, que el SNICS es el responsable de establecer y en su caso proponer conjuntamente con las demás dependencias e instituciones vinculadas, políticas, acciones y acuerdos internacionales sobre conservación, acceso, uso y manejo integral de los Recursos Fitogenéticos para la Alimentación y la Agricultura, protección de los derechos de los obtentores de variedades vegetales, así como para el análisis, conservación, calificación, certificación, fomento, abasto y uso de semillas promoviendo la participación de los sectores vinculados. Para atender las premisas de estas atribuciones, el SNICS creó en el 2002 al SINAREFI, cuya unidad funcional y modelo organizativo son sus Redes por cultivo; y que junto con "Variedades Vegetales" y "Certificación de Semillas" forman sus tres áreas sustantivas.

OBJETIVO

Vincular a los actores relacionados con los cultivos prioritarios en materia de conservación y uso sustentable, promoviendo el trabajo colaborativo mediante redes de cooperación por cultivo específico que eviten la duplicidad de actividades y contribuyan a fomentar el desarrollo sustentable e integral del sector agroalimentario del país.

METODOLOGÍA

Actualmente se reporta que solo 30 cultivos proporcionan el 95% de las necesidades de energía de la población y 4 de ellos: arroz, trigo, maíz y papa proporcionan más del 60% de la energía, lo que significa que la seguridad alimentaria del mundo depende de un pequeño número de cultivos, por lo tanto es de importancia primordial conservar la mayor diversidad de ellos. En este sentido, la pérdida de variabilidad genética supone una limitación de la capacidad de responder a nuevas necesidades y un incremento de la vulnerabilidad de nuestros cultivos frente a cambios ambientales o aparición de nuevas plagas o enfermedades, lo que pone en riesgo la seguridad alimentaria de la población.

Los RFAA son importantes de manera integral por las siguientes razones:

- Son la materia prima para la seguridad alimentaria de la población.
- Incrementan la calidad, productividad y estabilidad de los cultivos y los sistemas de subsistencia, así como de los procesos derivados de transformación agroindustrial.
- Incrementan la diversidad genética resistente a plagas y enfermedades.
- Contribuyen al desarrollo de nuevos cultivos que respondan a las nuevas necesidades del mercado.
- Contribuyen al desarrollo de variedades adaptadas a condiciones ambientales adversas haciendo frente al cambio climático.
- (Franks, 1999, Maxted et al., 2003).

La unidad funcional del SINAREFI son las Redes, las cuales son grupos interinstitucionales e interdisciplinarios para la atención de cultivos cuyo centro de origen y/o diversidad es México. Están constituidas por productores, investigadores, académicos, mercadólogos, antropólogos, estudiantes, comerciantes entre otros. Actualmente se han constituido 46 Redes.

La vinculación se sustenta en una estructura horizontal de participación, y corresponsabilidad de cada uno de los integrantes, conformándose por actores que trabajan

sobre algún cultivo y una línea de investigación específica de acuerdo con un plan estratégico elaborado al inicio de sus operaciones enfocado a actividades de conservación y uso sustentable de los RFAA; cada una de las Redes cuentan con un coordinador propuesto en consenso por el grupo, quien se encarga de recabar y compilar las propuestas de actividades para integrar un proyecto único, dar seguimiento del mismo y reportar los avances y resultados al término de cada ejercicio fiscal a los Evaluadores Técnicos responsables en el SINAREFI. Los mismos integrantes en su papel de corresponsables, se encargan de buscar nuevos actores que complementen las actividades que en el momento no puedan realizar o que no se tenga la capacidad.

El SNICS a través del Sistema realiza la coordinación normativa, dictamen y seguimiento de los proyectos que llevan a cabo las Redes, cuyo modelo organizativo se encuentra constituido, además de las Redes, por el Secretariado y el Grupo Permanente de Trabajo (GPT), el cual está constituido por integrantes de las diversas Redes expertos en el sector agrícola que son los encargados de evaluar, definir criterios y prioridades del Sistema (Figura 1).

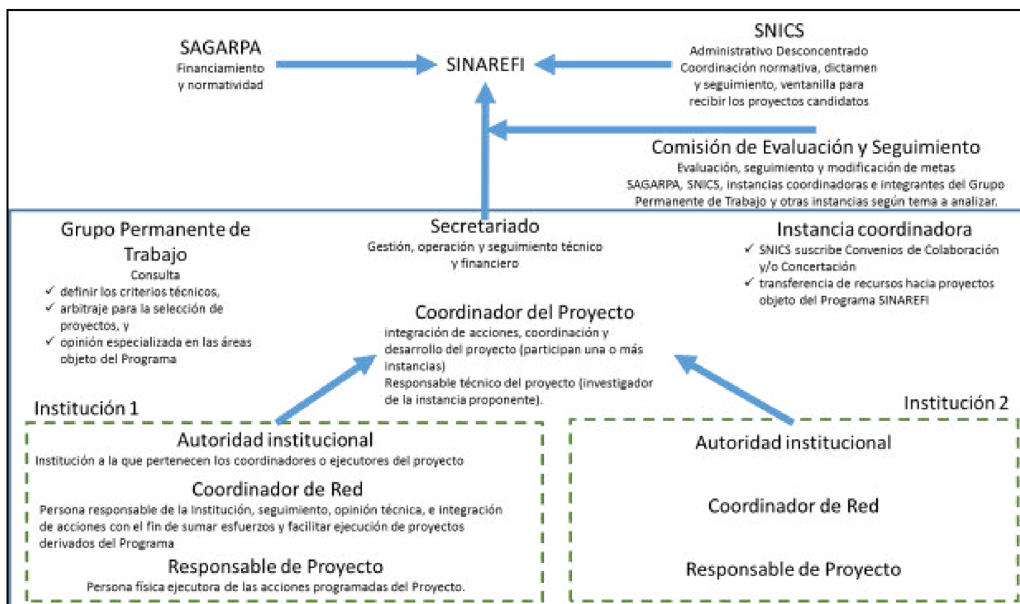

Figura 1. Modelo organizativo del Sistema Nacional de Recursos Fitogenéticos para la Alimentación y la Agricultura (SINAREFI).

RESULTADOS Y DISCUSIÓN

En la actualidad participan de forma directa e indirecta más de 60 instancias, 400 investigadores y más de 500 productores (Figura 2). Se atienden 45 cultivos nativos de México importantes para la alimentación y la agricultura y 1 Red Temática: Centros de Conservación (Cuadro 2) (www.sinarefi.org.mx).

Figura 2. Distribución de las instancias participantes en las Redes SINAREFI.

Tabla 2. Cultivos atendidos a través de Redes.

Básicos e Industriales	**Frutales**	**Hortalizas**	**Impulso**	**Ornamentales**
Agaváceas	Aguacate	Calabaza	Achiote	Cactáceas
Amaranto	Anonáceas	Camote	Romerito	Cempoaxóchitl
Algodón	Cacao	Chayote	Quelites	Dalia
Frijol	Ciruela	Chile	Verdolagas	Echeveria
Girasol	Guayaba	Jitomate	Yuca	Hymenocallis
Jatropha	Nanche	Papa		Nochebuena
Jojoba	Nogal pecanero	Tomate de cáscara		Orquídeas
Maíz	Nopal			Pata de elefante
Tabaco	Papaya			Tigridia
Vainilla	Pitaya y Pitahaya			
	Sapotáceas			
	Tejocote			
	Vid			

Cada Red generó un diagnóstico con el propósito de conocer el estado que guarda el cultivo que atiende, así como un Plan Estratégico donde se definen las acciones a adoptar en el corto, mediano y largo plazo para la conservación y uso sustentable del mismo.
Se promueven esquemas de mejoramiento participativo para la conservación in situ en 8 cultivos nativos de México (achiote, algodón, cacao, aguacate, guayaba, maíz, sapotáceas, tomate de cascara).
Conservación in situ de 52 razas nativas de maíz a través de “custodios1” mediante el programa “Incentivos a la Conservación” (Figura 3).

Figura 3. Distribución de custodios de las razas nativas de maíz, 52 razas conservadas in situ.

Creación de la Red de Centros de Conservación, integrada por cinco Centros de Conservación de Semillas Ortodoxas (CC-SO), tres Centros de Conservación de Semillas Recalcitrantes (CC-SR), 20 Colecciones de Trabajo, tres Colecciones in vitro y 26 Bancos Comunitarios (Figura 4). En conjunto resguardan más de 66,000 accesiones de germoplasma vegetal de los principales cultivos nativos de México y de otras especies importantes para la alimentación y agricultura a nivel nacional, las cuales se ha colectado por las Redes en los 31 Estados de la República Mexicana y el Distrito Federal (Figura 5).

Figura. 4. Distribución de los diferentes bancos de germoplasma que integran la Red de Centros de Conservación.

Figura 5. Distribución geográfica de las colectas realizadas.

Se creó el sistema de información "Germocalli", plataforma donde se encuentra la base de datos pasaporte de las accesiones colectadas a la fecha.

Se han implementado programas de regeneración de accesiones de las redes amaranto, frijol, sapotáceas, chile, tomate de cáscara, cactáceas, echeverias, frijol, papa y maíz.

Para evitar la biopiratería se tiene un acumulado de 215 variedades registradas en el Catálogo Nacional de Variedades Vegetales (CNVV) del SNICS de 21 cultivos, destacando 45 de nopal, 30 de cempoalxóchitl y 25 de xoconostle.

Generación de nuevos materiales vegetales que reúnan las características agronómicas que el productor requiere, se desarrollan programas de mejoramiento genético y del cual han derivado cerca de 30 nuevas variedades vegetales que se registran en la Gaceta Oficial de los Derechos de Obtentor de Variedades Vegetales del SNICS.

Identificación de valor agregado (nuevas fuentes de energía, nutracéuticos, usos), en los cultivos de jatropha, cacao, camote, achiote, quelites, verdolaga, romerito, jojoba, chayote entre otras.

Para la conservación y el aprovechamiento sostenible de la vida silvestre se promueve la reconversión de viveros a Unidades de Manejo Ambiental (UMAs), como una herramienta para la conservación y comercialización de especies que se encuentran dentro de la NOM-059-SEMARNAT-2010. A la fecha se ha colaborado con el registro de 17 UMAs siendo el Estado de Veracruz con mayor número de viveros reconvertidos.

Se han generado cerca de 70 publicaciones de carácter técnico, científico y de divulgación para el público en general con distintos tópicos sobre el aprovechamiento sostenible de los Recursos Fitogenéticos. (http://snics.mx/sinarefi/biblioteca/publicaciones.html)

Se promueven eventos de difusión tales como:

- Muestras gastronómicas.
- Talleres de actividades múltiples para niños y adultos: como manualidades, diseño de arreglos florales, identificación de especies nativas, elaboración de artesanías, entre otras.
- Exposiciones fotográficas.
- Congresos y simposios para los investigadores, técnicos, productores y público en general.
- Organización de dos feria de la Agrodiversidad y Agroproductos donde han participado más de 20,000 personas. La finalidad es dar a conocer la riqueza agrobiologica con la que cuenta nuestro país, así como sensibilizar a la opinión pública de la importancia de los Recursos Fitogenéticos. (http://www.youtube.com/watch?v=BFFclMwqwVM).

CONCLUSIÓN

La vinculación a nivel institucional con múltiples universidades y centros de investigaciones del país ha servido como plataforma para el intercambio de ideas y fortalecimiento de los recursos humanos y materiales a favor de la conservación y uso sustentable de los Recursos Fitogenéticos importantes para la Alimentación y la Agricultura.
El SINAREFI atiende 44 cultivos prioritarios para la Alimentación y la Agricultura, de los cuales México es centro de origen y/o diversificación. Se conoce el diagnóstico (estatus) que guarda cada uno de ellos en el país y se trabaja en la conservación y uso sustentable a través de un Plan Estratégico específico para cada grupo de trabajo.
La generación y promoción de las Redes contribuye a facilitar las interacciones entre los entornos científicos, tecnológicos, industriales, financieros, de mercado y agronómico, generando resultados importantes en materia de innovación y transferencia de tecnología que son aprovechados y adoptados por los más de 500 productores colaboradores del Sistema.
Las actividades de colecta han permitido identificar y resguardar 66,000 accesiones que corresponden a 698 especies de 125 géneros de los 45 cultivos atendidos por el Sistema.
La Red Centros de Conservación ha permitido fortalecer la estrategia e infraestructura para la conservación al corto, mediano y largo plazo de la diversidad fitogenética de México (resultado de las actividades de colectas) garantizando con ello el resguardo y la regulación del flujo de material a nivel nacional e internacional.
El contar con el sistema de información “Germocalli” permite el acceso rápido y seguro a las bases de datos generados.
El programa de “Incentivos a la Conservación” ha permitido mantener en las parcelas de los agricultores la diversidad genética de 52 razas nativas de maíz; estos materiales han sido y seguirán siendo fuente de material base para la generación de nuevas variedades con mejores características agronómicas (resistencia a plagas y enfermedades, mayor producción, resistente a sequía, entro otros).
Con el registro de materiales ante el Catálogo Nacional de Variedades Vegetales (CNVV) y la Gaceta Oficial de los Derechos de Obtentor de Variedades Vegetales, se contribuye a evitar la biopiratería de los Recursos Fitogenéticos en México.

El registro de Unidades de Manejo Ambiental (UMA´s) permite a los productores tener acceso a materiales que se encuentran dentro de la Norma Oficial Mexicana NOM-059-SEMARNAT-2010 en alguna categoría de riesgo o amenaza, lo que permite conservar, utilizar de forma sustentable y comercializar estas especies.

Se han publicado más de 70 publicaciones como resultado del trabajo de las Redes que se encuentran disponibles para el público en general, contribuyendo al acervo científico de nuestro país en materia de Recursos Fitogenéticos.

Mediante la implementación de diversos eventos de divulgación se ha logrado la sensibilización de la opinión pública sobre la importancia de la Conservación y Uso Sustentable de los Recursos Fitogenéticos.

El desarrollo de actividades a través del modelo organizativo del SINAREFI (Redes por cultivo) ha permitido potenciar los recursos asignados, reforzando las estructuras institucionales, favoreciendo el intercambio y evitando la duplicidad; impactando de forma directa en la seguridad y soberanía alimentaria del país.

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Effect of night temperature on titratable acidity and gas exchange of *Agave cupreata*

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ABSTRACT

We studied the effect of night temperature on the night-day cycle of titratable acidity (TA) and on gas exchange during the following day in *Agave cupreata*. Two experiments were set up with *Agave cupreata* plants kept at controlled temperatures (between 19 and 32.5 ºC) during the night and at ambient temperature during the day. In the first experiment, TA was determined every 3 hours during 24 hours; in the second experiment gas exchange was measured between 9:30 and 17:30.

TA accumulation during the night decreased with temperature, from 350 meq Kg^{-1} at 19.5 ºC to less than 100 meq Kg^{-1} at 32.5 ºC. The rate of TA degradation during the day appeared to be almost proportional to the amount of TA accumulated during the night, so that in all treatments TA reached it minimum value around 16:30. Concerning gas exchanges, transpiration varied between 1 and 2 mmol m^{-2} s^{-1} with wide variations but no significant differences between treatments. In all treatments, net CO_2 assimilation was negative in the morning and positive in the afternoon. At 19.5 ºC, CO_2 assimilation was around -12 µmol m^{-2} s^{-1} in the morning (between 10:45 and 12:30), but became positive and increased from 1 to 2.5 µmol m^{-2} s^{-1} in the afternoon (between 15:30 and 17:30). At 26 and 32.5 ºC, CO_2 assimilation was around -5 µmol m^{-2} s^{-1} in the morning and increased from 2 to 5 µmol m^{-2} s^{-1} in the afternoon.

Key words: CAM metabolism, temperature, *Agave cupreata*, titratable acidity, gas exchange.

INTRODUCTION

Agave cupreata (Trel. et Berger), also known as “Maguey chino” in the state of Michoacán, is an endemic species of the states of Michoacán and Guerrero, México, that grows between 1200 and 1800 m altitude on the slopes of the mountains around the Balsas river. It can be found in diverse environments, from rocky slopes to the understory of oak and pine forests. The climate varies between warm and temperate, depending on altitude. Precipitation is around 1000 mm/year, with a dry season between February and May.

Agave cupreata has been used for centuries for the production of Mezcal, but over-exploitation has led to the decline of wild populations. During the past 10 years, many

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producers have started cultivating it, either by replanting in its natural habitat or as a monoculture in cultivated land. However, many of these new plantations produce small plants with low sugar content. Among other factors, we think that this may be due to a limitation of photosynthesis in micro-climates with excessive night-time temperature.

To our knowledge, the physiology of *Agave cupreata* has never been studied. In this work, we describe the effects of night temperature on the dynamic of titratable acidity (TA) accumulation and degradation, taken as a measurement of CO_2 fixation and decarboxylation respectively, and on gas exchanges during the following day.

METHODOLOGY

This work consisted of two experiments, where *Agave cupreata* plants were kept overnight (from 19:00 to 7:00) at different temperatures, then at ambient temperature during the following day. We used 72 individuals, grown for two years in 2L polystyrene pots containing a perlite-peat substrate (without fertilization and rainfed) until they had reached about 25 cm diameter. Two months before each experiment, the plants were fertilized with 1 g/plant complete fertilizer (15-12-12 with micro-nutrients) and irrigated twice weekly.

In order to control temperature during the night we built 6 chambers, that consisted of a cubic iron frame (1.25 m side for 2 m^3 volume) covered with plastic wrap, a heating resistance (700 W) laid in zig-zag on a 1 m^2 frame at 80 cm height, and a bulb thermostat. The thermostats were installed at the height of the leaves. However, these thermostats appeared to present random bias, so that the temperature obtained was different from that programmed. The actual temperature was measured using an IR thermometer, twice every three hours.

The first experiment started on 13 October 2013. The average night temperatures obtained were: 19.6, 23.4, 25.9, 27.7, 29.0 and 32.5 ºC. Leaf samples (3 leaves taken at random in each chamber) were cut every 3 hours during 24 hours, starting at 19:00, and kept for later analysis of titratable acidity. Titration was done as follows: an approximately 3 g piece of tissue was cut from the middle section of a frozen leaf, weighed and diluted with an equal mass of distilled water. Acidity was titrated by adding 0.05 mM NaOH until pH=7.

The second experiment started on 25 February 2014. The average night temperatures obtained were 19.5, 26 and 29.5 ºC. Although leaf samples had been collected to follow TA they were lost due to a freezer failure. Gas exchange measurements were taken at 10:00, 12:30, 15:00, 16:45 and 17:30 using a LI-COR 6400 IRGA. Leaves were cut from the plant immediately before these measurements (preliminary tests have shown that gas exchanges are not affected for at least for a few minutes). The LI-COR 6400 was equipped with the fluorometer chamber, which has its own light source and measures a 2 cm^2 leaf area. Before each series of measurements, we set the light source to reproduce the ambient light intensity (between 1200 and 1800 µmol m^{-2} s^{-1} photons flux density for all measurements). We used the calculation routines provided by the instrument to calculate assimilation (CO_2 flux to the leaf) and transpiration (H_2O flux out of the leaf).

Analysis of variance was used to detect significant difference between treatments in TA measured at 7:00. The rate of TA degradation was calculated by linear regression between 10:00 and 16:00. Concerning gas exchanges, a separate analysis of variance was performed for each measurement time to detect statistically significant differences (5% confidence level).

RESULTS AND DISCUSSION

The night-day fluctuation of TA presented the classical pattern of CAM plants (Fig. 1.A): TA increased during the night, beginning at the end of the afternoon, and decreased during the day until the middle of the afternoon. Maximum TA was reached at the end of the night at cool night temperatures (19.6, 23.4, and 25.9 ºC), but earlier (between 22:00 and 4:00) at higher temperatures (27.7, 29 and 32.5 ºC). Maximum TA decreased regularly with night temperature (Fig. 1.B), as found on most CAM plants (Nobel 1988). Interestingly, TA degradation rate during the following day was approximately proportional to the TA reached at the end of the night (Fig. 2). In other words, TA degradation seemed to adapt so as to exhaust the pool of stored CO_2 around 16:30, independently of night-time temperature.

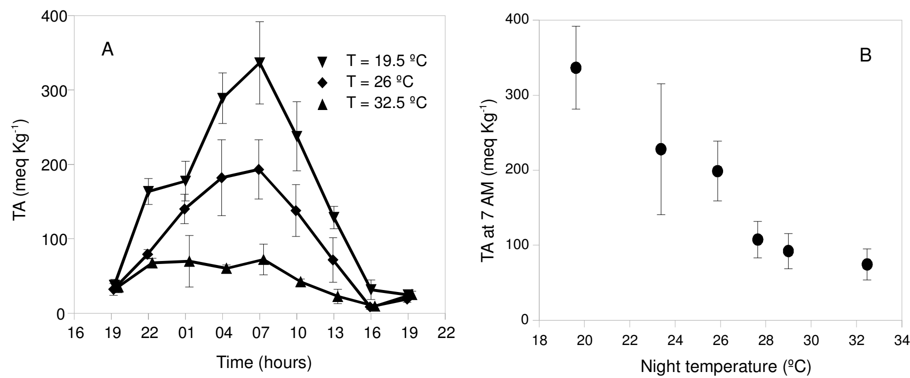

Figure 1. A: Fluctuations of titratable acidity (TA) during the night-day cycle at 3 different night temperatures; B: Relationship between average night temperature and titratable acidity at 7:00.

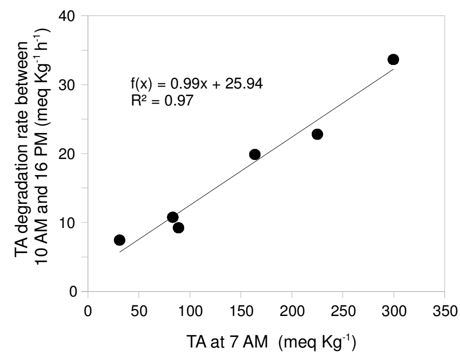

Figure 2. Relationship between titratable acidity accumulated at 7:00 and rate of degradation during the following day.

Transpiration did not show the typical CAM pattern of stomatal closure during the day and reopening at the end of the afternoon (Fig. 3.B). Instead, it was highly variable, with average values between 1 and 2 mmol m^{-2} s^{-1}. Although low, these values amounted to 25% of those we measured, for example, on strawberry (a typical C_3 plant) under the same conditions. In all treatments, net CO_2 assimilation was negative in the morning (between 10:45 and 12:30) and positive in the afternoon (between 15:30 and 17:30). At 19.5 ºC, CO_2 assimilation was around -12 µmol m^{-2} s^{-1} in the morning, but became positive and increased

from 1 to 2.5 µmol m^{-2} s^{-1} in the afternoon. At 26 and 32.5 ºC, CO_2 assimilation was around -3 µmol m^{-2} s^{-1} in the morning and increased from 2 to 5 µmol m^{-2} s^{-1} in the afternoon.

Given the equivalence between one mole CO_2 and 1 equivalent TA, and taking into account the measured specific leaf mass (0.275 g cm^{-2}), these rates can be converted to meq Kg^{-1} h^{-1} to compare them with TA dynamics. In the morning, the -12 and -3 µmol m^{-2} s^{-1} assimilation rate at 19.5 ºC and 26 to 29.5 ºC translates into losses of 15 and 3.75 meq Kg^{-1} h^{-1} respectively. These values can be compared to the 34 and 9 meq Kg^{-1} h^{-1} TA degradation rate at the corresponding temperatures: in both cases, between 40 and 44% of decarboxylated TA was lost through the stomas. A similar comparison between CO_2 assimilation in the afternoon (estimated between 14:00 and 18:00) and total TA variation shows that only 2 to 3% of CO2 was gained through the stomas after a 19.5 ºC night, but up to 25% after a night at 26 to 29.5 ºC.

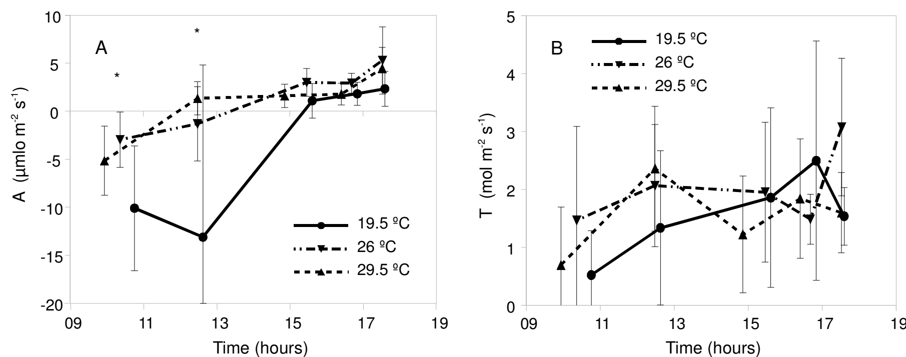

Figure 3. Evolution of assimilation rate (A) and transpiration during the day following a night at 19.5, 16 or 29.5 ºC. Times when statistically significant differences (5% confidence level) were found between treatments are indicated by (*) symbols.

CONCLUSION

Agave Cupreata presents the classical CAM features of night-day cycle of titratable acidity. However, incomplete stomatal closure during the day causes CO_2 loss through the stomas in the morning, only partially compensated by CO_2 uptake in the afternoon.

ACKNOWLEDGEMENTS

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San Lucas, a prehispanic workshop of maguey processing

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ABSTRACT

Archaeological, pedological and geomorphological field surveys combined with the analyses of digital airphotos, and geophysical methods, lead us to identify subtle differences in soils that indicated buried archaeological features in several cases.

The application of these methods were invaluable in order to obtain: 1) specific data concerning the extension, relative depth, and form of these archaeological features; 2) an accurate and precise site selection for archaeological excavation 3) information on the ecological context during the Late Postclassic (AD 1430-1521); 4) three-dimensional information about soil terrace properties and 5) potential past soil use and land capability classes.

The cumulative results of this research revealed the presence of a Late Postclassic Aztec household unit dated to approximately 1490 B.), comprised of three rooms. The abundance of ceramic materials indicated the domestic character of the structure, in spite of the predominance of very shallow, rocky soils characterized by their severe limitations making them generally unsuitable for cultivation of annual crops. However, the frequent presence of *malacates* (spindle whorls) and fiber scrapers recovered from the excavation and surrounding areas led us to infer long-term economic activities related with a workshop to *maguey* (*agave)* processing, including scraping the *pencas* (leaves) and spinning the fiber obtained.

Keywords: maguey, spindle whorls, fiber scrapes, agricultural terraces, Teotihuacan Valley.

INTRODUCTION

Domestic agricultural systems, represented by the construction of terraces on the slopes of the volcanic complex, formed the basis of one of the most important means of economic specialization as well as the most extensive form of intensive agriculture in piedmont areas in the prehispanic Basin of Mexico.

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The objective of this research is the characterization of soils and agricultural residential areas in a system of terraces located on cerro San Lucas, where a rural Late Postclassic village was established between 1350 and 1520 A.D (Evans, 1988).
The methodological proposal incorporated the systematic application of a sequence of prospection techniques to locate potential areas of study (Barba, 1990). The use of the gradiometer, ground penetrating radar and electrical resistivity equipment provided concrete data on the extent, depth, the shape and size of the observed anomalies. With the obtained data, extensive excavation was undertaken in a 14 x 20 m area, exposing a residential structure with dimensions of 13 x 7 m, consisting of three rooms identified as a food preparation area, storage area and a living area.
The ceramic material from the surface included large amounts of fragments of bowls, pots, jars and griddles, clearly indicating the domestic character of the unit. On the other hand, the presence of spindle whorls and fiber scrapers in relation to the chemical distribution patterns suggested intensive economic activities derived from agave planting, such as those in prehispanic workshops devoted to scraping the leaves and spinning fibers.
The results of this investigation provide a broader vision of prehispanic exploitation of natural resources and the development of agroecosystems in a semi-arid region of the Basin of Mexico.

METHODOLOGICAL DESIGN

Study area

Cerro San Lucas is located at 65 km northeast of Mexico City, in the Eastern sector of the Valley of Teotihuacan, Mexico (Figure 1). Represents a Late Quaternary horseshoe volcano and lithological terms, it is constituted by buried acidic lavas interdigited with deposits of pyroclastic. The climate of the area is predominantly semiarid (Bs) with annual precipitation lesser than 500 mm and soils characterized by xerophytic vegetation.

Digital imagery

Two flights over the study area were carried on, the first with aerial photography aircraft (3,000 meters above sea level) and the second with an aerostatic balloon (100 m above ground). During the balloon flights vertical and oblique images were obtained using several high-resolution digital cameras: Nikon D2x, D70, Kodak DCS 14n and Canon EOS 300D.

Geomorphological survey

Three maps with different levels of information morphogenetic, morphodynamic and potential archaeological sites maps of Cerro San Lucas, were generated. On stereo-pairs, acetates were placed in order to mark, by photointerpretation, the geomorphological units. In another acetates were marked the processes of water erosion on hillslope of type sheet, rills and gullies, surface and subsurface ones; and gravitational movements of type falls rocks, debris and soil. As well as cumulative hydric processes, such as not differentiated and gravitational alluvial deposits. Finally in a third one were marked traits associated with underlying prehispanic structures in the hillslope area.

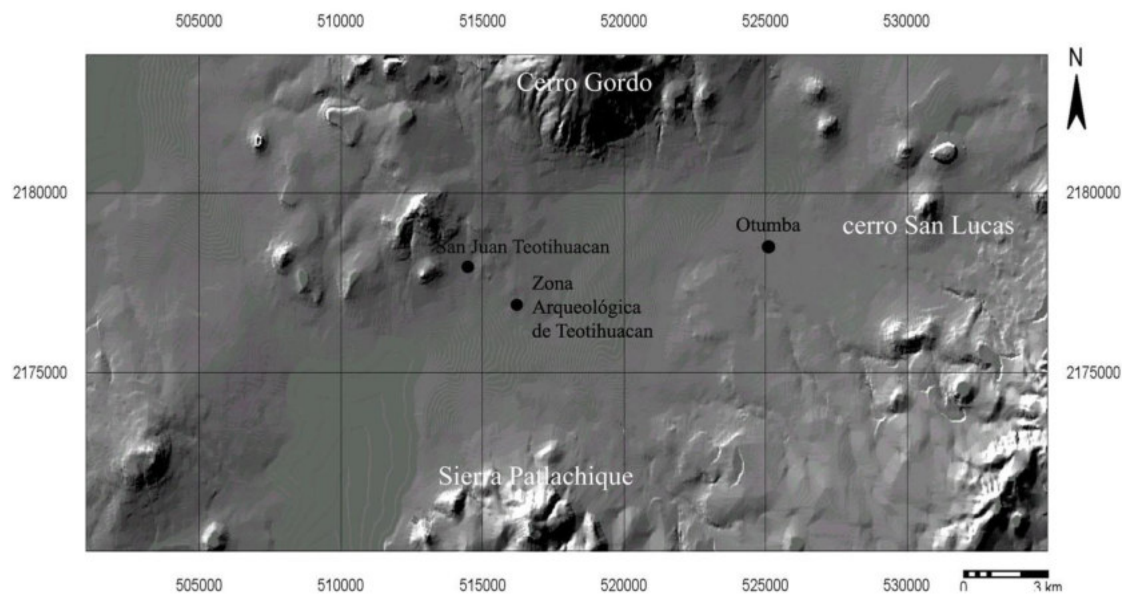

Figure 1. Localization of the study area.

Automatic image analysis

Automatic analysis of digital images in the visible range of the spectrum was undertaken by means of Image-Pro Plus 4.1. This software allowed us to distinguish the spectral properties of a series of geometric features distributed over the terraces that currently cover the volcanic cone. The variables considered for the analysis include shape, size, tone, color and texture.

Geophysical techniques

Magnetic gradient. The data were taken with the equipment Geoscan FM36 fluxgate to gradiometer, and the land was sampled at intervals of 0, 25 m in transects with direction NE 26° and a distance among them of 1 m, having obtained a total of 6400 readings of magnetic gradient.

Ground penetrating radar. The GSSI ground penetrating radar (gpr), model SIR-2 was used like a complementary method to the magnetic gradient; 42 lines of verification were carried out in the central grids, acquiring 840 m. of total continuous profiles.

Electrical resistivity. In order to have a correlation adapted between the data of magnetic gradient and gpr, the electrical method was employed in the central grids where the profiles with gpr were risen. The data were collected with an M.A.E equipment of 32 intelligent electrodes, which respectively rose 20 electrical lines in a dipole-dipole arrangement in sampled intervals of 0,5 m and 1 m. The total electrical data obtained was of 12800 in punctual mode.

Chemical prospection. A soil sample was taken each 1 m. through the complete grid. Studies about phosphates, carbonates, pH, protein residues, fatty acids and carbohydrates, were made in the chemistry laboratory.

RESULTS AND DISCUSSION

The data of magnetic gradient revealed the presence of anomalies in the two central grids. A grid was characterized by magnetization by induction and the other anomaly, seems to be produced by remnant magnetization. Verifications using georadar were made to obtain data of the dimensions and depth of the magnetic pattern. Radargrams show superficial reflections of medium to great amplitude, between 0, 2 and 0, 3 m. of depth produced by the stone walls. In depths of 1,2 m. a slight change in the dielectric characteristics of *tepetate* was registered produced by a humidity change and major homogeneity in its composition, this is produced by the diminution in the amplitude of the reflections. The chemical studies of soil that covered the excavated site show patterns that are a consequence of the anthropic use of the space, fatty acids, carbohydrates, carbonates, phosphates, protein residues and pH.

Excavation of the anomaly

An area of 14 x 20m was selected for excavation. A rectangular domestic structure (Figure 2) of approximately 13 x 7m was uncovered comprised of three rooms: a) food preparation area; b) storage area; and c) living area.

The ceramic materials recovered from both the surface and the excavation included four predominant forms: cajetes (bowls), ollas (pots), jarras (jars) and comales (griddles) indicating the domestic character of the structure. The presence of malacates (spindle whorls, and scrapers led us to infer economic activities related to maguey (*Agave* sp) processing, including scraping the pencas (leaves) and spinning the fiber obtained (McClung and col. 2008).

Figure 2. Area of excavation and domestic structure.

CONCLUSIONS

In this investigation the search for spaces destined for prehispanic agricultural and domestic activities in Cerro San Lucas benefited immensely from the application of different types of remote sensing. Distinctive characteristics of the soil and the environmental context of the habitation unit support the inference that this was a marginal area for agricultural production in prehispanic times.
The population which settled the area of Cerro San Lucas was undoubtedly dependent in large part on the production of nopal (*Opuntia* sp.) and maguey (*Agave* sp.) for subsistence and other economic activities

ACKNOWLEDGEMENTS

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Analysis of differentially expressed genes during the transition from the vegetative to reproductive stage in A*gave tequilana*

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Agave tequilana is a very important crop for the economy in Mexico, since it is the only agave species from which we can obtain tequila. Once agave plants pass from the vegetative to reproductive state, an inflorescence meristem develops indicating the optimum state for harvesting. The developing inflorescence depletes stored carbohydrates and in turn decreasing the efficiency of production of tequila and for this reason the inflorescence is removed manually. Since most agave species including *A. tequilana* are perennial, monocarpic plants, it is unknown as to how the bolting process is triggered or what changes occur at the genetic and molecular level during the transition from the vegetative to reproductive stage.

In order to study this process at the genetic level, analysis based on deep RNA sequencing (RNA-seq) was used to analyze genes that are expressed differentially during 4 phases of the transition from vegetative to reproductive phase. The phases are defined as: vegetative adult (Mv), initiation of bolting (Mch), inflorescence 10cm (In10cm) and inflorescence 30cm (In30cm).

Adjacent stages "Mv *vs* Mch", "Mch *vs* In10cm" and "In10cm *vs* In30cm" were compared in terms of differential expression patterns. 2686 differentially expressed transcripts were found during the transition, where the genes reported as inducers of flowering such as FT and SOC1, showed low levels of expression in the vegetative and 10cm stages and high levels in initiation of bolting and 30cm stages. Some genes reported as repressors of flowering showed inverse patterns of expression as expected. We found other genes that have similar expression patterns to *SOC1* and *FT* that might be acting as inducers or facilitating the transition to the reproductive stage, some are related to carbohydrate metabolism or flow of auxin whereas others were not annotated.

This work gives us an idea of what happens at the genetic and molecular level in the transition from vegetative to reproductive stage in *Agave tequilana*, laying the groundwork for future research to predict and control the floral transition and to determine optimal harvesting for increased production of tequila.

Key words: *A. tequilana*, bolting, transcriptome, expression analysis, RNAseq.

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INTRODUCTION

Agave tequilana is a semi arid, monocarpic plant species which produces a single inflorescence at the end of its life cycle then dies. *A. tequilana* plants take 6 to 12 years to mature depending on the environment, soil and agronomic treatments. *A. tequilana* Weber, var. azul is a domesticated crop, exploited commercially for the production of tequila. Initiation of bolting signals that plants are ready to harvest and this process must be controlled in order to reduce loss of stored carbohydrates. Although of economical and cultural importance, there is not much information or research at the molecular and genetic level on the process of bolting in *A. tequilana*. Due to its long life cycles and undesirable loss of stored carbohydrates by the developing inflorescence, we do not know how this process is triggered and what is happening at the genetic level inside the plant. Recently, Delgado Sandoval *et al.* (2012) described morphologically the stage close to the transition between the vegetative and reproductive stages. In the vegetative stage, the leaves of *A tequilana* grow as a triangle where the center leaves seem to grow faster than the others forming a parabolic shape, when the plant is ready to switch to the reproductive stage the leaves in the center grow more slowly than the others, producing what seems to be a "sinking" morphology at the center of the plant as shown in Figure 1.

The improved technology and increasingly low cost of transcriptome analysis based on deep RNA sequencing has become a very powerful and reliable tool to perform the identification and quantification of almost all transcripts present in a cell or tissue, either in a specific developmental stage or physiological condition. These advances allowed us to perform a transcriptomic analysis based on RNA-seq technology in order to have a better understanding about what is happening at the genetic and physiological level during the transition from the vegetative to reproductive stage in *A. tequilana*.

Our aim is to generate and characterize transcriptome data, based on RNA-seq at four distinct stages during the transition from the vegetative to the reproductive stage of *Agave tequilana.* Here we present preliminary data of this transcriptome analysis.

METHODOLOGY

Plant Material and sampling

We took 12 meristems from *Agave tequilana* plants located in "Real de Penjamo", Penjamo Guanajuato, Mexico. The plants correspond to 4 different stages of the transition: vegetative adult stage, initiation of bolting, inflorescence of 10cm and inflorescence of 30cm (Fig 1), with 3 biological replicates per stage.

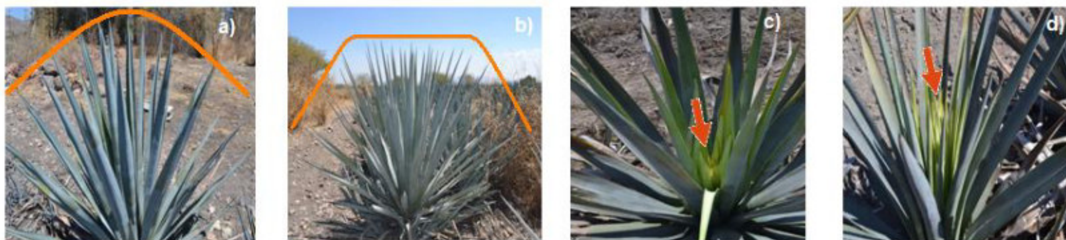

Figure 1. a) vegetative meristem. b) initiation of bolting. c) inflorescence of 10 cm. d) inflorescence of 30 cm. (adapted from Delgado Sandoval et al. 2012)

Plants where dissected to obtain just the apical meristem as shown in Figure 2 which was then frozen in liquid nitrogen.

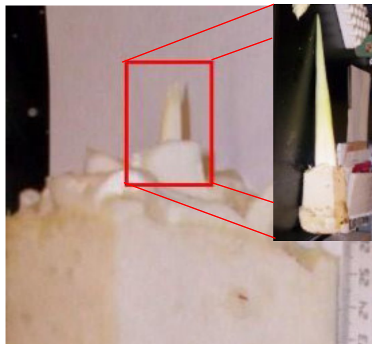

Figure 1. ***Agave tequilana*** **apical meristem.**

RNA extraction

RNA was obtained from the shoot apical meristem at 4 stages during the transition from the vegetative to reproductive stage as described by Delgado Sandoval *et al.* (2012).

Sequencing

The total RNA extracted was sent to the Advanced Genomics Unit (UGA) at CINVESTAV Irapuato to be sequenced using the Illumina MiSeq 2X250 platform.

Assembling and quality control

A global *De novo* assembling was performed as described in the Trinity protocol (Hass B J *et al.* 2013) and quality control was performed with Trimmomatic (Bolger *et al.* 2014).

Mapping and quantification

Each read per library was mapping to the transcriptome assembled as a reference, the aligned and quantification was performed RSEM (Li *et al.* 2011).

Differential expression and annotation

Differential expression analysis was performed with R thru the library edger (Reinhardt *et al.* 2010) taking as a cut of a false discovery rate (FDR) from 0.05. The annotation step was performed with Blast2go with an e-value from 0.1 e-5 against Refseq for plants.

RESULTS AND DISCUSSION

The *de novo* assembled transcriptome data reflected the high quality of the sequencing data obtained. 221,686 isoforms were reconstructed representing 0.250Gb assembled with a N50 statistic of 1669 bases, minimum length of 201 bases and a maximum of 15,804 bases. 31042 hits were obtained from different proteins in Refseq for plants which is around 14% of the reconstituted reads.

Differential expression was carried with the isoforms. In Table 1, we show how many transcripts are up and down regulated during the transition from the vegetative stage to the reproductive stage. As expected, we found some genes that are flowering inducers such as *FT* and *SOC1* that have low counts in the vegetative stage and when bolting initiates increase. We also identified some novel genes in agave that have interesting expression patterns during bolting (Figure 3) and seem closely linked to the transition stage. Some are related to carbohydrate metabolism, auxin efflux, transcription factors and also include non-annotated sequences, whereas others are down regulated and potentially act as repressors.

Table 1. Number of differentially expressed genes.

Comparation	Up-regulated	Down-regulated	Total
Mv vs Mch	738	830	1568
Mch vs In10cm	988	531	1519
In10cm vs In30cm	487	500	987

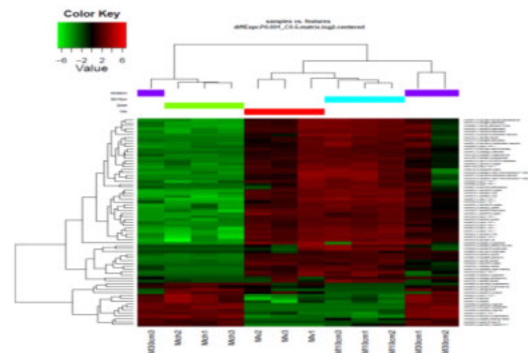

Figure 3. Heatmap of differential expression.

CONCLUSION

Our analysis provides detailed data on the changes in gene expression during bolting in *A. tequilana* and reveals genes involved in specific metabolic pathways important for the vegetative-reproductive transition. This will allow us to understand, predict and eventually control the floral transition in *A. tequilana*.

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Analysis of the "dark" side of the vegetative-reproductive transition in *Agave tequilana.*

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ABSTRACT

Agave tequilana is a monocarpic, perennial species, whose reproductive event takes place after six to seven years of vegetative development. During this time complex polysaccharides, called fructans are synthetized and accumulated in the "piña" or stem. The emergence of the inflorescence marks the maturity of *Agave tequilana* plants and denotes the optimum stage for harvesting for tequila production. Although the regulation of the transition to reproductive development is well understood in annual plants such as *Arabidopsis thaliana*, little is known about the factors involved in this transition in perennial species such as *Agave tequilana.* To address this problem, we studied at the morphological level and *in silico* changes associated with the reproductive transition. We have found in *A. tequilana* several orthologous genes involved in reproductive transition and we are beginning to analyze differential expression patterns.

Keywords: Agave, transcriptome, inflorescence, development, miRNAs

INTRODUCTION

Mexico is considered the center of origin of the *Agave* genus, because most species are found within its territory and a high percentage of endemism also exists (García-Mendoza, 2007). Within the *Agave* genus *Agave tequilana* is the most important species due to the generation of employment and the economic flow that generates its agroindustrial exploitation for tequila production. *A. tequilana* is a monocarpic and perennial species, whose reproductive event takes place after six to seven years of vegetative development. During this time complex polysaccharides, called fructans, are synthesized and stored in a structure called "piña", composed of stem and basal leaf tissue (Cortes-Romero et al., 2012). However, it has been shown that fructans of up to 9 degrees of polymerization may be synthesized in vascular tissues of leaves and then transported within the phloem to the "piña", where they could be converted into fructans with higher levels of polymerization (Praznik et al., 2013).

The emergence of the inflorescence marks the maturity of *A. tequilana* plants and denotes the optimum stage for harvesting for tequila production, at which time the plant has come to store the maximum concentration of sugars. A crucial step in the production of tequila is the removal of the inflorescence, as this will consume the sugars that were synthesized during

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vegetative development of the plant, so they cannot be utilized for the production of tequila. Since the emergence of the inflorescence in *A. tequilana* plantations is not homogeneous, the process of removing it, also known as "desquiotado", is a manual process that requires constant monitoring.

In *Arabidopsis thaliana* approximately 180 genes have been implicated in flowering-time control, these genes have been grouped into six pathways of perception of endogenous and exogenous signals: Vernalization, Autonomous, Photoperiod, Gibberellin, Ambient temperature and Age dependent (Fornara et al., 2010). The evidence suggests an interaction between the components within these pathways, as well as all paths converge to a set of genes, known as floral integrators, which inhibit or promote the transition to reproduction (Chuan et al., 2013).

Although the regulation of the transition to reproductive development is well understood in annual plants such as *Arabidopsis thaliana*, little is known about the factors involved in this transition in perennial species such as *Agave tequilana*. To address this problem, we will study at the molecular, morphological and physiological level the changes associated with the reproductive transition in leaves and shoot apical meristem tissues of *Agave tequilana* plants before and during the development of the inflorescence meristem.

METHODOLOGY

Morphological registration and collection of biological material

We measured the height, diameter and number of leaves of two, four and six years old *A. tequilana* plants in commercial plantations, where the age of the plants corresponds to years in the field. Additionally, we collected samples of leaf and shoot apical meristem of three different plants of two, four and six years of age for the molecular analysis.

Bioinformatic analysis of *A. tequilana* transcriptome

We carried out BLAST (Altschul et al., 1997) searches in the transcriptome of *A. tequilana* to find orthologs of genes reported as involved in reproductive development in other plants (Ávila-de Dios et al., in preparation). BLAST searches were done using as query those sequences reported in *Oryza sativa* (rice) and *A. thaliana* with an e-value of 10^{-5}. To identify miRNAs, we identified both immature and mature sequences using as a query sequences those reported in miRBase database (Griffiths, 2010) and Rfam (Burge et al., 2013).

RESULTS AND DISCUSSION

In search of a correlation between the size of *A. tequilana* plants and evidence for the initial stages of the vegetative to reproductive transition we measured the height, diameter and number of leaves of sixty plants of two, four and six years of age in a commercial field in Jalisco state. As shown in Table 1, there is a wide variation in the morphological variables analyzed in plants of the same age, this can be due either to the differences in the size of planted offsets or biological variation (Table 1).

Age of agave plants	Number of leaves	Height in centimeters	Diameter in centimeters
2	38 ± 9	87 ± 11.5	111 ± 16.7
4	45 ± 10	131 ± 16.6	179 ± 18.9
6	63 ± 23	149 ± 15.7	206 ± 26.3

Table1. Morphological measurements of 60 random *Agave tequilana* plants in a field.

Furthermore, through bioinformatic methods we were able to identify both the mature and immature sequences of miRNA 156 and miRNA 172, as well as at least eight genes that may be regulated by them in *Agave tequilana*. Both miRNA genes have been reported as key regulators of flowering induction by the aging pathway and in *A. tequilana* their *in silico* expression pattern is similar to that reported in other plants, that is to say the expression of miR156 decreases with the age of plant whereas miR172 expression is increased with the age of the plant (Fornara et al., 2010). Additionally we have found two orthologs for Flowering Locus T of *Arabidopsis thaliana*, similar to reports in *O. sativa.*

CONCLUSIONS

Using bioinformatic methods together with transcriptomic data from *A. tequilana,* we have identified several orthologs of genes involved in reproductive transition. However, we need to validate their expression pattern as well as their possible regulation by miRNAs using molecular techniques such as 5´RACE. Additionally, based on morphological results, it is necessary to analyze a wider age range during the development of *Agave tequilana* plants.

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EFFECTS ON SAPONIN, FLAVONOL AND ANTIOXIDANT ACTIVITY IN *in vitro* PLANTS OF *Agave salmiana*

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ABSTRACT

Maguey, *Agave salmiana*, is an important plant for "pulque" industry and functional foods but has several constraints of elite germplasm availability and homogeneous nutraceutical properties. A micropropagation protocol was established to generate *in vitro* plants from young germinated plantlets by axillary shoots. At the same time we evaluated the impact of this process in the phytochemical profile of the new plants.
The optimal induction of axillary shoots was observed in plantlets incubated on a solidified Murashige and Skoog (MS) medium supplemented with L2 vitamins and 0.04 mg l^{-1} 2,4-Dichlrophenoxyacetic acid (2, 4 –D) and 10 mg l^{-1} 6-benzylaminopurine (BAP). The *in vitro* protocol took 16 weeks, obtaining an efficiency of 87.5% after acclimatization under controlled conditions. The total phenolic (TP) content, antioxidant (AOX) capacity and identification of phenolic compounds and saponins were performed in wild type (WT) plants, *in vitro* (IN), *ex vitro* with irrigation (EXW) and *ex vitro* at normal environmental conditions (EXN).
The highest TP content were in IN and EXN plants, however AOX capacity for IN plants was 3-fold higher than EXN and EXW plants, and more than 3-fold higher compared to WT plants. Two different glycosylated flavonols were detected in EXW (quercetine) and EXN (kaempferol). Saponins such as hecogenin (0.418-4.321 mgEHe g^{-1}), tigogenin (18.821-31 mgEHe g^{-1}), manogenin (0.288-0.861 mgEHe g^{-1}) and chlorogenin (0.339-2.042 mgEHe g^{-1}), in different glycoside form were detected and quantified. Tigogenin was only found in the plants that pass through *in vitro* process, being more concentrated in IN plants.
In summary, we successfully microprogated and regenerated *A. salmiana* plants from seeds and they contained different amount of their TP, flavonoids and saponins and AOX capacity compared with WT.

Keywords: *axillary shoots, micropropagation, tigogenin, hecogenin, kaempferol*

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INTRODUCTION

The agaves are succulent plants native from Mexico, southwest region of U.S.A., Central America and Canary Islands. Around 75% of the species can be found in Mexico, and 74% of these are endemic (García, 2007; Martínez-Salvador et al. 2005). The *Agave* species with major revenue produced in Mexico belong to magueys “pulqueros” (Siacon, 2014). This group is represented by the species of *Agave americana*, *Agave atrovirens*, *Agave mapisaga* and *Agave salmiana* (Cedeño, 1995; García, 2007).

Agave salmiana have sexual and asexual reproduction strategies (Arizaga and Ezcurra, 2002). Ramírez-Tobías et al. (2011) determined that 50% of seedlings can be more vigorous than offshoots in *A. salmiana*, however the availability of seed is limited. The use of micropropagation is an option for the lack of germoplasm and has several advantages such as to obtain populations with elite characteristics, stress tolerance, pathogens free, and stable genetic background (Domínguez-Rosales et al. 2008). Many reports have highlighted that *Agave* species contains phytochemicals with bioactivity (Almaraz-Abarca et al. 2009; Guerra de León et al. 2007; Gutiérrez et al. 2008; Morales-Serna et al. 2010).

The aim of this study was to establish a strategy of micropropagation for *A. salmiana*, evaluate the impact of this process in the total phenolic compounds and saponins content, antioxidant activity and determine which relation is between the bioactivity and the content of secondary metabolites.

METHODOLOGY

Seeds of *A. salmiana* were germinated *in vitro*, establishing an optimal germination protocol for further micropropagation. Three weeks old plantlets were multiplied by axillary shoots method. After remove roots, one single plant was cultured in jars with 20 ml of solid culture medium MS modified with L2 vitamins (MS+L2) (Philips and Collins, 1979). After two weeks, 6-benzylaminepurine (BAP) and 2, 4 –Dichrolophenoxyacetic acid (2, 4–D), were added to new MS+L2 solid culture medium (Santacruz-Ruvalcaba et al. 1999). The cultures were transferred to a room with temperature set to 27°C with a photoperiod of 16:8h light:dark. The number of axillary shoots and presence of callus, after 60 days of culture were observed (Santacruz-Ruvalcaba et al. 1999). Once were multiplied, they were put in acclimatization medium and let the plants growth for 30 days. Rooted plants obtained from the previous steps were transferred to field. Irrigation was applied twice per week for the half of plants and the other half was not irrigated to simulate normal field conditions.

Total phenolic (TP) concentration was determined using the Folin-Ciocalteu reagent according to the method of Zheng and Wang (2001) and antioxidant (AOX) capacity was determined using the oxygen radical absorbance capacity assay (García-Pérez et al., 2011). Saponins and phenolic compounds were detected and identified using HPLC-MS-TOF and quantified using HPLC-PDA-ELSD. Phenolic compounds were quantified as aglycones of kaempferol, quercetin or myricetin and saponins were quantified as aglycone hecogenin equivalents.

RESULTS AND DISCUSION

An efficient micropropagation protocol was established to regenerate plants of *A. salmiana*. Table 1 summarizes the results of axillary shoots generation in *A. salmiana*. The combination of plant growth regulators to produce the high axillary shoots was 10 mg l^{-1} of BAP and 0.04 mg l^{-1} of 2, 4-D, obtaining 14 shoots per explant after 60 days.

Table 1 Number of axillary shoots generated after 60 days by a combination of 2,4-D and by axillary shoots generation technique.

BAP (mg l^{-1})	2, 4 - D			
	0.00	**0.01**	**0.025**	**0.04**
0.0	0.66	0.25	0.00	1.66
0.5	3.00	2.75	0.00	0.00
1.0	1.33	2.66	2.33	0.33
5.0	2.33	6.00	10.33	0.00
10.0	3.50	2.00	7.33	14.00

* BAP: 6-Benzylaminepurine. 2-4-D: 2, 4 – dychlorophenoxyacetic acid

This protocol allows us to regenerate whole plants from wild type genotypes in 16 weeks using the germinated young plantlets. This protocol was 40% more efficient in term of time and 25% more efficient in term of plants/explant than the proposed by Chen et al. (2014) for *A. Americana*.

Table 2 Phenolic compound and saponin content. Phenolic compounds are quantified as aglycones of the corresponding flavonol (mg/g dw) and saponins in Hecogenin equivalents (mg EHe/g dw) NQ = no quantifiable metabolite.

Compound	EXW	EXN	IN	WT
Kaempferol 1	0.290	0.384	0.163	0.147
Kaempferol 2	0.000	0.132	0.000	0.000
Quercetin 1	0.117	0.000	0.000	0.000
Manogenin 1	0.000	0.000	0.000	NQ
Manogenin 2	0.861	NQ	0.474	0.288
Chlorogenin 1	1.104	2.042	0.678	0.339
Chlorogenin 2	0.970	1.707	1.101	0.595
Gentrogenin 1	0.000	1.190	1.437	0.891
Tigogenin 1	18.821	22.625	31.007	0.000
Hecogenin 1	3.785	4.231	3.168	0.418
Hecogenin 2	5.227	3.561	0.968	0.000

Free phenolic compounds were detected in all extracts of *A. salmiana*. WT plants show higher content of phenolic, with 13.06 mgEGA/g dw and EXW had the lower concentration with 9.02 mgEGA/g dw. All the extracts show antioxidant activity. The IN plants shows the higher antioxidant activity (369.84 µmolTE/g dw). Between WT (130.39 µmolTE/g dw), EXW (143.38 µmolTE/g dw) and EXN (184.13 µmolTE/g dw) samples, there was not significant statistically difference. The differences could be because the presence of different molecules in response of the environment from which the plants came from (Almaráz-Abarca et al., 2013). It could be identified a total of three different flavonols and 8 different saponins in the extract (Table 2). The number of flavonols identified coincide with *A.*

americana and *A. sisalana*, which had reports from 2 to 4 different flavonols, but mismatch with other species, such as *A. victoria-reginae* and *A. striata*, which number of identified flavonols ascends to 11 and 14 (Almaráz-Abarca et al., 2013). Tigogenin was the saponin more abundant compared with the other saponins. Hecogenin glycoside 2 was not found in WT. The tigogenin glycoside 1 and hecogenin glycoside 2, were a kind of marker to identify the plantlets that were generated in *in vitro* and the wild-type. The tigogenin generated the higher concentration in the IN samples, confirming the response of the secondary metabolites to an environment of micropropagation with high moisture (Barreto et al., 2010).

CONCLUSSION

Agave salmiana was micropropagated by axillary shoots from seeds. The process of micropropagation generated 14 buds per explant, with an efficiency of 87.5% in 16 weeks. The micropropagation causes changes in the antioxidant activity of the extracts but not had the same effect in the phenolic compound composition.

The saponins hecogenin and tigogenin appeared only in plants that went through the micropropagation process. The tigogenin content was much more higher compared to other saponins, presenting the highest content in the IN. This suggest that the process of micropropagation could be a platform for the generation of steroidal compounds in agave, avoiding to wait long periods of time to generate biomass for industry like pharmaceutical, that employs this kind of compounds as precursors.

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Development of an *Agrobacterium tumefasciens* mediated transformation protocol for two *Agave* species by organogenesis

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ABSTRACT

The goal is to develop an efficient protocol of genetic transformation in *Agave tequilana* and/or *Agave desmettiana* mediated by *Agrobacterium tumefasciens*, using apical meristems of bulbils as explants for both species. Since genetic analysis in this genus is currently limited, genetic manipulation constitutes a valuable tool for numerous lines of research and applications of scientific and industrial interest. The strains of *Agrobacterium* used were LBA4404 and GV2260, and transformation was carried out by co-culture with the bacterium, the selective agent was phosphinothricin (PPT), included in the multiplication medium. Regeneration was more efficient in *A. desmettiana* and explants co-cultured with pB7WG2D1 produced higher numbers of shoots and developed more rapidly. *A. tumefasciens* strain GV2260 is less aggressive for explant tissue and may be advantageous for the development of an efficient transformation protocol. Currently better preliminary results have been observed in comparison to previous reports in *Agave salmiana*.

Key words: Agave, transformation, bulbils, organogenesis, *Agrobacterium tumefasciens*.

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INTRODUCTION

In spite of the economic importance of Agave species, research at the genetic, molecular and biotechnology levels has not been developed strongly. The long life cycles, inaccessibility of inflorescences, large genomes, and the fact that they are monocarpic and in most cases are semi-domesticated or wild, has made it difficult to develop Agaves as model species.

New and useful tools such as next generation sequencing, expression analysis for some species, *in silico* analysis of expression by *in situ* hybridization and RT-PCR, functional analysis of enzymes involved in the synthesis of oligofructans, antibody immunolocalization and various histologic methods have been implemented. Currently however, due to the lack of an efficient method for genetic transformation of Agave species, functional analysis of genes of interest has been performed in the heterologous system of *A. thaliana* (Abraham et al. 2010).

The only formal previous report was carried out in *Agave salmiana* (Flores et al. 2007) and describes a protocol based on somatic embryogenesis from dedifferentiated callus tissue, where co-cultivation with *Agrobacterium tumefasciens* and particle bombardment was tested. The transformation of somatic embryos derived from callus formed on leaf explants via co-culture with *A. tumefasciens* was the most efficient method for producing GUS positive plants, with a transformation efficiency of 2.7%. Although successful, the method described for *A. salmiana* is time-consuming and laborious and therefore not optimal for carrying out extensive genetic analyses.

Here we describe preliminary results in the development of methodology to transform *A. tequilana* and *A. desmettiana* based on co-culture of bulbil meristem explants with *A. tumefasciens* and regeneration via organogenesis.

METHODOLOGY

Materials

Bacterial strains and plasmid

Meristems from bulbils of *A. tequilana* and *A. desmettiana* were used as explants for transformation using the LBA4404 and GV2260 strains of *A. tumefasciens* with either pSR387 (Alatorre-Cobos et al. 2012) or pB7WG2D, 1 (Abraham-Juarez et al. 2010) which both carry the constitutive promoter CaMV35S, the green fluorescent protein (GFP) and Phosphinothricin (PPT) selectable markers. The pSR387 vector also carries the GUS reporter gene and the pB7WG2D1 vector the AtqKNOX2 gene.

Obtaining explants and disinfection:

Approximately 2 month old bulbils of *A. tequilana* and *A. desmettiana* were used to obtain meristem explants based on the protocol established by Nava-Cedillo (1988).

Method of transformation:

A. tumefasciens strains with reporter genes and controls were plated in solid selective medium (YEB) at 28°C for 48 hours, independently grown were selected and resuspended in liquid selective medium (MS), supplemented with 100 uM acetosyringone (Flores et al. 2007). Bacterial cells were concentrated by centrifugation and resuspended in liquid medium, in this *A. tumefasciens* suspension the explants were immersed for 20, 30, 45 and 60 minutes and then co-cultured on solid MS with acetosyringone for 48, 72 and 96 hours. Subsequently the explants were transferred to selective medium supplemented with hormones BAP (9 mg / ml), IBA (0.6 mg / ml) and acetosyringone (Flores et al. 2007).

Selection was carried out in medium containing PPT at different concentrations. Explants co-cultured with LBA4404 and GV2260 strains not harboring pSR387 or pB7WG2D1 were used as negative controls. Regeneration by organogenesis was carried out as described by Nava-Cedillo (1988). Presence of transgenes will be detected by Polymerase Chain Reaction (PCR) and GUS/GFP assays.

RESULTS AND DISCUSSION

Bulbil explants from *A. tequilana* and *A. desmettiana* were suitable for the *in vitro* regeneration of plantlets according to the scheme in Figure 1.

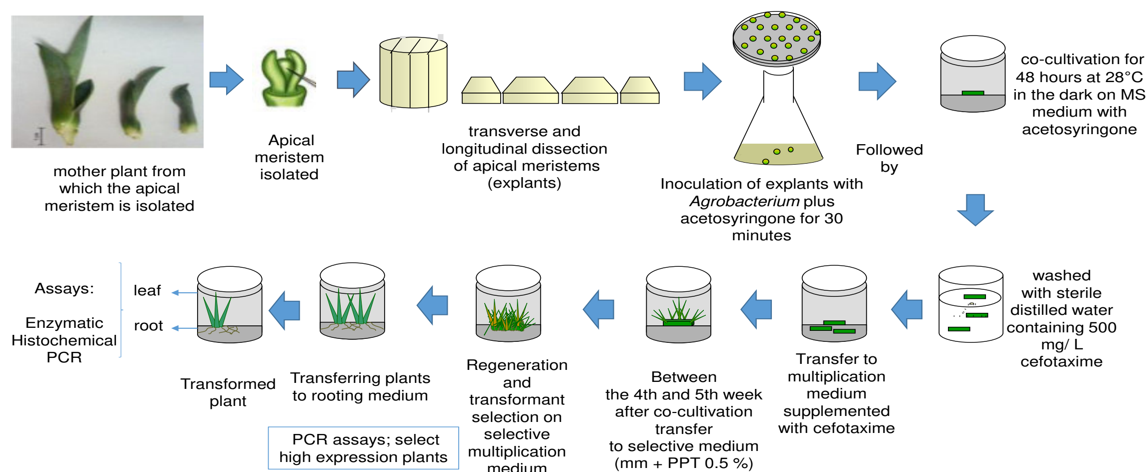

Figure 1. Diagram of the stages of *in vitro* culture and co-cultivation of *Agave* with *A. tumefasciens*.

Different combinations of strains of *A. tumefasciens* transformed with the plasmid pSR387 or pB7WG2D, 1 allowed shoot formation in various explants although growth inhibition is greater in explants from *A. tequilana* than *A. desmettiana*.
Agave desmettiana also produced a greater number of shoots per explant in comparison to *A. tequilana* and the *A. desmettiana* shoots also developed faster.
Negative control and non-transformed shoots showed stronger symptoms of stress and more necrosis. The number of necrotic explants/shoots increased as time of co-culture in the presence of the *Agrobacterium* increased. Elimination of bacteria following prolonged co-cultivation was also more difficult. Shoots obtained from explants co-cultured with the LBA4404 strain (both plasmids) showed a stronger purple coloring and more rapid death of shoots on selective medium in comparison to those transformed with strain GV2260.

CONCLUSIONS

Bulbils are a viable source of explants for organogenesis in both *A. tequilana* and *A. desmettiana* and a good source of material to develop transformation protocols. Interestingly, regeneration is more efficient in *A. desmettiana* which also naturally reproduces exclusively by bulbils. Interesting differences between the two *A. tumefasciens* strains used, LBA4404 and GV2260 containing the same plasmid were also observed. Explants co-cultured with pB7WG2D, 1 produced higher numbers of shoots and developed

faster. Strain GV2260 is less aggressive for explant tissue and may be advantageous for the development of an efficient transformation protocol. Initial PCR positive *A. desmettiana* plantlets encourage us to continue improving this methodology.

ACKNOWLEDGEMENTS
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HISTOLOGIC ORIGIN OF MICROPROGATED SHOOTS OF *Agave tequilana*

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ABSTRACT

Agaves in Mexico have had great economic and cultural importance to Mesoamerican inhabitants since ancient times. One of the most important uses of these plants is the production of alcoholic drinks, including tequila, obtained from *Agave tequilana* whose demand drives the production of healthy and homogeneous plant material. Biotechnology has opened doors to new production processes as *in vitro* propagation, which offers advantages over conventional methods, such as sanitation of plants, conservation of commercial variants and the reduction in harvest time; besides genetic improvement, because of the genetic variability present in this plant. The present study was carried out to determine the origin of micropropagated shoots via axillary shoot proliferation, using histological techniques to identify the possible presence of organogenesis. It was demonstrated that the process of micropropagation by proliferation of axillary shoots, presumably is not unique to this plant production protocol, because organogenesis occurs during it, which can cause undesirable variation; proliferation of axillary shoots is a process that generates less variation. Searching for genetic homogeneity for industrial processes becomes a challenge to the industry as somaclonal variation can be led in the loss of genotypes of commercial interest; therefore axillary shoot proliferation protocols must be adjusted to avoid shoots production via organogenesis.

Keywords: axillary shoots and organogenesis, histology, micropropagation, somaclonal variation.

INTRODUCTION

Among the most conspicuous plants of the Mexican landscape, especially in arid and semi-arid areas, the agaves are considered keystone species in these regions, both for its abundance and the amount of resources they provide to other organisms (García-Mendoza, 1992; 2007). Historically agaves in Mexico have had great economic and cultural importance to Mesoamerican groups, one of the most outstanding uses of these plants is the production of alcoholic drinks, including tequila, obtained from *Agave tequilana*, alcoholic beverage whose demand boosts the production of healthy plants and homogeneous material (Santacruz-Ruvalcaba *et al*., 2014). Biotechnology has opened doors to new production processes as is micropropagation, offering advantages over to conventional propagation, such as sanitation of plants, preservation of variants of commercial interest, reduction in

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harvest time and also the plants can be submitted to improvement, since genetic modification can be presented spontaneously and induced (Santacruz-Ruvalcaba *et al*., 2008), which depends to the system of micropropagation, so it is important to identify if there is presence of organogenesis during axillary shoot proliferation. Therefore in order to know the origin of shoots generated by different propagation paths, the aid of histological techniques are needed, since many of the internal structures of plants result imperceptible if they are not subject to special treatment. Curtis-Patiño (1986) mentioned that studying the internal structures three basic steps should be followed: 1) preparing the tissues, 2) use efficiently the microscope and related equipment, to study and interpret the structures in plant material, 3) record microscopic images to illustrate and discuss the results. This procedure with the help of differential staining techniques are an important role in the study of plant tissues, since the main difficulty is that once the cuts are made, there is a lack of contrast between the cell and the surrounding medium, the simplest way to contrast them is by increasing the use of colors. The stains in general terms contrast cellular structures and helps make it more visible (García, 1990).

METHODOLOGY

Agave tequilana cultivar azul samples were taken from *in vitro* established plants, previously micropropagated via axillary shoot proliferation and organogenesis according to available protocols (Portillo and Santacruz, 2006; Robert *et al*., 1987). Samples from 1.0 to 1.5 cm were selected for histological cuts, embedded in polyethylene glycol 1450 (PEG) at a concentration of 1: 4 (PEG: deionized water) until evaporation, passing them to pure PEG during 24 h (Monroy *et al*., 2010). Once the samples were solidified in PEG, cuts of 15 µm were sectioned by a rotation microtome with metal blades. Sections were subjected to a staining treatment; carmine acetic to stain cell nuclei due to its affinity for acids, and astral blue to stain cell walls and cytoplasm. Following the double staining, each sample was observed under microscope to compare the different structures.

RESULTS AND DISCUSSION

The shoots obtained by axillary shoot proliferation were generated asynchronously and abundantly (Figure 1a), which difficult an accurate identification of their origins, as the appearance also indicated possible origin through organogenesis. PEG facilitated having histological sections of good quality. Organogenic centers adjacent to axillary buds meristems were found, which allowed assuming that not all shoots via axillary shoot proliferation are unique to this process. Such observed cells in organogenic centers were very similar in appearance to those of the axillary buds meristems, with evidently mitotic activity (Figura 1b). The formation of organogenic shoots on *Agave* has been reported previously, both direct (Aureoles-Rodríguez *et al*., 2008) and indirect (Monroy *et al*., 2010; Robert *et al*., 1987).

The results show that one micropropagation method in particular, may involve others, in this case axillary shoot proliferation and organogenesis, which suggests that somaclonal variation, can be higher; thus, these results also indicate the importance to observe and adjust the processes of micropropagation, especially when less genetic variation is desired, since every process generates it in a differential way. As recommendation, molecular analysis to

test somaclonal variation of axillary shoots will be necessary to perform in order to determine the significance of this variation, which is a parallel activity currently under research to be integrated to this study.

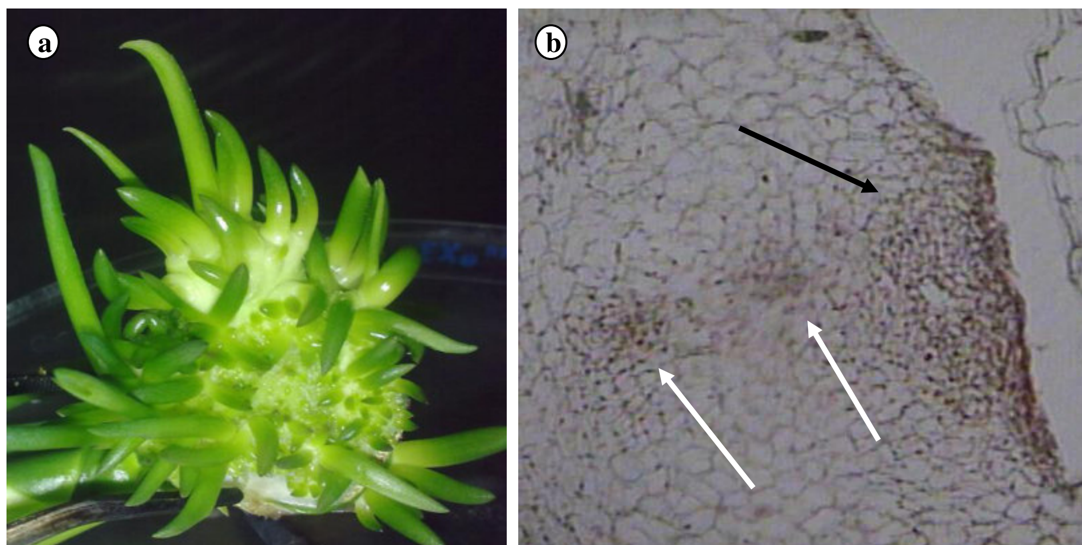

Figure 1 a) Putative axillary shoot proliferation of *Agave tequilana*, b) Histological section of axillary bud (black arrow) of *A. tequilana* accompanied by two organogenic centers (white arrows) after stimulation of shoot proliferation.

CONCLUSION

Histological techniques allowed to located organogenic cell groups during axillary shoot proliferation in *Agave tequilana*, which presumes that organogenic shoots are also generated. In order to reduce as much as possible the organogenic processes, it is important to look over the micropropagation method to minimize genetic variation. The information of somaclonal variation in axillary shoots through molecular analysis will help to control it.

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In Vitro polyploidy induction of two Agave species

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ABSTRACT

Polyploidy is a process involving chromosomal duplication, it has been considered of great importance in the evolutionary history in different organisms. In plants it was estimate that over 60% of angiosperm species have undergone a process of polyploidization, many crops important economically are polyploid, for example potato, banana, maize, triticum and others.
Polyploids acquire different traits compared with their diploid parent who should enable adaptation to new habitats or acquiring new characteristics of agronomic interest. The goal of this work, was to study the process of polyploidization in *Agave*, to understand those changes arise after polyploidization as a tool for genetic improvement.
In this work, during clonal micropropagation of *Agave tequilana* and *Agave angustifolia* the mitostáticos oryzalin effect was evaluated in different concentrations (2.5, 5.0, 7.5 µM). To select putative polyploid shoot, a correlation between nuclei size and the ploidy level was estimated. The nuclei from species with different ploidy levels (*A. tequilana* and *A. angustifolia* (2n = 2x), *A. fourcroydes* Var Kitam ki (2n = 3x), *A. fourcroides* Var Sa ki and A. *fourcroydes* Var. Yaax Ki; (2n = 5x)) was isolated.
There is a hight correlation 0.92 among the ploidy level with the size of the nuclei, *A. tequilana* and *A. angustifolia* nuclei showed similar size 10.8 microns and 11.2 µm in average respectively, to *A. fourcroydes* var. Ki Kitam the average was 14.4 µm, while nuclei *A. fourcroydes* var. Yaax ki and *A. fourcroydes* var Saa Ki were similar, showing 16.2 and 15.5 µm respectively. The Shoots of *A angustifolia* and *A. tequilana* were exposed to the oryzalin during four weeks on the multiplication phase. The presence of oryzalin decreases the production of shoots. The concentration showed the highest response in terms of productions shoots polyploid was 5.0 µM, We detected a shoots with increased nuclei size.

Keywors: Tissue culture, polyploidy induction, Agave

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INTRODUCTION

Ploidy is defined as on increase in the number of chromosome sets in multiples of haploid number, usually represented as (nx), where a nucleus with two complete sets of chromosomes are called diploid (2x), three complete sets of chromosomes is triploid (3x) and so on (Levin 1983). Based on the origin of its genomes two types of polyploidy are recognized, the autopolyploids (single species involved in genomic bending) and allopoliploídes (two species involved in genomic bending). In the nature the autopolyploids could be originate during cell division of somatic cells (mitosis) or by the union of unreduced meiosis gametes (Yang et al. 2011; Levin 1983), meanwhile the allopoliploids can be arise through, interspecific hybridization with a set of non-homologous chromosomes, followed by a duplication of the genome, or first chromosomal duplication followed by interspecific hybridization (Yang et al. 2011). In plants it has been estimated that over 60% of the species of angiosperms have experienced a process of polyploidization (Buggs 2008; Buggs et al. 2008), likewise the most economically important crops (wheat, corn, canola, banana, agave, cotton) are polyploids (Leitch et al. 2008). Polyploids can be experimentally induced *in vitro* by explant treatments with mitostátics agents such as colchicine (Sun et al. 2009), oryzalin (Miguel et al. 2011), and trifluralin (Dhooghe et al. 2011). The goal of this work was to demonstrate that in *Agaves* is possible to induce polyploidy by the use of mitostatics agents, during a specific stages of cultivation *in vitro*. The newly formed polyploid may be used to generate knowledge about the consequences of the process of polyploidization.

METHOD AND MATERIALS

Vegetal materials

Shoots of *A. angustifolia* (2n = 2x = 60) and *A tequilana* (2n = 2x = 60) were subcultured every 4 weeks, in multiplication medium Murashige and Skoog (MS) supplemented with (8.8 µM 6-BAP and 1.1 µM 2, 4-D, 1.5 g / l of agar, 1.5 g / l gel rite, pH = 5.75).

Induction of polyploid

A stock solution 1mM oryzalin (SIGMA-ALDRICH) was prepared in DMSO 10%, of these different volume was took to achieve the treatment concentration (2.5 µM, 5 µM and 7.5 µM). The oryzalin solution was sterilized by filtration and added to the culture medium previously sterilized. Shoots of *A. tequilana* and *A. angustifolia* (three replicates of 25 shoots each concentration) were cultured for 4 weeks in multiplication medium. The shoots generated were transferred to fresh multiplication medium without mitostatic agent.

Selection of putative polyploid by correlation of core size with ploidy level.

In order to establish a correlation of the core size and the ploidy level, nuclei was isolated from species with different ploidy levels (2n = 2x *A. tequilana* and *A. angustifolia*, 2n = 3x *A. fourcroydes* Var. Kitam ki. 2n = 5x *A. fourcroides* Var. Saa ki and *A. fourcroydes* Var. Yaax ki,) as reported Palomino et al. (2003). The 20 µl of the suspension of nucleus were placed on a slide and stained with 20µl carbolfucin for 30 min, once stained excess drained for allowed to dry, once put a drop of acrylic recina (poly-mount, Polysciences, Inc. cat . 08381) and the coverslip is placed. The diameters of 50 nuclei three plants of each ploidy level were directly measured the used of an ocular micrometer Axiostar plus the 100 X

microscope field. The images were captured with a camera PC 1089 with a 4.5X magnification.

RESULTS

Compared with the control, the response decreases with increasing concentration of oryzalin, in *A. angustifolia* at concentrations of 0.0, 2.5, 5.0 and 7.5 mM, the response was 100, 67, 63 and 57%, while *A. tequilana* was 32, 25 and 14, 5.0% respectively (Table 1). The mitostatic concentration affects the rate of multiplication in both species, however in *A. tequilana* multiplication rate at concentrations of 0.0 mM was 3 shoots per explant, while in treatments 2.5, 5.0 and 7.5 mM was 1 shoot per explant. In *A. angustifolia* due to abnormalities of the explant to 5 and 7.5 uM oryzalin was not possible to determine the multiplication rate (Table 1).

Table 1. Effect of the oryzalin in the induction of poliploid *in vittro*.

	Concentration	%Response	News Shoots /Shoots assessed	Polyploids
A. *tequilana*				
	0.0 µM	32 a	74/10	0
	2.5 µM	25 b	25/10	0
	5 µM	14 c	14/10	0
	7.5 µM	5 d	5/5	0
A. angustifolia				
	0.0 µM	100 a	300/10	0
	2.5 µM	67 b	52/10	0
	5 µM	63 c	22/10	1
	7.5 µM	57 d	12/10	0

Letters represent significant differences P≥.05

The shoots and the conglomerated structures obtained were transferred a new medium without oryzalin. In *A. angustifolia* 52, 22 and 12 shoots from exposure to 2.5, 5.0 and 7.5 uM oryzalin respectively were generated. While for *A. tequilana* generated 25, 14 and 5 respectively at the same concentrations.

With respect to the nuclei size, as a polyploidy indication, the nuclei of *A. tequilana* and *A. angustifolia* (2x) showed similar size (10.8 µm and 11.2 µm in average respectively; Figure 1 A and D), meanwhile *A. fourcroydes* Var. ki Kitam (3x) showed the average of 14.4 µm (Figure 1 B) finally the nuclei of *A. fourcroydes* Var. A. Yaax ki (5x) and *A. fourcroydes* Var Sa ki (5x) were similar 16.2 µm and 15.5 µm respectively (Figure 1 C and F). There is a correlation of R^2= 0.92 of ploidy level with the size of the nucleus.

In *A. tequilana* any shoots evaluated showed an increase in nuclei diameter. In *A. angustifolia* 10 shoots generated from each treatment with oryzalin were analyzed, on treatment with 5 µM of oryzalin was observed that the size of the nuclei (14 µm) of shoots 5.0M1 (Figure 1 E) was greater than the average of the nuclei diploid 10.8 µm and 11.2 µm (Figure 1 A and D) and lower than the pentaploids 16.2 and 15.5 µm (Figure 2.4 C and F). This shoots was considered a tetraploid.

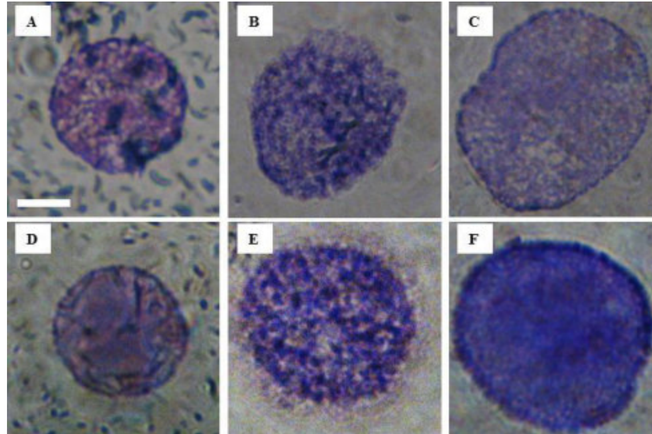

Figure 1. Nuclei size of *Agave* species with different ploidy levels. A) *A. tequilana* 2x, B*) A. fourcroydes* Var. 3x Kitam ki, C) *A. fourcroydes* Var. 5x Yaax ki, D) *A. angustifolia* 2x, E) induced tetraploid of *A. angustifolia* 5 µM In Vitro (5.0M1) and F*) A. fourcroydes* Var sa ki 5x. Bar = 5 µm

DISCUSSION

Evidence suggests that in plants, several polyploid species show several changes (genetic, molecular, anatomical, etc.) that generate new feature compared to their parental diploids (Abbott and Lowe 2004, Adams 2007). The induction of in vitro polyploid is a method for exploring these changes in a shorter period of time and faster as occurs in nature. The establishment of the method of inducing polyploidy depends on different factors such as the type of explant, mitostatic agent concentration, the application method, time of exposure, and genetic factors (Dhooghe et al. 2011).

The analysis of the effect of diferent concentration of mitostatic on the poliploid of shoots formation, our results sowhed that increasing the concentration of mitostatic, decreases the response (Table 1). This fact has been observed in other studies where different concentrations of oryzalin (Dhooghe et al. 2011) are evaluated and has been attributed to the toxicity of mitostatic agent.

The methodology widely used to assess the ploidy level is flow cytometry, however, is a tool that requires specialized materials and equipment to carry it out, so it is necessary to use alternative tools to evaluate and select putative polyploid in a quick and easy. Several studies reported the use of, stoma size and cell, stomatal density and trichomes to select putative polyploidy the use flow cytometry to confirm the ploidy level (Aina et al. 2012). Furthermore, Bourdon et al. (2011) suggest a correlation with core size ploidy level. In *Agave* has been reported that the size of the haploid genome (1C value) is 7.5 pg and the DNA content increases proportional to ploidy level (Robert et al. 2008) this also suggests an increase in size of the nucleus with the ploidy level, in *Agave* found that there is a correlation of nuclei size with increasing ploidy level of (Figura 1).

Induction of *in vitro* polyploidy in *Agave* will be a valuable tool to understand the process of polyploidization and their consequence in order to apply in several genotypes to improvement of fiber quality or sugar content. If we are able to duplicate the genome of this, we would obtain allopolyploids and thus the two polyploidization process in *Agave*. In terms of comparison with the naturally induced, we could correlate which of the two processes is what has happened in *Agave*. Likewise, in later studies evaluated whether the induction of polyploidy confers benefits (increased fiber or sugars content) for genetic improvement and

from an evolutionary point of view, to understand the role of polyploidy in the diversification process.

CONCLUSIONS

A correlation with nuclei diameter and the ploidy level was found in the analyzed species with different levels of ploidy,

The mitostatic used generate several morphological changes in the news shoots.

Increasing the concentration of oryzalin reduces the response and shoots multiplication.

The treatment with oryzalin 5 µM showed the higher response in terms of obtaining polyploid shoot.

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Effect of nitrogen and concentration of nutrients in the development of seedlings of *Agave duranguensis* Gentry

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ABSTRACT

The Agave seedling quality is affected by several factors. Among these factors, one of the most important in the production of Agave seedlings vigorous and resistant to transplantation, is largely related to the chemical composition of the nutrient solution, thus the aim of this investigation was to evaluate the effect of nitrogen ($N\text{-}NO_3^-$) in five concentrations 0, 2.5, 5, 10 and 15 mol of N m^{-3} and three levels of osmotic potential (OP) : --0.037, -0.73 and -0.11 MPa. Four samplings were performed, the first at 30 days and the remaining three every 30 days, after 56 days of the planting. The variables measured were: plant height, stem diameter, basal diameter, number of leaves, dry weight of root, stem and leaves. The main factor affecting the growth and quality of seedlings was $N\text{-}NO_3^-$, while the OP present reduced influence. As the concentration of $N\text{-}NO_3^-$ was increased in the nutrient solution the variables seedling height, basal diameter, number of leaves, dry weight of seedling increased. Seedlings with higher growth and better quality were obtained with the treatment of 15 $N\text{-}NO_3^-$mol m^{-3} and an osmotic potential of -0.11 MPa.

Key words: Agave mezcalero, Durango, fertilization, nutrition

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INTRODUCTION

Durango is one of the Mexican States that holds the designation of origin of mezcal, published in the Official Gazette of September 5, 1994. The high economic potential of this activity for the state, promoted since then, various attempts to establish agro industries for mezcal production for national consumption and international markets. Unfortunately, these attempts have not been favorable, because the production of mezcal in the southeastern region of Durango is rustic and raw materials (Agave) are obtained by the collection of wild plants, never cultivated plants from commercially plantations (Valenzuela 2003). Since 1997, there have been several attempts for reforestation and the establishment of commercial plantations, unfortunately the availability of quality plant material of Agave is low, even though it is one of the most important factors to achieve high performance in Agave production. A suitable technology for greenhouse production, transplant and maintenance in nurseries for a period of 24 to 30 months is required in order to obtain plants of high quality of adequate size and vigorous and resistant to transplantation. The Agave seedling quality is affected by several factors, among them the most important is nutrition. The nutrient requirements of Agave, are similar to other plants, often nitrogen (N) is the most limiting element for growth in both agricultural and natural areas. To date, there exists limited information on the factors associated with nutrition of Agave plants as well as their development and cultivation. Nobel (1996) mentioned that most Agaves are adapted to temperate climates and semi-arid soils, showing a good adaptability to the scarcity of water and soils with low nutrient content, even when the reproductive cycle is from 14 to 16 years. Chirinos (1992) indicated that Agave is a plant that requires thin, calcareous and low soil nutrient content, even in those which the reproductive cycle requires 14 to 16 years. Nobel (1998) noted that the content of N and P in the agave plants are high but low in Ca compared to the leaves content of cultivated plants, while Berry and Nobel (1985) noted that sprouts of *Agave deserti* Engelm were very sensitive to salinity, but resistant to high concentrations of N and Ca. Ramirez (2003), indicated that the first fertilization to the Agave plant has been made through applications of ammonium sulfate, urea, single calcium superphosphate, triple superphosphate or calcium mixtures whose composition is the most common 15-15-15, 16-16-16 or 17-17-17. Studies by Nobel and Hartsock (1986) and Nobel et al. (1988, 1989) on the influence of nitrogen and other elements in Crassulacean Acid Metabolism (CAM) plants stressed that nitrogen stimulates the net uptake of CO2. Nobel et al. (1988) found that the application of nitrogen and phosphorus, in an independent form in *Agave lecheguilla* Torr, increased the number of unfolded leaves and its net CO2 exchange rate; while fertilization with potassium and boron did not show differences in unfolded leaves, but it did so in nocturnal CO2 assimilation. The best dose of N, P, K and B were 100, 500, 100 and 100 kg/ ha^{-1}, respectively. Valenzuela and Gonzalez (1995) found that in *Agave tequilana* Weber the stem or "piña" production was duplicated compared to the control, fertilizing with dose of 120N-80P-60K ha^{-1}, while using the dose of 120N-120P-60K ha^{-1} the yield was lower. To date, there is limited information on the factors associated with nutrition of seedlings as well as its development and field crop, therefore, this project aims to determine the effect of nitrogen and nutrient concentration (osmotic pressure) in seedlings of *Agave durangensis* Gentry.

EXPERIMENTAL METHOD.

This research was conducted in the municipality of Vicente Guerrero, Durango, Mexico. Seeds of *Agave durangensis* were collected in the region of La Michilia, Municipality of Súchil, Durango. Peat moss was employed as a substrate (Premier) for filling the trays. The seeds were germinated at a temperature of 25° C, then placed in each well for sprouting. Once planted, the trays were moved to the greenhouse. In the preparation of the nutrient solution treatment (SN) Steiner (1984) was used as a base, by increasing the nitrogen (N-NO_3^-) maintaining the mutual relationship between the other anions ($H_2PO_4^-$ and SO_4^{-2}); cations were maintained in the same relationship. The osmotic potential was obtained by the product of the total concentration (mol m^{-3}) by the factor 0.024, the resulting unit was atmosphere (Steiner, 1984), this became a Mega Pascal (MPa). The treatments were composed of 15 nutrient solutions resulting from the combination of five levels of N-NO_3^- and three levels of OP. All treatments were administered the same amount of micronutrients: 1 mL of stock solution per liter of SN; this solution had a content of 1600 ppm Mn, 875 ppm B, 23 ppm Zn and 11 ppm Cu. In addition, 1 mL of a solution of 4000 ppm of Fe per liter of SN in the chemical form of chelated iron was added.

The application of the SN was performed every other day without irrigation after the application. The amount of SN applied to each experimental unit was 250 mL in each irrigation. The experimental unit was constituted by 200 seedlings (a tray). The distribution of treatments was established under a completely randomized design with a factorial arrangement with three replications. The measured variables were: plant height, stem diameter, basal diameter, number of leaves, dry weight of root, stem and leaf. For data analysis the SAS (StatisticalAnalisisSystem) algorithm was used (SAS Institute, 2004).

RESULTS AND DISCUSSION

Effect of nitrogen.

Different levels of N-NO_3^- in the nutrient solution showed highly significant differences for the variables height of plant (AP), number of leaves (NL), basal diameter (DB) and dry weight of leaf, root, stem and plant, but showed no effect on stem diameter (DT), stem length (LT) and dry weight of stem (PST) (Table 1).

The variable seedling height, showed highly significant differences in the last two samples (90 and 120 application days) as a response to the effect of the concentration of N-NO_3^-in the nutrient solution (Table 1), the highest seedling was reached for the treatment of 15 mol of N m^{-3} for the last two samples, while in the first two samples showed no response to factor N. In relation to the effect of AP variable OP over the four samples showed an highly significant effect (Table 1). For the first two samples the greatest effect on AP were the treatments -0.073 and -0.11 MPa, while in the third and fourth sampling the most AP was for the treatment of -0.11 MPa, going more negative the OP as the AP increased.

For the variable NL the N factor was highly significant from the second sampling. The highest NL was obtained for treatments of 10 and 15 mol of N m^{-3} (Table 1). Regarding the factor OP, a highly significant effect was found in the NH from the first sampling. The OP where the highest NL was obtained was -0.11 MPa.

The DT and LT variables showed no significant differences by increasing levels of N–NO_3^- (Table 1). This behavior was similar to the OP factor.

Regarding the DB variable, differences were highly significant at varying levels of $N–NO_3^-$ (Table 1) from the first sampling. In general, increasing seedling age increases the difference between treatments. The behavior was very similar to that obtained in AP. The effect of the factor OP in the development of DB revealed no significant differences in the first two samplings, while in the third and fourth sampling the effect was significant. Most DB is obtained from the third sampling and it was for the treatment -0.11 MPa.

Table 1. Effect N concentration and osmotic potential of the nutrient solution in height, leaves, stem length, stem diameter, basal diameter and seedling dry weight Agave variables.

							dry weight (gr)				
DDIAS	Levels N meq L^{-1}	Height (cm)	Leaves > 0.5 (cm)	Stem length (cm)	Stem diameter (mm)	Basal diameter (mm)	Leave	Stem	Root	Aerial part	Plant
30	0	1.67 a	2.00 a	1.84 a	1.82 a	5.00 ab	0.057 ab	0.004 ab	0.013 a	0.061 a	0.074 a
	2.5	1.61 a	1.78 a	0.82 a	1.71 a	4.97 ab	0.051 b	0.006a	0.014 a	0.057 a	0.070 a
	5	1.62 a	1.89 a	0.98 a	1.47 a	5.59 a	0.053 ab	0.003 ab	0.015 a	0.056 a	0.071 a
	10	1.63 a	2.11 a	0.93 a	1.54 a	5.65 a	0.064 ab	0.002 b	0.015 a	0.067 a	0.082 a
	15	1.70 a	1.89 a	1.06 a	1.64 a	3.90 b	0.068 a	0.004 ab	0.014 a	0.073 a	0.086 a
	DMS	0.37	0.61	0.42	0.86	1.11	0.016	0.004	0.006	0.017	0.020
	PO (MPa)										
	- 0.037	1.64 ab	1.66 b	0.86 a	1.84 a	4.84 a	0.049 b	0.004 a	0.011 b	0.053 b	0.065 b
	- 0.073	1.80 a	2.00 ab	0.89 a	1.65 a	4.95 a	0.058 ab	0.004 a	0.013 b	0.062 ab	0.075 b
	- 0.11	1.48 b	2.13 a	1.02 a	1.42 a	5.26 a	0.068 a	0.004 a	0.017 a	0.072 a	0.090 a
	DMS	0.24	0.40	0.27	0.56	0.73	0.011	0.003	0.004	0.011	0.013
60	0	1.82 a	2.55 b	1.08 a	1.5 a	7.26 b	0.089 b	0.004 a	0.023 a	0.093 b	0.116 b
	2.5	1.73 a	2.77 ab	1.00 a	1.34 a	7.37 b	0.090 b	0.002 a	0.022 a	0.091 b	0.114 b
	5	1.83 a	2.44 b	1.32 a	2.02 a	7.12 b	0.088 b	0. 004 a	0.022 a	0.092 b	0.114 b
	10	1.78 a	3.44 a	1.01 a	1.58 a	7.23 b	0.095 b	0.003 a	0.031 a	0.098 b	0.130 ab
	15	1.91 a	3.00 ab	1.28 a	1.41 a	8.07 a	0.13 a	0.002 a	0.027 a	0.128 a	0.156 a
	DMS	0.43	0.73	0.52	0.93	0.17	0.025	0.005	0.013	0.025	0.033
	PO (MPa)										
	- 0.037	1.80 ab	2.73 b	1.82 b	0.99 b	6.92 a	0.090 a	0.002 a	0.026 a	0.092 a	0.117 a
	- 0.073	1.98 a	2.80 b	1.32 a	1.92 a	7.70 a	0.104 a	0.003 a	0.027 a	0.107 a	0.134 a
	- 0.11	1.66 b	3.00 a	1.28 a	1.80 a	7.62 a	0.099 a	0.004 a	0.024 a	0.103 a	0.127 a
	DMS	0.28	0.18	0.34	0.61	0.77	0.017	0.003	0.008	0.016	0.022
90	0	1.83 b	2.77 b	1.71 ab	3.11 ab	8.03 b	0.093 b	0.009 ab	0.032 c	0.101 b	0.134 b
	2.5	1.72 b	2.88 b	1.70 ab	2.10 b	7.86 b	0.108 b	0.006 b	0.040 bc	0.113 b	0.153 b
	5	1.80 b	2.56 b	1.58 b	2.68 ab	7.49 b	0.104 b	0.009 ab	0.033 c	0.113 b	0.146 b
	10	2.30 ab	3.11 ab	1.53 b	2.84 ab	8.60 ab	0.138 b	0.010 ab	0.051 ab	0.148 b	0.198 b
	15	2.81 a	3.78 a	2.31 a	3.50 a	9.87 a	0.300 a	0.0158 a	0.056 a	0.314 a	0.371 a
	DMS	0.61	0.73	0.68	1.27	1.50	0.121	0.009	0.016	0.124	0.122
	PO (MPa)										
	- 0.037	1.85 b	2.73 b	1.86 a	3.10 a	7.74 b	0.110 a	0.012 a	0.037 a	0.122 a	0.159 a
	- 0.073	1.97 b	2.93 ab	1.69 a	2.80 a	8.40 ab	0.176 a	0.008 a	0.046 a	0.184 a	0.230 a
	- 0.11	2.47 a	3.40 a	1.74 a	2.64 a	8.97 a	0.159 a	0.009 a	0.044 a	0.168 a	0.216 a
	DMS	0.40	0.48	0.44	0.83	0.99	0.079	0.006	0.010	0.814	0.080
120	0	2.12 b	3.00 b	1.77 ab	2.92 a	9.50 b	0.134 b	0.010 a	0.042 b	0.143 b	0.186 b
	2.5	2.17 b	3.22 b	1.77 ab	2.28 a	9.21 b	0.145 b	0.010 a	0.044 b	0.551 b	0.199 b
	5	2.26 b	3.44 b	1.91 ab	2.11 a	9.41 b	0.156 b	0.0067a	0.047 b	0.162 b	0.210 b
	10	2.18 b	3.77 ab	1.40 b	2.40 a	9.86 b	0.170 b	0.008 a	0.052 b	0.179 b	0.231 b
	15	2.76 a	4.55 a	2.21 a	3.37 a	11.92 a	0.362 a	0.0126 a	0.107 a	0.375 a	0.482 a
	DMS	0.49	0.79	0.76	2.04	1.96	0.061	0.010	0.030	0.061	0.084
	PO (MPa)										
	- 0.037	2.28 b	3.40 b	1.80 a	2.57 a	8.98 b	0.156 a	0.009 a	0.048 a	0.165 b	0.213 b
	- 0.073	2.26 b	3.56 b	1.71 a	2.30 a	8..95 b	0.202 a	0.007 a	0.063 a	0.209 a	0.272 a
	- 0.11	2.56 a	3.93 a	1.92 a	2.97 a	10.01 a	0.222 a	0.013 a	0.064 a	0.235 a	0.299 a
	DMS	0.27	0.32	0.50	1.34	1.01	0.040	0.007	0.019	0.406	0.055

* Means with the same letter are not statistically different. $\alpha \leq 0.05$

Statistical analysis of data from the dry weight for the different organs of seedlings show Agave $N–NO_3^-$ response in biomass formation, except the stem (Table 1). The leaf dry

weight (LDW), stem (PST) and seedling (PSP) showed highly significant differences from the second sampling, in response to the concentration of $N–NO_3^-$. The root dry weight (RDW) and PSP generally increased as the concentration of $N–NO_3^-$ increased in the SN. The organ that showed greater sensitivity to the effect of N was the synthesis of leaf tissue, the dry weight of the root system was the one with minor changes (Table 1).The PO had no significant effect over the organs dry weight of seedling in the first three samples, but in the fourth sample tested organs were affected by PO to the extent that the SN had more concentrated nutrients (-0.11 MPa). These results are similar to those found by Nobel and Hartsock (1986), Nobel et al. (1988, 1989) on the influence of nitrogen and other elements in the CAM plants, which stimulated the uptake of CO2 levels, increasing the number of leaves unfolded and duplicate the yield of pineapple compared to plants without nutrient application (Valenzuela and González, 1995).

CONCLUSIONS

1. The increase in the concentration of N in the nutrient solution, was the main factor that determined the growth and quality of seedlings of *Agave durangensis*.
2. The parameters that define the quality of seedlings (plant height, leaf number and dry weight of seedling) augmented by increasing the N concentration in the nutrient solution. The highest values of biomass accumulation were held within the treatment of 15 mol $m^{-3} N–NO_3^-$.
3. The best treatment for the accumulation of biomass was obtained with all osmotic potentials of -0.036, -0.073 and -0.11 MPa.

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BIOCONTROL OF SOFT ROT AGAVE BY BACTERIOPHAGES

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ABSTRACT

Cultivation of several species of the genus *Agave* is used as raw material for the production of beverages such as *A. tequilana* and *A. cupreata*. However, the cultivation of these species is threatened by pests and phytosanitary problems, among them are the weevil (*Scyphophorus acupunctatus*), wilt (*Fusarium oxysporum*) and soft rot (several bacterial species). Especially rot causes great losses in agave fields for the production of tequila and mezcal in the states of Jalisco and Michoacán in Mexico. Results of chemical control of soft rot have not been successful. On the other hand, bacterial viruses (bacteriophages) are employed for efficient fight off bacterial diseases in plants. So the aim of this work was to isolate and characterize bacteriophages associated with the causal agent of soft rot for *A. tequilana* and *A. cupreata*. Samples of crops with agave soft rot were performed on sites of Jalisco and Michoacán. Bacteriophages were isolated from phytopathogenic and virulent bacterial isolates and were characterized by transmission electron microscope and restriction patterns. Three morphotypes of bacteriophages were isolated from four bacterial species involved in soft rot of agave. The bacteriophages lysed differential magnitude the bacterial cultures *in vitro*. These results suggest the possible application for biocontrol of bacteria involved in soft rot of agave, and this would help to the sustainable production of the raw material for tequila and mezcal.

Keywords: Bacterial viruses, *Bacillus* sp, Plant pathogenic bacteria.

INTRODUCTION

Agave tequilana Weber var. Azul is the plant from which tequila is obtained, booze representative of Mexico, where more than 100000 hectares of agave are currently grown in the states of Jalisco, Nayarit, Michoacán, Guanajuato and Tamaulipas.Similarly, *A. cupreata* is an important plant for the production of mezcal in Michoacán (Martínez-Palacios y col. 2011). The propagation of blue agave is mainly through asexual reproduction however, this

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monoculture system has had serious phytosanitary consequences for the tequila industry, increasing the incidence of diseases caused by fungi such as *Fusarium oxysporum* (Vega-Ramos et al. 2013) and bacteria *Pectobacterium*, *Pantoea*, *Pseudomonas*, *Bacillus*, *Arthrobacter* and *Streptomyces* (Rincón-Enríquez et al. 2014; Jiménez-Hidalgo et al. 2004), causing losses estimated in around 40 percent of agave plantations affected by these microorganisms. Presently, the use of copper-based agrochemicals and antibiotics are the main tools used by the producers of agave to combat these pests. However, the use of bacteriophages to control bacterial diseases has been in recent years a field of great interest in the area of plant health with a great potential to replace the use of currently used agrochemicals. For example, in other bacterial pathosystems, bacteriophages have been used with successful results (Frampton y col. 2012). For example in the pathosystem apple (*Malus domestica*) - fire blight (*Erwinia amylovora*), bacteriophages were isolated and assessed successfully decreasing the symptoms of blight in apple by applying the virus (Gill y col. 2003.). Also, in the pathosystem tomato (*Solanum lycopersicum*) - bacterial wilt (*Ralstonia solanacearum*) application of bacteriophages in plants inoculated with *R. solanacearum* showed a significant decrease of the disease (Fujiwara y col. 2011). Bacteriophages can be used effectively as part of integrated management strategies for plant diseases. Also, the use of these presents several advantages over the use of copper compounds and antibiotics, because of the relative low manufacturing cost in addition to the high specificity for the host, which makes them good candidates for use as biocontrol agents (Jones y col. 2007). Therefore, the objective of this work was to isolate and characterize specific bacteriophages of pathogenic bacteria associated with soft rot bud in *A. tequilana* and *A. cupreata.*

METHODOLOGY

Collection of soil samples

183 soil samples (20 cm deep) from the base of healthy agaves and rot symptoms were collected, from different sampling points located in the states of Michoacán (8 sites) and Jalisco (6 sites).

Bacterial host strains

For isolation of bacteriophages 11 bacterial isolates obtained from diseased plants from the same sampling sites (named BV) were used. Bacterial isolates were characterized and analyzed to identify virulence factors such as the production of cellulase enzymes. The genera to which belonged some of these bacterial isolates were: *Pantoea*, *Pseudomonas*, *Bacillus*, *Arthrobacter* y *Streptomyces* (Rincón-Enríquez et al. 2014).

Isolation, propagation and characterization of bacteriophages

The isolation, propagation and characterization of bacteriophages was conducted following the procedure described by Solís et al. (2014). Briefly, this procedure consisted of: isolation which was performed from soil samples by enrichment by using 100 g of soil sample in 100 mL of nutrient broth and 600 µL of each of the bacterial cultures (16 h/ 30°C) and incubated for 18-24 h at 30°C. Subsequently, cultures were centrifuged at 10,000 g × 20 min and supernatants were recovered and filtered through 0.22 µm membrane. The presence of phages was confirmed by a test double-layer soft agar by mixing 100 µL of bacterial culture, 100 µL of filtered supernatant enrichment and 5 mL of nutrient agar (0.7%), incubated at 30°C for 18 h and examined for the presence of lysis areas or plaques. After single plaques were isolated and propagated in liquid medium until high viral titers (>$1x10^8$ plaque forming

units). Pure bacteriophages were used to test the range of infection and with these data a cluster analysis was performed using the Unweighted Pair Group Method algorithm using Arithmetic averages (UPGMA) through the statistical program StatGraphics Centurion XV. Subsequently, purification of the viruses was performed by centrifugation (70-80000Xg 4-6 h) and washing (0.1 M ammonium acetate pH 7) for subsequent analysis of bacteriophages in a transmission electron microscope (TEM) Jeol JEM-1010 (grid Fombar-coal coated copper 300 mesh, stained with 5 µL uranyl acetate).

RESULTS AND DISCUSSION

183 soil samples were collected, of which 77 were tested, and 40 virus isolates were obtained, all clearly showed the formation of plaques which correspond to the replication cycle of lytic bacteriophages, also a high diversity of phages was observed on the size of the plaques formed in double plate assays (Figure 1 A, B, C and D). Cluster analysis of infection-rank test showed two aspects: three viral isolates from 648 and 649 strains were able to lyse up to 8 different bacterial strains, and these were of broad spectrum (Figure 1F); while on the side of the bacterial species, the 648 strain was the one that showed higher susceptibility to infection, as 20 viral isolations lysed this strain (Figure 1G). Moreover, the large number of isolates were obtained in soil samples collected from agave plants with soft rot.

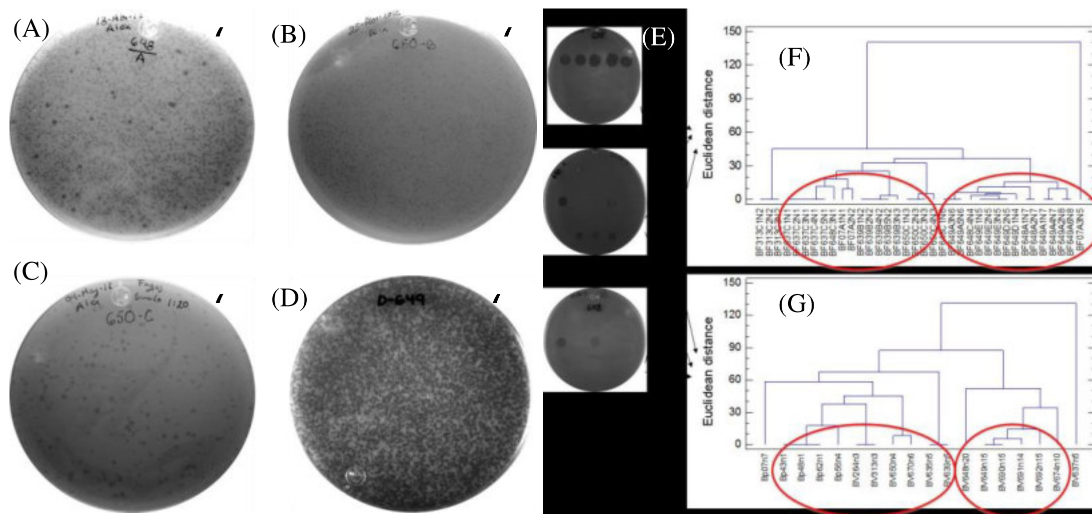

Figure 1. Diversity, density and virulence of bacteriophage associated with bacteria involved in soft rot of agave. Bacteriophages isolated using: (A) 648 strain: two types of lytic plaques are observed. (B) 650 strain: shows a uniform high amount of plaques which suggest a package that is a single virus. (C) 650 strain: 1:20 dilution, different plates with different sizes are observed, suggesting the presence of more than one type of bacteriophage. (D) 649 strain: high number of plates with the same morphology. (E) Infection assay range: in the same bacterial strain different serial dilutions of virus isolation are evaluated. (F) The bacteriophages showed a differential response to infect their bacterial hosts: the last two digits on each label the X axis shows the number of bacteria that phages can to lyse. (G) Some bacterial species were more susceptible to several bacteriophages: the last two digits on each label the X axis show the number of phages that can lyse a strain bacterial.

The characterization of the bacteriophages by TEM revealed that all virus isolates are of the

Siphoviridae family except 637-C-3, which corresponds to the *Podoviridae* family and the 313-C-1 which likely corresponds to *Myoviridae*. These families belong to the order *Caudovirales* or virus with glue, which are exclusive to bacteria and archeas, likewise, this is the group of viruses with more distribution and abundance of all the viruses and represent the majority of the bacteriophages described to date, they are generally lytic, but may present a lysogenic cycle. Bacteriophages belonging to the *Siphoviridae* family have isometric capsids with long noncontractile tails, as λ phage group, while *Myoviridae* has contractile tails. Whereas the *Podoviridae* family bacteriophage has isometric capsids and short tails not contractile (Figure 2).

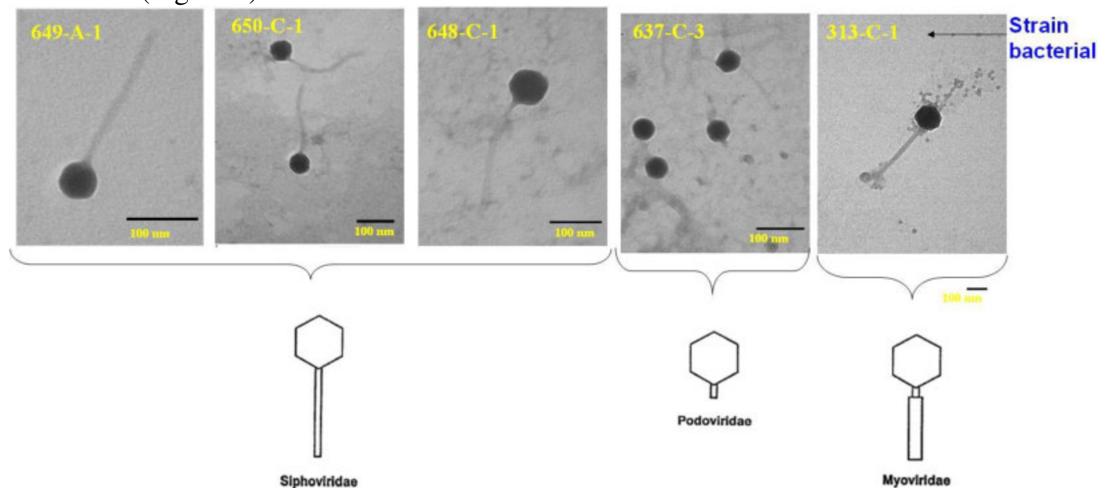

Figure 2. Transmission electron micrographs of bacteriophages from different families involved in the lysis of bacteria associated with soft rot of agave.

The results show the presence of specific bacteriophages for bacteria associated with soft rot of agave in soil samples; these bacteriophages have remained viable in soil samples and suggest alternative biological control for soft rot of agave.

CONCLUSION

Forty viral isolates were characterized and identified in the order Caudovirales of families *Siphoviridae*, *Myoviridae* and *Podoviridae*. All isolates of bacteriophages are lytic for the bacteria associated with soft rot of agave (*A. tequilana* and *A. cupreata*).

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MYCORRHIZATION OF *Agave cupreata* FOR PREVENTION OF WILT CAUSED BY *Fusarium oxysporum*

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) promotes plant growth on most cases through the transport of nutrients from the soil to the roots. In recent decades, they have also been used as biological control agents against various plant pathogens and the results show decreased incidence and / or severity of the disease due to the mycorrhizal symbiosis. Therefore, the aim of this study was to evaluate the effect of mycorrhizal plants *Agave cupreata* in bioprotection against *Fusarium oxysporum. A. cupreata* plants seven month old, mycorrhizal and non-mycorrhizal under greenhouse conditions were used to evaluate the effect of bioprotection against *F. oxysporum.* The severity of the disease (SE) based on the observed damage in the leaves (chlorosis and wilting) of plants *A. cupreata* was evaluated by an ordinal scale, at 20 and 90 days after inoculation of the plant pathogen. The results showed colonization of fungi (AMF) in agave plants, regarding bioprotection, plants of *A. cupreata* presented initial wilt symptoms and severity of disease averaged 1.3 to 1.82 (first yellow leaf low, plant damage between 8-21%) with no significant differences (Kruskal-Wallis, $p \leq 0.05$) between plants with and without AMF at 90 d after inoculation with *F. oxysporum* strain FPC. This result was probably due to the physiology of the plant, so more time action pathosystem (mycorrhized agave-*F. oxysporum*) is required to assess the bioprotection of AMF against *F. oxysporum.*

Keywords: *Mycorrhiza, bio-protection, wilt agave.*

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INTRODUCTION

Agave cupreata is a species of economic importance to Mexico, because from the fermentation of stems ("pineapples") of adult plants produced mezcal (Martínez-Palacios y col. 2011). During 2013, mezcal region from Michoacán entered the Denomination of Origin of Mezcal (DOM); which will bring benefits to the mezcal industry. Also, an increase in the cultivated area *A. cupreata* expected, so better agronomic and phytosanitary management will be important to maintain the sustainability of this crop (Martínez-Palacios y col. 2011).
However, the increase in crop area could increase the incidence of disease, as has happened in plantations of A. tequilana (Vega-Ramos y col. 2013). One of the phytosanitary problems that significantly affect productivity agave is wilt, caused by the plant pathogenic fungus *Fusarium oxysporum*. The infection process of *F. oxysporum* starts at the roots, then a curl of leaf, death of leaf tips is presented and finally a wilting occurs throughout the plant (Qui-Zapata y col. 2011; Vega-Ramos y col. 2013). This problematic occurs in plant producing regions such as mezcal A. cupreata. Conventional control of F. oxysporum based fungicides has not been effective, also generate environmental problems, damage the soil and increase production costs (Bernal-Alcocer y col. 2005). Therefore, alternatives are required to control power *F. oxysporum* and thus decrease loss of agave. The use of microorganisms has been an alternative for biological control of various plant pathogens, as in the case of arbuscular mycorrhizal fungi (AMF), which are obligate symbionts that need roots plant to complete its life cycle. From this bidirectional symbiosis, the mycorrhizal fungi promotes plant growth by absorbing nutrients (P and N) of the soil to the roots of the plant and the plant turn provides the AMF from a source carbon (Smith and Read, 2008). Furthermore AMF are effective as bioprotectors against various phytopathogens. The results of several studies suggest that in most cases there is a reduction in the incidence and / or severity of disease due to the mycorrhizal symbiosis. For example, Steinkellner y col. (2012) found a bioprotector effect *Funneliformis mosseae* in tomato varieties (*Solanum lycopersicum* L.) against *F. oxysporum* f. sp. *lycopersici* (Fol); Hage-Ahmed y col. (2013) also showed that commercial AMF (Symbivit®) reduced the severity of disease caused by Fol. Hu y col. (2010) by inoculating cucumber (*Cucumis sativus* L.) and a native *F. caledonium* AMF consortium found decreased incidence of F. oxysporum inoculation with native consortium. Jaiti y col. (2007) showed that inoculation with native AMF consortium date palm (*Phoenix dactylifera* L.) decreased plant mortality effect of *F. oxysporum* f. sp. *albedinis*. In Maradol papaya (*Carica papaya* L.), Hernandez-Montiel y col. (2013) found significant difference in reducing the severity of disease caused by *F. oxysporum* when papaya plants were inoculated with a consortium of AMF. These works show that AMF may have bioprotector effect against *Fusarium*. Wherefore, the aim of this study was to evaluate the effect of mycorrhization of *Agave cupreata* by four consortia AMF native and a commercial inoculum (mycorrhizal INIFAP®, MI) in bioprotection against *Fusarium oxysporum*.

METHODOLOGY

A. cupreata seeds were placed *in vitro* conditions for the production of agave seedlings. After ten days, agave plants were placed in trays (38 wells) for growth on a substrate mixture of sand-perlite (4: 1, v: v) sterilized (121°C for 6 h), then was inoculated with 100 spores of AMF under study and placed in the greenhouse until 7 months. After three months after planting, seedlings were fertilized with low in phosphorus nutrient solution (Jarstfer and Sylvia, 1992) biweekly. From the sixth month after planting was stopped applying nutrient solution and watered with distilled water until the end

of the experiment. At the seventh month agave plants were inoculated with *F. oxysporum* at a final concentration of $1x10^4$ cfu g^{-1} (250 mL container with a mixture of sand-sphagnum peat-perlite substrate 4: 1: 1, v: v: v) and remained confined under conditions of growth chamber (8 h photoperiod at 30°C). The evaluation of the experiment was performed at 20 and 90 days after inoculation with the pathogen.

AMF and *Fusarium oxysporum* inoculum

Four native consortia from rhizosphere of *A. cupreata* Michoacán were used: El Huizachal (EH, N 19° 25' 31.4", O 101° 12' 51.4"), Cerro del Metate (CM, N 19° 34' 22.3", O 100° 56' 27.5"), Paso Ancho (PA, N 19° 30' 1.7", O 100° 54' 51.1") y Agua Dulce (AD, N 19° 32' 4.4", O 101° 04' 39.4") and commercial inoculum "Micorriza Inifap" (MI), based de *Glomus intraradices*. F. oxysporum strain FPC at CIATEJ collection was used, which causes wilt in A. tequilana (Qui-Zapata y col. 2011) and previously evaluated as phytopathogenic in A. cupreata (Trinidad-Cruz y col. 2013).

Experimental design and statistical analysis

Experimental design was completely randomized with 12 treatments: 6 levels of AMF: CM, PA, EH, AD, MI without AMF; 2 levels of pathogen: with and without *F. oxysporum*. Each *A. cupreata* plant was a experimental unit and nine replicates of each treatment were evaluated. The degree of severity (SE) of the disease was evaluated following the ordinal scale proposed by De Cal y col. (2000), briefly: 1 = healthy plant; 1.1 to 1.9 = one yellow leaf; 2 = 2.9 more a yellow leaf and a dead leaf, 3 to 3.9 = dead lower leaves and upper yellow leaves; 4 to 4.9 = lower leaves dead and upper leaves wilted and 5 = plant dead. Data from this scale were analyzed using the Kruskal-Wallis test ($p \leq 0.05$) and confidence intervals for the median ($p \leq 0.05$) were calculated using the statistical package StatGraphics Centurion XV.

RESULTS AND DISCUSSION

At 20 days after inoculation (DDI) with *F. oxysporum* significant differences (Kruskal-Wallis, $p \leq 0.05$) between the control treatment with *F. oxysporum* and *F. oxysporum* treatments without (Figure 1 **i**) were found. Subsequently 90 DDI, significant differences between CM, EH and MI treatments more *F. oxysporum* with respect to treatments without plant pathogen were found (Figure 1 **ii**). Was observed that the severity of disease (SE) on the foliage of plants of *A. cupreata* advanced slowly over time. Due to this, the damage at the root caused by *F. oxysporum* (FPC) in agave was visually analyzed in plants with and without AMF (Figure 1 **iii**). Smaller root damage due to FPC was observed in plants with AMF (CM + FPC) compared to non mycorrhizal seedlings (C + FPC) (Figure 1 **iii**). In this regard, Gardezi y col. (2001) evaluated the effect of a native consortium (*Glomus* sp. Zac-19) and *G. aggregatum* in plant bioprotection gladiola (*Gladiolus grandiflorus*) against *Fusarium* sp and found that root rot in plants AMF significantly decreased with respect to plants without AMF.

Montoya-Martínez (2014) *A. tequilana* plants inoculated with native consortium (the rhizosphere of *A. cupreata*) and a commercial for evaluating the effect of AMF bioprotector against *F. oxysporum* strain FPC, found that in plants with and without AMF (100 days after inoculation) showed initial symptoms of disease as curl and necrotic at the tips of the lower leaves and indicated that *A. tequilana* a longer-term development is needed in order to have greater accuracy in results. This result is reinforced in a study of Avila-Miranda y col. (2010) evaluating the virulence of strains of *F. oxysporum* in *A. tequilana* plants and observed initial symptoms (chlorotic leaf curl and wilt) from 200 days after inoculation. So

it physiology agave plants, can delay the symptoms of wilt and therefore the period of observation of these experiments should be extended to longer.

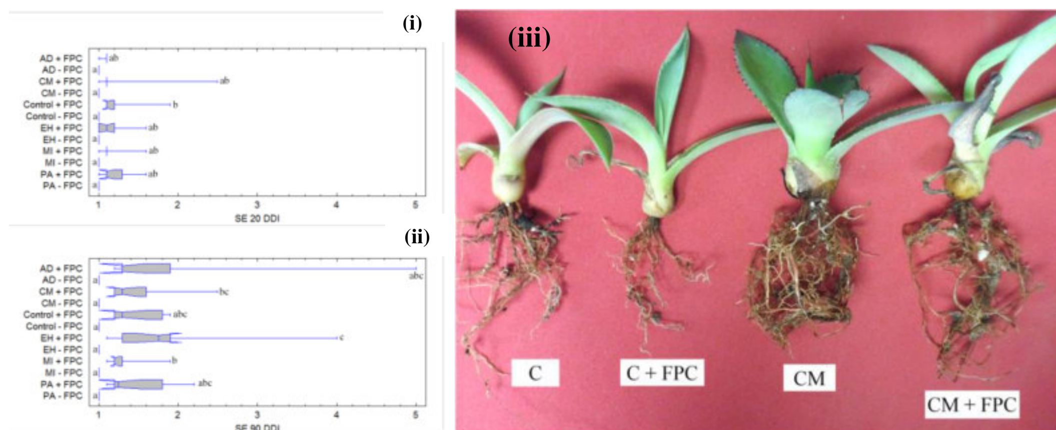

Figure 1. Effect of mycorrhization on mezcal agave plant wilt three months after inoculation with *F. oxyporum* (FPC). **(i)**, **(ii)** Box and whisker graphs of the distribution of the severity scale wilt (SE) of *A. cupreata*, at 20 and 90 days after infection (DDI). Control = without AMF; + FPC, -FPC: with and without *F. oxysporum*; AD: Agua Dulce; CM: Cerro del Metate; EH: El Huizachal; PA: Paso Ancho; MI: Micorriza commercial. **(iii)** Symptoms of wilt in plant *A cupreata* roots inoculated with F. oxysporum at 90 DDI. C: without AMF; C + FPC: without AMF + *F. oxysporum*; CM: Cerro del Metate; CM + FPC: Cerro del Metate + *F. oxysporum*; Bar = 5 cm.

CONCLUSION

Mezcal agave plant (*Agave cupreata*) with and without AMF had similar foliar wilt symptoms to 90 days after inoculation with *Fusarium oxysporum.* A root level damage agave mycorrhizal plant was lower compared to without mycorrhiza plants.

ACKNOWLEDGEMENTS

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Climate change reaches the Tequila country

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ABSTRACT

Climate change is an overwhelming fact that is changing the way of life of the living beings. The years 1998 and 2005 have been the hottest years since 1850, being human activities as almost certainly causing the planet to warm. The main challenge for scientists and politicians is ensuring the food security, not only for the world's poorest people, but for all. In this context, it is well known that abiotic stress such as drought but also excess water and temperature stress (heat and cold) have a great impact on phenology and potential yields.
On the other hand, plants exhibit a wide range of sensitivities to temperatures extremes. There is an ideal temperature at which each plant grows and develops the most competently, and there upper and lower limits. When the temperature varies outside these limits, there is no growth at all. Often a change of a few degrees substantially affects the plants growth and developmental processes, particularly reproduction.
The aim of this work was to analyze the temperature trends over the last 25 years on the tequila country and to study the putative effect of heat on the flowers and the female gametophyte of *Agave tequilana*.
Raw temperature data, for the municipalities of Tequila, Autlán, Cocula and Arandas from 1989 to 2013. Records of temperatures were kindly provided by CONAGUA – Jalisco.
It was found that in all four localities there was a significant rise of temperatures in the year 1998. For example, in Tequila the difference between the overall mean of the colder years (1989-1997) *vs* the overall mean of the hottest years (1998-2013) was 1.1°C.
Also, the putative negative effect of hot temperatures on the development of flowers and embryo sacs of *A. tequilana* could be observed, frequently causing loss of reproductive viability.

Key words: *Agave tequilana*, high temperature, sterility, abnormal embryo sac, abnormal flowers.

INTRODUCTION

Climate change is an overwhelming fact that is changing the way of life of the living beings. The Intergovernmental Panel on Climate Change (IPCC) reported that the years 1998 and 2005 have been the hottest years since 1850 (IPCC, 2007) and said that, human activities are

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almost certainly causing the planet to warm. The main challenge for scientists and politicians is ensuring the food security, not only for the world's poorest people, but for all. In this context, it is well known that abiotic stress such as drought but also excess water and temperature stress (heat and cold) have a great impact on phenology and potential yields.

Plants exhibit a wide range of sensitivities to temperatures extremes. There is an ideal temperature at which each plant grows and develops the most competently, and there upper and lower limits. When the temperature varies outside these limits, there is no growth at all. Often a change of a few degrees substantially affects the plants growth and developmental processes, particularly reproduction. During the life cycle of flowering plants, reproductive development is considered a significant and delicate process and abiotic stresses, particularly heat and cold, have a detrimental effect on the early stage of male gametophyte in important crops such as rice, wheat, maize, barley, sorghum, and chickpea (Boyer and Westgate, 2004). Heat is one of the leading abiotic stresses that have a significant impact on growth and development of plants (Sha Valli Khan et al. 2014).

Hot or cold temperature stresses can be detrimental to all phases of plant development (Zinn et al. 2010). While temperature stress has been extensively studied and reviewed, most of the literature emphasizes insights from experimental accessible tissues, such as roots and leaves. By comparison, studies on sexual reproduction are often more difficult because gamete development and fertilization are complex processes that occur during a short window of time, and are predominantly hidden within tissues of the flower. Excess heat causes male and female abnormalities, from morphology to sterility (Zinn et al. 2010).

The aim of this work was to analyze the temperature trends over the last 25 years on the tequila country and to study the putative effect of heat on the flowers and the female gametophyte of *Agave tequilana*.

METHODOLOGY

Raw temperature data, for the municipalities of Tequila, Autlán, Cocula and Arandas from 1989 to 2013, kindly provided by CONAGUA - Jalisco was ordered, analyzed and graphed. Plant material consisted of embryo sacs extracted from immature flower buds taken from mature *Agave tequilana* plants collected in the zone of Denomination of Origin Tequila in the State of Jalisco, México. Embryo sacs were processed following Stelly et al (1984) and observed by using a Leica® DMR microscope (Wetzlar, Alemania) coupled to an Evolution QEi® camera. Images were taken using the Image Pro® software (Media-Cybernetics, Bethesda, USA), and microphotographs were processed with Adobe Photoshop Software version CS6, and evenly adjusted for better contrast.

RESULTS AND DISCUSSION

It was found that in all four localities there was a significant rise of temperatures in the year 1998 (Figure 1). For example, in Tequila the difference between the overall mean of the colder years (1989-1997) *vs* the overall mean of the hottest years (1998-2013) was 1.1°C.

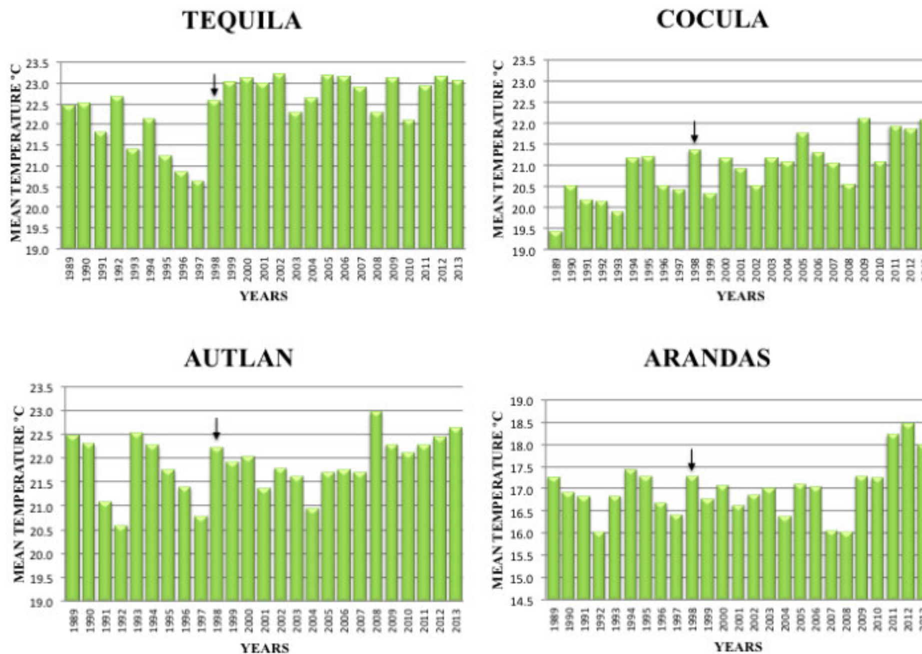

Figure 1. High temperature trends in the localities of Tequila, Cocula, Autlán and Arandas in the state of Jalisco, México from 1989 to 2013.

In regard to the putative effect of heat on female gametophytes, a high number of abnormalities were found in embryo sacs collected in the year 2010 (Figure 2). Many embryo sacs were empty of cells. Others, stopped their development at the very beginning. In the year 2012, apparently healthy and normal flowers with normal embryo sacs were pollinated with viable pollen, however, no seeds were produced.

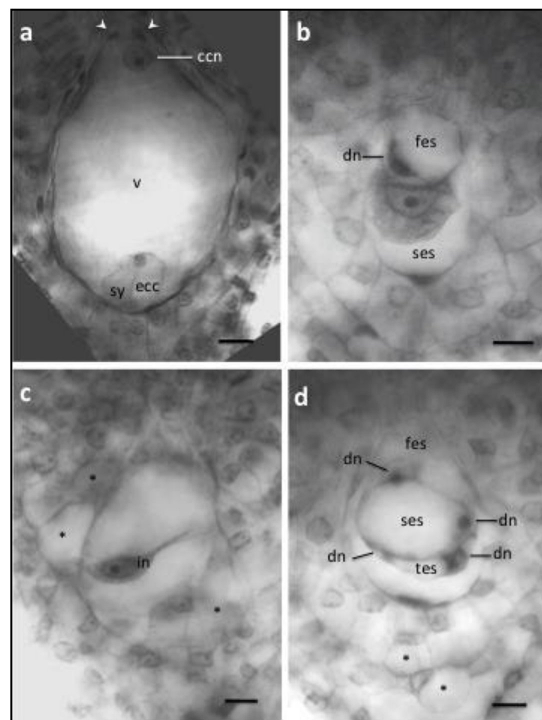

Figure 2. Putative abnormalities caused by heat on the embryo sac of *Agave tequilana*. a) Normal embryo sac. b, c and d) Abnormal embryo sacs. ccn=central cell nucleus, v=vacuole, sy=synergid, ecc=central cell, dn=degenerating nucleus, fes=first embryo sac, ses=second embryo sac, in=indeterminate nucleus, tes=third embryo sac, black dots in c and d=abnormal nucellar tissue, white arrow heads in a=antipodals. Bars=20 μm.

Other embryo sac abnormalities could be observed, such as abnormal and disrupted nucellar tissue (Figure 2c), formation of two or three embryo sacs (Figures 2 b and d) and indeterminate and degenerated nuclei (Figures b, c and d).

Also, flowers with an abnormal morphology were frequently collected. There were plants with abnormal flowers and female gametophytes along with plants bearing normal flowers and gametes, meaning that there are plant genotypes showing some tolerance to heat stress (Figure 3).

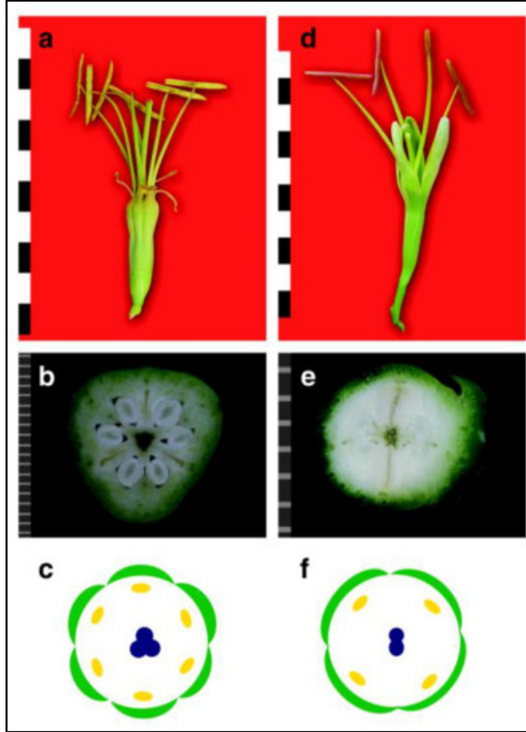

Figure 3. Normal and abnormal flowers of *Agave tequilana* showing the putative effects of hot temperatures in the state of Jalisco, México. a, b and c=normal flower with six anthers. d, e and f)=abnormal flower with four anthers. Bars in a and d=centimeters. Bars in b and e=millimeters.

In *A. tequilana* as in other plant species, hot or cold temperature stresses have a significant effect on the reproductive (gametophytic) phase, either in the male or in the female gametophyte such as in canola (Polowick and Sawhney, 1988; Young et al. 2004) and tomatoes (Peet et al. 1998).

CONCLUSION

Putatively heat stress affects the flowers and the megagametophytes of *Agave tequilana*, however, there exist the possibility of finding heat tolerant plants with viable embryo sacs, which are indicative of high temperature tolerance (Bita and Gerats, 2013). Even when the flower stalk and the resulting fruits and seeds are not useful for the tequila industry, these plants can be selected and included in programs for genetic improvement of this important species of agave.

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NOTE ADDED WHILE IN PRESS

The US National Oceanic and Atmospheric Administration released the October 2014 Global Climate Report where the January-October combined global average temperature is reported as the warmest period surpassing the previously mentioned record for 1998, being October 2014 as the highest on record for the month since record keeping began in 1880 (NOAA, 2014).

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MICROPROPAGATION OF *Agave victoriae-reginae* (T. MOORE) IN A TEMPORARY IMMERSION SYSTEM

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ABSTRACT

Agave victoriae-reginae T. Moore is an endemic species from Mexico which due to its high ornamental value has been submitted to an extensive illegal collection for commercial trade. Currently, it is listed as extinction endangered species by the Mexican government. In the present study the *in vitro* multiplication of *A. victoriae-reginae* was attempted for the first time by using a new temporary immersion system. Murashige and Skoog´s medium supplemented with Kinetin (Kn), 6-benzylaminopurine (BA), and Thidiazuron (TDZ) was tested at various concentrations, being the immersion frequency of one minute a day. Results showed that the best proliferation response was reached in a culture medium supplemented with 0.53 mg/L IBA and 0.1 mg/L BA, resulting in 7 shoots per initial explant after nine weeks of culture. This micropropagation system constitutes a promissory alternative to repopulate and conserve the *A. victoriae-reginae* natural populations for present and future.

Keywords: *Agave victoriae-reginae, conservation, endemic, in vitro, temporary immersion, micropropagation*

INTRODUCTION

Agave victoriae-reginae is an endemic plant from Mexico, which is catalogued as an extinction threatened species according to the NOM—059—SEMARNAT—2010 and the Appendix II by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Those plants had been subjected to intensive exploitation due its great value, mainly as exceptional ornamental plants. As result, their wild populations have been drastically reduced due to over collection and severe perturbation of their native habitat. All these facts make *A. victoriae-reginae* an important plant to be conserved by all means, including the *in vitro* culture.

Previous reports have established *in vitro* regeneration protocols for *A. victoriae-reginae* propagation whether by indirect somatic embryogenesis (Rodriguez-Garay et al., 1996) or

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by direct organogenesis design (Martínez-Palacios et al., 2003; Ramírez-Malagón et al., 2008). These authors cultured their *A. victoriae-reginae* plants on a semi-solid substrate including a gelling agent, e.g. agar, which is one of the most expensive ingredients in those culture media. Handling of that vegetal material and its periodic transfer to new media is time consuming and causes contamination and tissue damage (Weathers and Giles, 1988).

To overcome these difficulties associated with semisolid media; the propagation system based in temporary immersion provides an interesting approach. This system consists in the immersion of plant tissue during specific time periods in the culture medium (Etienne and Berthouly, 2002). The aim of this research was to develop an efficient protocol for the *in vitro* propagation of *A. victoriae-reginae* plant in a new temporal immersion system.

METHODOLOGY

Plant material

Leaves (5–8 cm length) of *Agave victoriae-reginae* plants, previously *in vitro*-grown by Dr. Rafael Ramírez Malagón on MS medium containing agar (0.8%), were inoculated into MS liquid medium (Murashige and Skoog, 1962).

Shoot induction

Explants were inoculated into translucent and autoclavable glass bottles (15x15x50 mm), containing 50 ml MS liquid medium, which operates on the principle of temporary immersion. In this system, plants are placed on a plastic net that separates the vegetal material from the liquid media. For shoot induction twelve treatments were evaluated. The basal MS medium was supplemented with different levels of cytokinins; such as Kinetin (Kn), 6-benzylaminopurine (BA), and Thidiazuron (TDZ), and the auxin indole butyric acid (IBA), according to Ramírez-Malagón et al. (2008) and Martínez-Palacios et al. (2003) (Table 1). The media pH was adjusted to 5.6-5.8 in all cases.

Table 1. Description of plant hormones used for the shoot induction of *Agave victoria-reginae* in a temporary immersion system.

Treatments	Plant hormones (mg L^{-1})			
	IBA	BA	Kn	TDZ
T1	0.53	0.1	-	-
T2	0.53	0.5	-	-
T3	0.53	1	-	-
T4	-	0.1	-	-
T5	-	0.5	-	-
T6	-	1	-	-
T7	-	-	1	-
T8	-	-	3	-
T9	-	-	5	-
T10	-	-	-	0.1
T11	-	-	-	0.2
T12	-	-	-	0.3

One shoot was added per vessel and every experiment was repeated four times. Bottles were randomly placed in the growth chamber and maintained at 25°C under a 16-h photoperiod provided by cool white fluorescent lamps (23-26 µmol m^{-2} seg^{-1}). Temporary immersion cultures were established with immersion of explants for 1 min every 24 h. Variance analysis was conducted to evaluate the results, and Tukey´s test was used to separate the data means.

RESULTS AND DISCUSION

New adventitious shoots emerged from initial explant after nine weeks of culture for all treatments. Analysis of variance indicated that medium composition significantly affected the shoot number developed per explant.

The treatment containing 0.53 mg/L IBA and 0.1 mg/L BA (T1) showed the best multiplication rates, with around of seven shoots per explant. The rest of treatments not exceeded three shoots per explant, being less efficient than treatments T10 and T11 (Figure 1).

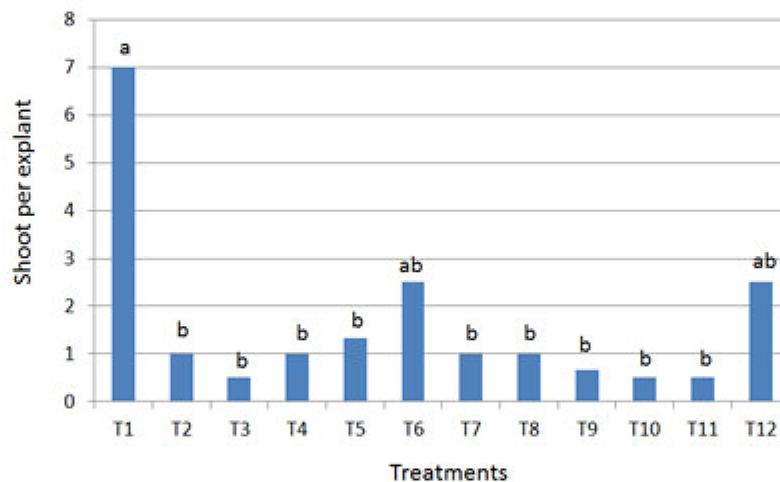

Figure. 1. Plant hormone effects on *Agave victoria-reginae* shoot induction growning in a temporary immersion system. T1 to T12 correspond to the twelve treatments evaluated using MS medium supplemented with different plant hormones combinations. The bars with different letters indicate significant differences between treatments at $p<0.05$ according to Turkey's test.

After nine weeks of culture, the agave plants remained with vigorous appearance and without any sign of hyperhydricity. The necrotic tissue presence was lower than 5% for all evaluated treatments (Figure 2).

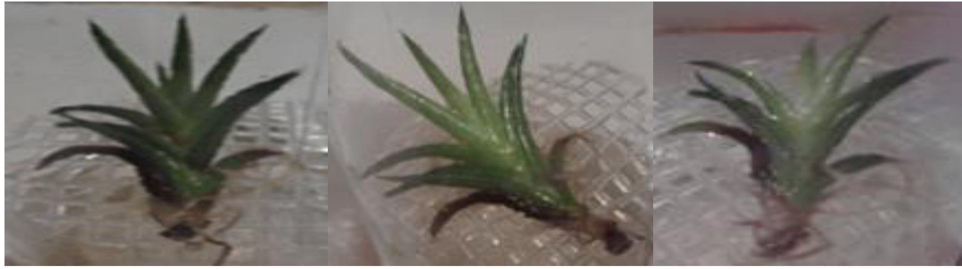

Figure. 2. *Agave victoriae-reginae* plants after nine weeks grown on temporary immersion system.

Up to our knowledge, there are a few reports about the *in vitro* regeneration of *A. victoriae-reginae*. In those previous studies, the addition of BA favored the shoot proliferation even when only 1.1-2.2 axillary shoots were regenerated from *A. victoria.reginae* stems (Martínez-Palacios et al., 2003). Likewise, only 5.5 axillary shoots per explant of *A. victoriae-reginae*, using 2.46 µM IBA and 2.22 µM BA after 60 days of culture in semisolid medium, were induced (Ramirez-Malagón et al. 2008). Nonetheless, no reports were found about the *A. victoria reginae* shoot multiplication using a liquid culture system. In this regard slightly greater plant yields were obtained in the present study. In general, liquid culture systems can provide much more uniform conditions into the plants containers, due to the occurrence of a close contact and uniform entrance of nutrients and plant growth regulators to the explants. Also, the culture atmosphere is renewing in each immersion resulting in better growth rates (Etienne and Berthouly, 2003). In addition, the liquid system is useful to increase the scale of production and also enables its future automation reducing the production costs per plant. This fact converts the temporary immersion systems in a relevant tool to accelerate the conservation programs of extinction threated species.

CONCLUSIONS

This study demonstrated that it was possible to regenerate, in a practical way, *A. victoriae-reginae* vigorous shoots by using a temporal immersion system. The MS culture media supplemented with 0.53 mg/L IBA and 0.1 mg/L BA (T1) was found to be the most effective for inducing vigorous shoots of *A. victoriae-reginae*, obtaining 7 shoots per explant and suggesting the possibility of large-scale multiplication of this important endangered agave species.

ACKNOWLEDGMENTS

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Tequila and mezcal distillation technology: Similarities and differences

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The goal of tequila and mezcal distillation is to concentrate and to separate ethanol and chemical compounds that impart to each product its characteristic sensory character. Tequila and mezcal elaboration processes have similar stages: raw material preparation, agave cooking, fermentable sugars obtaining, fermentation, distillation and finishing processes but for every step there are specific conditions that determine the production of chemical compounds that cause differences between they.
Distillation of these alcoholic spirits can be carried out by using differential and/or continuous fractionation with different conditions such as reflux, cutoffs of heads and tails, utilizations of equipment manufactured with copper or with stainless steel and heat supply rate, for instance. To know concentrations profiles of regulated compounds along distillation and relevance of heads and tails cutoffs is important in order to control the process and to meet official specifications, to keep sensory character and to improve process efficiency. These factors are reviewed as well as a thermodynamic factor, the activity coefficient, which can help us to understand the behavior of concentrations of regulated compounds along distillation. The role of materials of construction of distillation equipment on quality of distillates is analyzed because of the effect of copper as a catalyst of favorable reactions and finally is showed profiles of concentrations of regulated chemical compounds along distillation of mezcal, which can be considered to establish the size of heads and tails.

Palabras clave: tequila, mezcal, distillation, alembic, continuous column.

INTRODUCTION

Making of tequila and mezcal, both Mexican distillates with designation of origin and with international acceptance, includes some stages characterized by its technological advancement which for tequila is higher than for mezcal process. Both processes include agave cooking (wet for tequila and dry for mezcal), sugars extraction, fermentation and distillation. Tequila production is regulated by official norm NOM-006-SCFI-1994 and mezcal by norm NOM-070-SCFI-1994.

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To carry out a distillation of fermented agave juice or from any fermented substrate, it is necessary to involve the following principles (Stichlmair and Fair, 1998): a) Creation of a two phase system, b) Mass transfer between both phases, c) Phases separation to obtain a different composition in each one. Developing of these steps demands a determined energy amount that is supplied like heat which is after removed by the condenser. Distillation needs an intimate contact between liquid and vapor phases, in such a condition, the chemical compounds in liquid phase transfer to vapor phase.

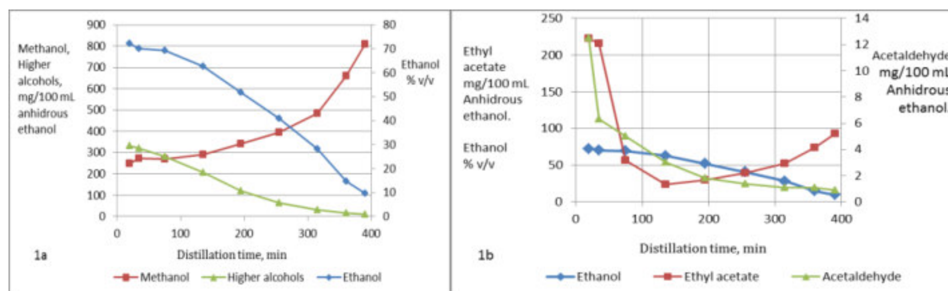
When we want to separate the less volatile components of a mixture, the distillation is called *stripping* and the separation of the more volatile compounds is called *rectification* or *enriching*. Separation of liquid mixtures with an important amount of volatile compounds, similar to those found in alcoholic spirits, involves mass transfer phenomena and thermodynamics of solutions, related to vapor-liquid equilibria, which is essential to solve and to estimate the efficiency of separation and the concentration of some compounds that are sensory significant. Fermented must and ordinario in tequila and mezcal processes are complex multicomponent mixtures because of the big amount of polar chemical compounds and can be considered nonideal mixtures and from a thermodynamic point of view calculations can be corrected introducing the dimensionless *activity coefficient*.

DISTILLATION PROCESSES IN TEQUILA PRODUCTION.

Batch distillation.

Tequila and mezcal can be distilled using a batch, or continuous scheme or under a combined arrangement batch-continuous or continuous-batch. In Jalisco, the main tequila producer state, most of companies use batch distillation but today increases the number of companies that make stripping or rectification or ethanol recovery from bottoms stream (vinasses) by using continuous columns. In batch distillation, the aim of the stripping is to obtain a distillate called *ordinario*, with 25-30% ethanol v/v. Some companies separate the first volume of distillate, called *heads*, and this cutoff is recycled to fermented must or pumped to the heads and tails storage tank. Stripping finishes when stream distillate has an ethanol content of 5-6% v/v. Goal of ordinario distillation is to increase the ethanol content up to 55% v/v and the product of this stage is called *rectificado*, which has most of the sensory compounds. In this step are also separated heads and the distillation continues up to the distillate has an ethanol content around 25% ethanol v/v. The distillate obtained after this cutoff is called *tails*.

Energy supply rate to alembic determines distillation time and ethanol concentration profile in distillate stream and the latter the profile of regulated compound as methanol, isoamyl alcohol, ethyl acetate and acetaldehyde. See Figures 1a and 1b. These profiles can be used to establish cutoffs of heads and tails remarking that acetaldehyde, ethyl acetate and isoamyl alcohol are found mainly in heads and higher methanol concentrations are found in tails.



Figures 1a, 1b. Regulated compounds along tequila batch distillation.

Continuous distillation.

Only few researches on tequila distillation using continuous column (CC) have been published. Prado (2002) evaluated continuous distillation using ordinario and feeding a 8 inches diameter and 20 trays copper CC. Experimental treatments included to feed at tray 17, after changing to tray 14. Alembic distillation with same ordinario was taken as reference. Rectified products were obtained with a 64.7%, 84.3% and 56.0% ethanol v/v content, respectively. GC-MS analysis were carried out and chemical groups of congeners in rectified products were identified I, see Table 1.

Table 1. Groups of chemical compounds identified in rectified products.

Compound	Treatment			
	Ordinario	Alembic	Column run 1	Column run 2
Ethanol	23.6	56.0	84.3	64.7
Higher alcohols	207.0	209.4	199.2	201.9
Methanol	286.0	263.2	343.8	248.3
Acetaldehyde	18.5	1.6	17.2	5.6
Ethyl acetate	87.4	44.5	65.1	47.2
Furfural	1.5	1.2	2.5	1.3

Small differences in number of congeners are attributed to the low amount of trays used in the enriching section of the column however was confirmed the feasibility to use of a CC to rectify ordinario without some reflux stream. Dávila (2004) evaluated continuous distillation by using a pilot packed column. Results showed an increase from 12.3 up to 64.4 and 74.5% ethanol v/v and concluding that this technique is feasible to recover heads and tails as well. Castro (2014) studied regulated compounds by using a 50 trays CC and feeding fermented must. Sampled trays in enriching section showed it is possible to draw off product with a similar ethanol content to the obtained using alembic. These results are congruent to obtained at pilot plan level. Concentration profiles of regulated compounds in enriching section of CC showed acetaldehyde and ethyl acetate have highest values in top trays; methanol increases slightly its concentration as increases ethanol content and isoamyl alcohol is concentrated at trays with an ethanol content about 70% v/v, underlying here the effect of an important thermodynamic factor as the activity coefficient, which was determined for every chemical compound using ternary mixtures at different v/v ethanol content (35%, 55%, 75 %), see Figure 2. The behavior of activity coefficient is useful to explain profiles of chemical compounds concentrations along distillation column trays.

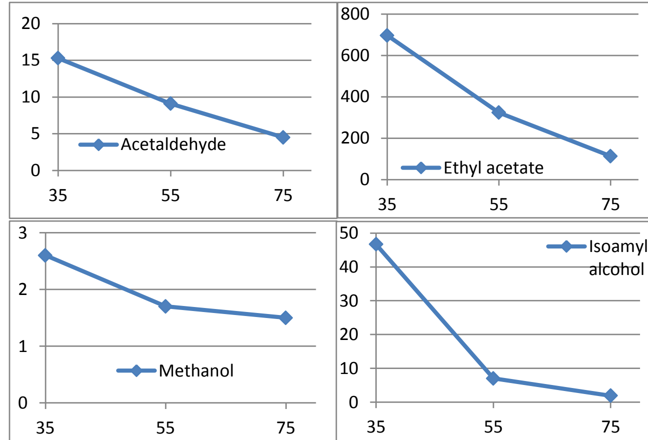


Figure 2. Activity coefficients for regulated compounds in ethanol-water mixtures.

Materials of construction of distillation equipment.

Alembics and columns have been traditionally manufactured with copper, but today, stainless steel has gained a higher relevance; besides, the role of copper has been understood because of its favorable effect on the sensory character of tequila and mezcal. This effect promotes removal of unpleasant smells (Nedjma, Hoffmann, 1996) due to thiols presence in distillates, however, some products obtained in copper alembic show higher copper concentration than the maximum allowed (2 mg/L, NOM-142-SSA1-1995).

DISTILLATION PROCESSES IN MEZCAL PRODUCTION.

Presently, most of mezcal is produced by using traditional alembics manufactured with copper, with a capacity from 400 to 800 L and distillation in two stages has substituted progressively the one stage distillation in order to meet official regulations. Mezcal distillation is carried out with *agave* bagasse and firewood as heat source. Distinctive sensory notes in mezcal include soil, smoke (Quiroz Marquez, 1997) and acidity. Regulated compounds exhibit a similar behavior than that found in tequila (see Figure 3) but irregular ethanol profile is caused because of the addition of water in the upper pipe of the boiler of alembic, which produces a reflux that increases at the moment the ethanol content in distillate stream.

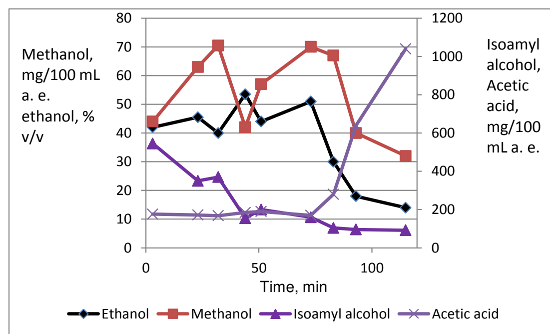


Figure 3. Regulated compounds in mezcal rectification.

CONCLUSIONS

Concentration of regulated compounds along tequila and mezcal distillation show similarities: highest concentrations of isoamyl alcohol, ethyl acetate and acetaldehyde are found in heads volume; methanol and acetic acid are found mainly in tails. Cutoffs of heads and tails are significant to regulate final concentrations in rectified products. Activity coefficient of regulated compounds explains to a great extent, their concentrations profiles along distillation. Alembics and continuous columns copper fully manufactured, produce tequila and mezcal with favorable sensory notes but with higher copper concentrations than those manufactured with stainless steel-copper. Mezcal industry exhibits a distillation process with a large amount of technological improvement opportunities in process and quality control areas.

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SELECTION OF BACTERIA ABLE TO GROW INTO FERMENTED AGAVE WORT TO MODIFY ORGANOLEPTIC FEATURES OF TEQUILA

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ABSTRACT

Tequila is an important alcoholic beverage obtained from the distillation of fermented juice from the blue agave plant (*Agave tequilana* Weber var. azul). The tequila commercial success is proportionated by its organoleptic characteristics, which depend on volatile compounds produced, principally, during fermentation stage. These compounds are the result of microbial metabolism, basically from yeasts. Into usual tequila process, at the end of fermentation stage, the yeast activity ceases. However, some bacteria, such as lactic strains, are able to develop a second fermentation. During this bacterial transformation, the principal conversion is malic acid to lactic acid and carbon dioxide, together with this metabolic reaction a synthesis of other volatile compounds is generated. This type of change produces a less acidity and enhances organoleptic characteristics of the alcoholic beverage. Our study is based on the bacterial capacity to metabolize malic acid contained into the agave wort. A selection of native bacteria isolated from fermented juice agave was performed. From assays applied, ethanol tolerance and growth capacity using malic acid as a carbon source, two bacilli were selected by their metabolic abilities. These two strains grew when inoculated into the fermented agave wort. Both bacilli were Gram positive and catalase negative, presumptively they could be lactic bacteria. These initial results are evidence that isolated native bacteria could be used to perform a second fermentation and improve organoleptic characteristics of tequila.

Key words: tequila, lactic bacteria, malolactic fermentation, organoleptic characteristics, blue agave

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INTRODUCTION

Tequila is a representative distilled spirit of Mexico, which competes with other types of alcoholic beverages worldwide. For example, in the year 2013, tequila industry generated 33 million dollars only for the concept of exportation. During the period between January and June of 2014, 124 million liters of tequila to 40% alcohol volume were produced, which represents an increase of 9% with respect to the same period of 2013 (Cámara Nacional de la Industria Tequilera, 2014). The consumption in the domestic and international markets of this generous beverage is due to its own organoleptic characteristics. Organoleptic characteristics depends on different stages of tequila process, nevertheless fermentation step is crucial in the development of volatile compounds, principal factor of the flavor, aroma and bouquet of the tequila (Cedeño-Cruz and Álvarez-Jacobs, 2007). The principal architect on this stage is the yeast. During juice agave fermentation, the nutritional and environmental conditions are changing, due to ethanol formation and carbohydrates disappearance, until they become unfavorable to yeast development and, finally, the yeast activity ceases, which indicates the final of fermentation stage. However, some bacteria, such as lactic strains, are able to develop a second fermentation using compounds contained in the fermented agave wort. For example, in the post-fermentation of wine, lactic bacteria perform a malolactic fermentation that enhances organoleptic characteristics of the wine after alcoholic fermentation was finished (Alexandre *et al*., 2004).

In this work are presented studies related with the post-fermentation capacity of native bacteria as selection criteria of strains, which will use to design a second fermentation process into tequila production. During the second fermentation, an improved of organoleptic characteristics of the tequila has been seen when there is a delay in the distillation of the fermented wort.

METHODOLOGY

Strains and culture conditions. Native bacteria isolated from fermented agave wort were used. Strains in all tests were incubated at 30°C. Native strains were maintained in MRS agar (de Man *et al*., 1960).

Ethanol tolerance. MRS broth was used as a basal medium. Content ethanol was adjusted of basal medium at 5% and 10% v/v. A control test was used without addition of alcohol.

Capacity to growth with malic acid. MRS broth was prepared changing glucose by malic acid, concentration 20 gL^{-1}.

Capacity to growth with malic acid and ethanol. MRS broth was prepared changing glucose by malic acid as a basal medium. Content ethanol was adjusted of basal medium at 5% and 10% v/v.

Capacity to growth into fermented agave wort. Fermented agave wort obtained from a tequila factory was filtrated with 0.22 µm membranes. Filtrated wort was inoculated with the different six bacterial strains.

Determination of bacterial growth. Optical density was used to determine bacterial growth at 600 nm using a Genesys 20 visible spectrophotometer.

Characterization of native bacteria. Gram staining method and catalase test.

RESULTS AND DISCUSSION

Criteria for selection of native bacteria were thought to design a second fermentation step after alcoholic fermentation. First, the hypothesis that the second fermentation could be a malolactic transformation is based on the agave plant metabolism. *Agave tequilana* belongs to CAM (Crassulacean Acid Metabolism) plants, which use this water-efficient form of photosynthesis to adapt to xeric environments (Gross *et al*., 2013). In general, CAM photosynthesis consists of the nocturnal carboxylation of phosphoenolpyruvate by using atmospheric or respiratory CO2, giving rise to oxaloacetate, a reaction mediated by the enzyme phosphoenolpyruvate carboxylase. The oxaloacetate is then reduced by malate dehydrogenase to malate, which is subsequently transported into the vacuole and stocked in the form of malic acid, generating the typical nocturnal acidification of CAM plants (Matiz *et al*., 2013). Then malic acid will be contained in the agave juice. Second, at the end of fermentation stage, ethanol concentration becomes inhibitory for yeasts.

Then, bacteria can be inoculated to metabolize compounds found into fermented agave wort and transform them in metabolites, such as volatile compounds, that contribute to better organoleptic characteristics of the final product. But, it is necessary to have bacteria able to develop in these drastic conditions for the yeasts.

The first assay was alcohol tolerance of bacteria, concentrations tested were 5% v/v and 10% v/v. The first concentration was approximated to usual ethanol content obtained at the end of alcoholic fermentation in the tequila production. The second concentration ensures there will be no bacterial inhibition by ethanol action in any wort used to tequila production. Six bacterial strains were tested, of which five were able to grow at 10% of ethanol. This implies that bacteria there will have no problem to transform alcoholic wort.

The second bacterial skill was its capacity to use malate as carbon source. Results indicated that five bacterial strains were able to use malate to grow as the only carbon source. The strains were the same that grew in ethanol.

The next experiment was planned to measure bacterial growth on the basal medium with malate, as a carbon source, and ethanol. Only two colonies achieved to grow on 10% of ethanol, named BF1 and BF2 (Figure 1). These two strains that were identified as bacilli Gram positive and catalase negative. According to the results, strains BF1 and BF2 were considered the better potential candidates to perform a malolactic fermentation into fermented agave wort, because their ethanol and malic acid tolerance.

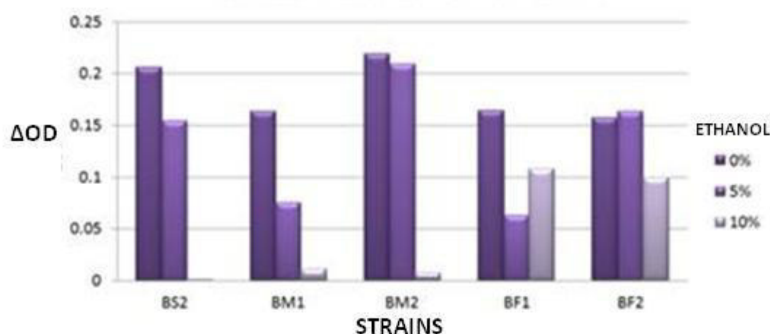

Figure 1. Growth of native bacteria into malate at different ethanol concentrations.

Finally, bacterial behavior was verified inoculating the native bacteria in fermented agave wort. The results obtained were satisfactory, five native strains grew into fermented wort, within which were BF1 and BF2. Nevertheless, the strain identified as BM2 presented the higher increase of biomass. This strain presented a poor growth at 10% v/v of ethanol, but its behavior in this last test open the question about its possibility to be a useful microorganism to achieve a malolactic fermentation. Strain BM2 had the highest increase of biomass at 5% v/v of ethanol, which is near alcoholic concentration at the end of fermentation of agave juice.

CONCLUSION

Two interesting native strains were detected, who have the capacity to grow into superior levels of ethanol and malic acid than, usually, found in fermented wort in the tequila production. Moreover, a third strain presented the highest biomass increase when it was inoculated at concentrations of 5% v/v of ethanol, which is an average concentration in fermented agave worts. These findings open a possibility to use the isolated native bacteria to modify tequila organoleptic profile. Nevertheless, more work is necessary to make a final conclusion. At this time, worts fermented by bacteria are characterized to determine the profiles of volatile compounds synthesized and contrast with the original profile before bacterial treatment. Preliminary studies indicate that there are changes in the composition of organoleptic compounds.

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Comparison of fermentative capacity of *Saccharomyces cerevisiae* and non-*Saccharomyces* between flask and reactor level under controlled conditions

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ABSTRACT

The study of non-*Saccharomyces* yeasts and their use in fermentation processes for the production of alcoholic beverages has been in constant increase for its importance in the generation of desired volatile compounds in the final product.

Recent studies in tequila show that some non-*Saccharomyces* yeast are capable of performing the fermentation step in pure culture. In this study the fermentative abilities and the production of volatiles are evaluate at flask and biorreactor level in agave juice under controlled laboratory conditions for a *S. cerevisiae* strain and two non-*Saccharomyces* (*Kluyveromyces marxianus* and *Pichia kluyveri*) to demonstrate the importance of scaling up, particularly with non-*Saccharomyces* yeasts.

K. marxianus and *S. cerevisiae* not presented problems in scaling, showing higher yields in reactor compared to flask level. In contrast, *P. kluyveri* was unable to ferment in reactor as observed in flask level. After the kL_a measurement, was observed that the oxygen dissolution in the reactor was different compared to flask. Adjust the kL_a at reactor level allows an adequate growth and finally perform the fermentation.

Detailed yeast fermentative capacity analysis showed that low ethanol tolerance of non-*Saccharomyces* was not the main factor responsible of the absence of these yeasts at the end of fermentation as mentioned in wine, but rather, is due the nutritional conditions or the environmental factors not allow their growth and survival during all the fermentation.

Keywords: non-*Saccharomyces*, Fermentation, Scale up, Agave juice, Tequila.

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INTRODUCTION

The scale up at industrial level of conditions tested in laboratory is complex because it is difficult to repeat the same physical and chemical conditions in higher volumes. The key parameters are: the diameter ratio of the reactor, the aeration, the oxygen transfer to the medium (kL_a), the dissolved oxygen level and the agitation to maintain the same profile (Waites y col. 2001).

The respiratory metabolism of yeast is oxygen dependent; the fermentation process takes place under anaerobic conditions, since it is not dependent on oxygen the yeast produce ethanol. *S. cerevisiae* yeast has been found in most fermentation processes, this specie is adapted along its evolution to optimize growth rate in environments rich in easily assimilated nutrients such as sugars and amino acids. The yeast *S. cerevisiae* can metabolize high levels of glucose and fructose by respiration so as by fermentation. All this makes this species present some metabolic peculiarities, such as the Pasteur Effect and the Crabtree effect, which must be taken into account when studying and trying to modify the production of ethanol during fermentation (Deken, 1966).

The Crabtree effect is described in *S. cerevisiae* and in a limited number of other yeasts species. It occurs in presence of high amounts of glucose even in presence of sufficient quantities of oxygen, most of consumed sugar are intended for the production of ethanol by the fermentative pathway. On the other hand Pasteur effect is observed when oxygen inhibits anaerobic process and simultaneously decreases the rate of glucose conversion to ethanol. *K. marxianus* has been reported as Crabtree negative yeast (Van Urk et al., 1989), while *P. kluyveri* has not been characterized.

METODOLOGY

Yeast strains *S. cerevisiae* AR5, *K. marxianus* DU3 y *P. kluyveri* GRO3 were obtained from the CIATEJ culture collection. Fermentations were carried out in 250 ml shake flasks (100 ml of fermentation volume, 30°C, 100 rpm) and in a 3L biorreactor with 2L of culture medium. The fermentation medium was composed by *Agave tequilana* Weber juice, supplemented with ammonium sulfate (0.5 g/L) and adjusted to 100 g/L of reducing sugars. Fermentations were started after inoculation of 1×10^6 cells yeast/mL previously growth. Samples were taken at different times from each bioreactor and flask to determine cell number using a Neubauer chamber, sugar concentration (Miller, 1959) and volatile compound synthesized during the fermentation using gas chromatography (Arellano y col. 2011).

kL_a measurement: To measure both the flask and the reactor kL_a, first all the oxygen was displaced performing aeration with nitrogen. The flask measurement was performed with a non-invasive system that allowed taking readings from the outside through a dissolved oxygen probe NOMA Sense O_2 Trace Oxygen Analyzer. While in the reactor the measurement was performed using the dissolved oxygen electrode Applikon.

RESULTS AND DISCUSSION

kL_a measurement: After kL_a measurement value of 0.0565 ± 0.0028 in flask with 100 mL medium agave juice and 100 rpm at 30°C was obtained. At reactor level without aeration

with agitation (250 rpm) and working volume of 2L a kL_a value of 0.0010 ± 0.0001 was obtained, and performing an aeration of 0.5 vvm the kL_a value was 0.0392 ± 0.0003.

Evolution of population and sugar consumption: the AR5 population (*S. cerevisiae*) and sugar consumption in flask and biorreactor showed similar behavior (Figure 1). The DU3 strain (*K. marxianus*) showed lower growth in the reactor and the sugar consumption was slower. The fermentation performed with GRO3 (*P. kluyveri*) without aeration showed no growth and neither fermentation. The results of kL_a obtained showed different values between flask and bioreactor, as follows was decided aerate the biorreactor to match the aeration occurred in the flask. After biorreactor aeration during 12 hours to support the growth of GRO3 (*P. kluyveri*), the population evolution was similar to others yeasts (Figure 1), but the sugar consumption was slower. Although it has been reported *K. marxianus* as Crabtree negative yeast shows similar behaviors to those observed with *S. cerevisiae*.

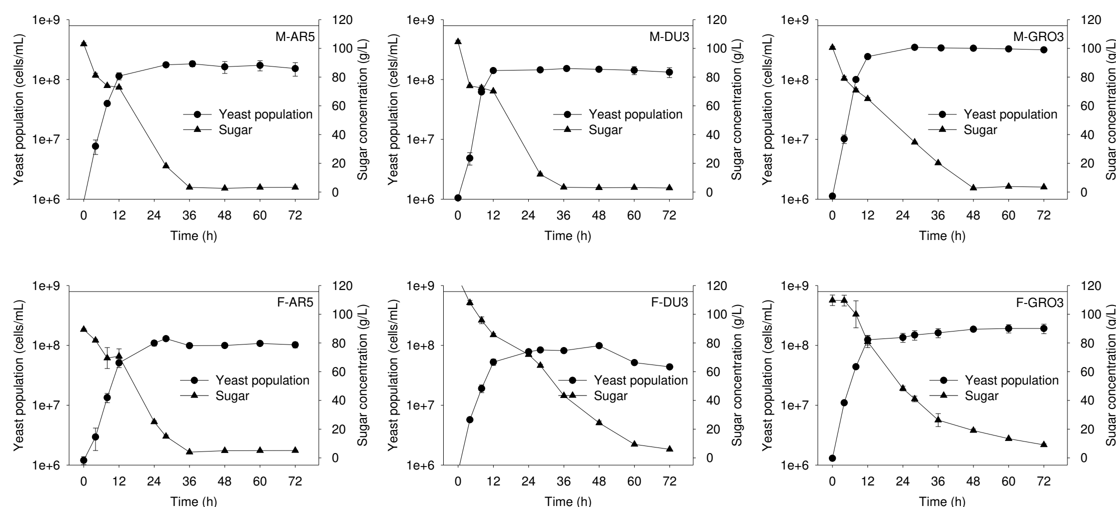

Figure 1. Evolution of the yeast population and sugar concentration during fermentation with AR5 strain (*S. cerevisiae*) in pure culture to laboratory level in flask and 3L bioreactor.

Volatile compounds production: In Figure 2 a higher ethanol production was observed at biorreactor level and acetaldehyde conversely the highest production was obtained at flask level (which demonstrated an incomplete fermentation). AR5 and DU3 yeast strains produce esters concentrations for example the ethyl ester had similar values between both systems, whereas GRO3 produced higher level in flask. Higher alcohols production was different between the strains and the conditions tested, highest concentrations were obtained using DU3 and the lowest was obtained using GRO3.

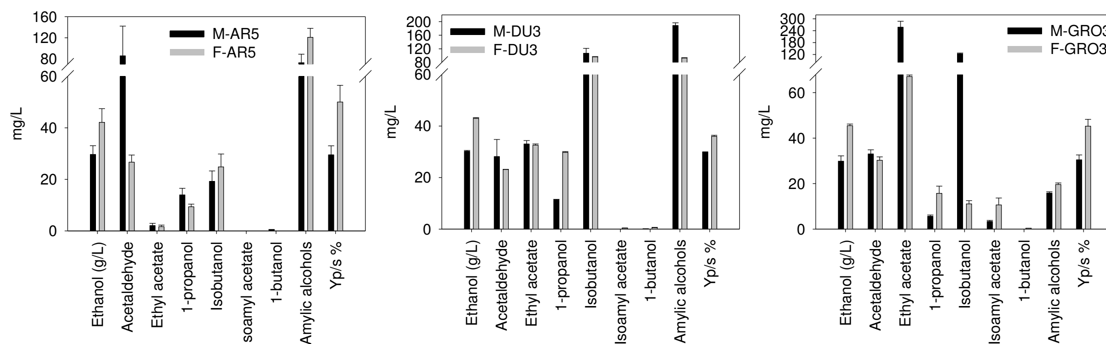

Figure 2. Concentration of major volatile compounds and alcohol yield about substrate (yp/s) produced by the AR5 (*S. cerevisiae*), DU3 (*K.marxianus*) and GRO 3 (*P. kluyveri*) strains in pure culture to laboratory level in flask and 3l bioreactor.

CONCLUSION

The recent interest in the study of non-*Saccharomyces* lies in its capacity to produce flavors (Ciani et al 2010). While in this work not only highlights its higher volatile compounds production capacity was obtained compared with *Saccharomyces*, also demonstrates that under the right conditions, the non-*Saccharomyces* strains are able to produce similar ethanol yields than the *Saccharomyces*. *P. kluyveri* (GRO3) showed higher oxygen requirement than the AR5 (*S. cerevisiae*), consequently, aeration is required to improve the fermentation with this strain.

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Growth capacity of yeast on *Agave salmiana* from San Luis Potosí State

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ABSTRACT

The present study aimed to investigate the effect of *Agave salmiana* var crasispina juice on yeast growth. This agave specie produces high level of steroidal saponins. The mezcal producers are used to add commercial yeast, but the fermentation is not completely carried out. Commercial yeast and wild yeast isolated from a mezcal factory where used. Both yeasts were grown using agave juice obtained from agave with different treatments (agave quiotilla, agave quiote and agave treatment). Also, nutrients as magnesium sulphate, calcium chloride and ammonium phosphate were added to the agave juice to evaluate if the agave juice requires nutrients for the yeast growth. The results obtained were that the wild yeast endured the saponin inhibition and grew 5 times more than a commercial yeast in the different agave juices, inclusive without adding nutrients only the ammonium phosphate where required to increase growth.

Keyword: *Agave salmiana*, yeast, saponin, growth inhibition.

INTRODUCTION

Agaves have great economic and cultural importance for several native communities in Mexico, and for centuries people have used these plants as sources of food, fuel, shelter, and fiber, but also in traditional alcoholic beverages. These plant species belong to the family *Agavaceae*, of which there are some 300 species in the world, over 250 of which are found in Mexico (Arellano et al. 2012). The use of agave fermented beverages has persisted in Mexico for over 500 years. Tequila and mezcal, both distilled from agave, have become Mexican symbols due to the fact that both beverages have a "Denominación de origen" (protected designation of origin) according to the Norma Oficial Mexicana (NOM, Official Mexican Regulations) regulations NOM-006-SCFI-2005 for tequila and NOM-070-SCFI-1994 for mezcal (Monterrosas-Brisson et al. 2013).

During the mezcal production process it is possible to use different kinds of agave species and it depends on the availability in each production region, principally *Agave duranguensis* in Durango state, *Agave salmiana* in San Luis Potosí State and in some places in Zacatecas

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and Guanajuato states, *Agave tequilana* in some places in Zacatecas state, *Agave cupreata* in Guerrero and Michoacán states, *Agave angustifolia* in Oaxaca state and others agaves are used but in small proportions. In the Mexican state San Luis Potosí, the *Agave salmiana* var crasispina is used for mezcal production, this agave species grows in a dessert place, the agave produces saponins as a defense versus pathogenic organisms (Coleman et al, 2010), and these compounds inhibit the fermentation during the mezcal processes. The mezcal producers cut the agaves at different ripeness degrees. The mezcal producer use commercial yeast as starter of the fermentation stage, but the fermentation is not carried out completely because the yeast population decreases. The aim of this work was to establish the growth capabilities of yeast in agave juice obtained from different agave ripeness states and also adding nutrients considering the yeast nutrients need.

METHODOLOGY

Microorganisms

Commercial yeast strain was obtained in specialized store. One yeast strain was isolated from a spontaneous fermentation in San Luis Potosí State and belongs to the collection of CIATEJ. All strains were grown on YEPD (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose). The yeasts where maintained at -80°C until used.

Agave cooked and milling

Agave samples with different ripeness degrees where taken in San Luis Potosí state. Agave treated (mature), agave quiote (middle mature) and agave quiotilla (tender) were taken. The agave samples were cut in pieces and put into an autoclave, the cook temperature were maintained at 121°C during 10 h adding steam. Before, the agave samples were maintained inside until the temperature decreased to 30°C. For the agave juice extract were used a lab sugarcane mill. The agave juices were maintained at -20°C until use.

Clarified agave juice

The agave juices were filtered using activated charcoal. A 500 mL glass column was packed with activated charcoal and three agave juice volumes were filtered at 60°C.

Growth conditions

The first experiment was carried out using the juice obtained from agave plants with different ripeness degree and from different agave parts, the heart (the central part), the leaves and the quiote (the higher central of the agave for the flowering). Additionally, *Agave tequilana* juice (from a tequila factory) was used as control. For this experiment commercial yeast was used. Previous to the growth kinetics, the commercially yeast was grown in YEPD medium for 12 h. After, the yeasts were centrifuged at 10,000 rpm, the pellet was washed twice with physiological solution and finally resuspended in physiological solution. The yeast cells were counted and inoculated at a final concentration of $20x10^{6}$ cell mL^{-1} in each

agave juice. All the agave juices were adjusted to 60 gL^{-1} of reducing sugars and sterilized at 121°C for 15 min.

The second experimental design for the propagation kinetics was performed using *Agave salmiana* juice (agave quiotilla juice, agave quiote juice and agave treated juice), two yeast strains (commercial yeast and yeast isolated from mezcal processes), nutrient addition (magnesium sulfate 0.5 gL^{-1}, calcium chloride 0.1 gL^{-1}, diammonium phosphate and 2.0 gL^{-1}). All the agave juices were adjusted to 60 gL^{-1} (8 brix grades). The cultures were made in 500 mL flask with 100 mL of agave juice at 30°C and 250 rpm. The flasks were previously sterilized at 121°C for 15 min. All the experiments started with $20x10^6$ yeast cells mL^{-1}. The growth kinetics was made for 24 h, samples were taken to determine cell population and reducing sugars (modified method from Miller, 1958).

RESULTS AND DISCUSSIONS

The growth kinetics were performed using commercial yeast in different types of agave juices obtained from the mezcal factory, also was used *Agave tequilana* juice, used in the tequila production, so that it would be considered as a control because it contains low levels of saponin. The growth kinetics time was 24 h in which the commercial yeast (used in the mezcal factory) could not increase its population and the highest population was minor to $40x10^6$ cells mL^{-1} (Figure 1). Meanwhile, in the *Agave tequilana* juice, the commercial yeast grew satisfactory, reaching a maximum of $189x10^6$ cells mL^{-1}. The yeast concentration in the *Agave salmiana* juices was 4 times lower as compared with the yeast population obtained with the *Agave tequilana* juice. A low yeast population could be a problem in the mezcal industry because it can be the reason for low ethanol yield during the fermentation stage.

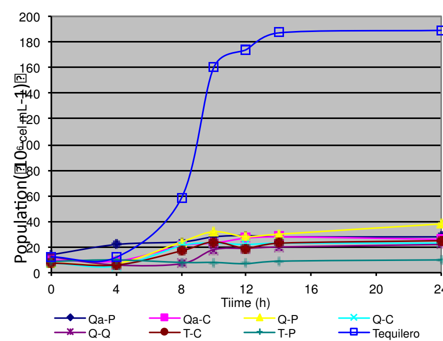

Figure 1. Yeast growth used baker yeast in different agave juices. Qa-P, quiotilla agave leaves; Qa-C, heart quiotilla agave; Q-P, quiote agave leaves; Q-C, heart quiote agave; Q-Q, quiote of agave quiote; T-C, heart agave treated; T-P, leaves of agave treated; Tequilero, agave used for tequila production.

With these results, it was discarded that the commercial yeast viability as a cause of growth failure because the yeast grown higher in *Agave tequilana* juice. Therefore, the presence of a growth inhibitor or lack of nutrients needed for reproduction of yeast in the juice became more evident. Because of this, was proceeded to examine possible nutrients and inhibitors as the furfural present in the juices. The arsenic and the cyanide were almost at similar level minor than 0.2 mg L^{-1} in all the agave juices used, moreover, the *Agave tequilana* juice had 4

times more furfural than the *Agave salmiana* juice, indicated those toxic compounds were not the reason for the poor growth.

In order to find solutions for the low yeast population the agave juice was modified; first the *Agave salmiana* juice was clarified removing long-chain compounds; second micronutrients were added and third a yeast strain belonging to CIATEJ, with an inhibitor resistance was used. Figure 2 shows the results obtained using different agave juices. It was observed that the commercial yeast did not show an acceptable growth in any of the agave juices, while CIATEJ´s yeast increased its population in the three media, growing up to 289 million cells mL^{-1} in treated agave juice. It was also observed that the yeast growth were more affected in agave quitilla juice and even CIATEJ´s yeast required more time to grow to similar levels than in the other juices..

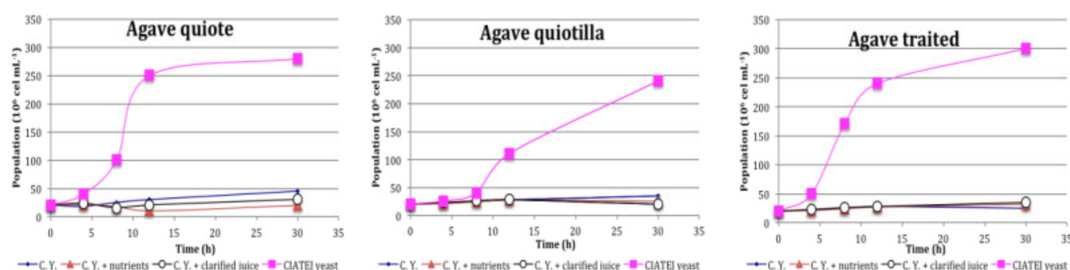

Figure 2. Population yeast grown using commercial yeast, different agave juices, nutrients and clarified agave juice (C.Y. commercial yeast; CY + nutrientes, commercial yeast with nutrients added; C.Y. + clarified juice, commercial yeast in clarified agave juice; CIATEJ yeast).

The results obtained show that it is possible to use CIATEJ´s yeast for fermentation, as was mentioned by Steensels and Verstrepen (2014), the authoctonous wild yeast could be better adapted to the properties of the fermentation must. However, it is still necessary to do research to know its properties and fermentation characteristics such as: fermentation kinetic parameters (efficiency, performance, speed of alcohol production and substrate consumption), and amounts of diverse generated organoleptic compounds.

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Study of the production of ethanol and esters by *Saccharomyces* and no-*Saccharomyces* in a continuous fermentation

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ABSTRACT

S. cerevisiae is widely used in the fermentation stage in the tequila process; however, in wine it has been observed that non-*Saccharomyces* yeasts, temperature, nutrient availability and aeration influence esters and ethanol production. The aim of this study was to evaluate the production of esters and ethanol by the yeasts *S. cerevisiae*, *K. marxianus* and *P. kluyveri* during continuous cultures at different conditions.

The highest yeast population was achieved during the aeration condition, while the lowest population was observed at 20°C. The addition of nitrogen increased the ethanol production in *S. cerevisiae* and *P. kluyveri*, however, *K. marxianus* reached higher ethanol concentration at the end of the stage when the media was aerated. For the esters production, the non-*Saccharomyces* yeasts produced higher concentrations levels of ethyl acetate in all the conditions studied comparing to *S. cerevisiae*, reaching the highest concentration when the temperature was low. Additionally, *P. kluyveri* was the only yeast able to produce isoamyl acetate. The low temperatures favored the ethyl caprylate and ethyl caprate production in all the yeast species used; the highest concentrations were observed in *S. cerevisiae*, on the contrary, aeration caused low esters concentration.

In continuous fermentations using agave juice, this study confirmed that non-*Saccharomyces* yeasts produce higher concentration of acetate esters than *S. cerevisiae* and higher concentrations of these compounds are produced at lower temperatures. On the contrary, aeration affects negatively the production of esters in both *S. cerevisiae* and non-*Saccharomyces*.

Keywords: *Saccharomyces*, non-*Saccharomyces*, ethanol, esters, continuous fermentation

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INTRODUCTION

Fermentation is a key stage in the complex process of the alcoholic beverages production such as tequila. Its main and common feature in all beverages is the intervention of living organisms, principally yeasts which transform sugars into ethanol and other compounds in smaller quantities as the esters; which play an important role in the final product characteristics.

In the tequila industry, the small and medium-sized companies are still using spontaneous fermentation where yeasts (*Saccharomyces cerevisiae* and non-*Saccharomyces*) and bacteria (mostly lactic acid and acetic acid) are current in mixed populations and have a significant impact on sensory characteristics of the final product. In bigger companies use selected yeasts is common; generally, a *Saccharomyces cerevisiae* strain is selected. However, in wine, it has been observed that non-*Saccharomyces* yeasts during controlled fermentation increase ester production. Furthermore, it has been reported that factors such as temperature, nutrient availability and aeration affect esters and ethanol production.

The aim of this study was to evaluate the production of ethanol and esters by *Saccharomyces* and non-*Saccharomyces* yeast species during continuous cultures at different study conditions.

METODOLOGY

Yeast strains *S. cerevisiae*, *K. marxianus* and *P. kluyveri* were obtained from the CIATEJ culture collection. Each yeast strain was used for alcoholic fermentations using continuous cultures to change the conditions studied. The fermentations were carried out in a 3 L bioreactor with 1.5 L of *Agave tequilana* Weber (blue variety) juice, supplemented with ammonium sulfate (0.5 g/L) and adjusted to 100 g/L of reducing sugars. Cultures were started in a batch mode, by inoculating 1×10^6 cells/mL and incubating at 30°C and 250 rpm for 12 h. After that, the culture was fed with sterilized agave juice medium.
Samples were taken during each condition, to determine cell number using a Neubauer chamber and volatile compound synthesized during fermentation were quantified by gas chromatography. Exhaust gases (CO_2, and O_2), temperature and pH were online measured during the fermentations.

RESULTS AND DISCUSSION

In this study was observed that in all the yeast strains tested, the highest level of cells was reached during the aeration condition, obtained a population of 272×10^6 cells/mL with *P. kluyveri* and followed by *K. marxianus* with 190×10^6 cells/mL, while *S. cerevisiae* achieved just 128.5×10^6 cells/mL. A low level in the populations was observed at 20°C, probably because the cell division and the growth rate become slower.

Table 1 shows the ethanol concentration produced for each yeast strain and was observed, that the higher ethanol production was achieved using *S. cerevisiae* in all conditions studied.

The addition of ammonium favored ethanol production in *S. cerevisiae* and *P. kluyveri*, achieving concentrations of 337 and 43 mg/L per million cells respectively. In previous studies with *S. cerevisiae* was observed, that the addition of ammonium source increased the ethanol production (Arrizon and Gschaedler, 2007). *K. marxianus* reached the high ethanol level (88 mg per million cells) at the final stage of the study aeration condition.

Table 1. Ethanol concentrations during continuous fermentations with different conditions. Values were normalized per million cells. Conditions: B: basal conditions, N: ammonium pulse; A: aeration; different temperatures.

	Ethanol (mg/L)		
Sample	*S. cerevisiae*	*K. marxianus*	*P. kluyveri*
B	283.0	75.0	35.0
N1	**337.0***	76.0	**43.0***
N2	276.0	79.0	38.0
N3	273.0	64.0	35.0
A1	274.0	46.0	35.0
A2	240.0	52.0	25.0
A3	134.0	**88.0***	27.0
30°	298.0	63.0	33.0
25° 1	260.0	66.0	27.0
25° 2	280.0	65.0	15.0
25° 3	257.0	49.0	24.0
20° 1	257.0	37.0	26.0
20° 2	214.0	37.0	15.0

*highest concentration

For the esters production, the table 2 shows that non-*Saccharomyces* yeasts produce higher concentrations of ethyl acetate in all the conditions tested; achieving higher concentrations (0.36 and 0.24 mg/L per million cells for *K. marxianus* and *P. kluyveri* respectively) at low temperature fermentation. This effect was observed in wine fermentation using *S. cerevisiae* (Molina et al. 2007). Additionally, *P. kluyveri* was the only yeast with the ability of produce isoamyl acetate. Low temperatures increased the ethyl caprylate and caprate production in all yeast strain tested; the highest concentrations were observed with *S. cerevisiae* (0.027 mg/L per million cells in both compounds). Otherwise, aeration caused decrease in esters production in all yeasts; as it had been reported in previous studies with *S. cerevisiae* (Valero et al. 2002).

Table 2. Esters production during continuous fermentations in different conditions. Values were normalized per million of cells. Conditions: B: basal conditions, N: ammonium pulse; A: aeration; different temperatures.

	Ethyl acetate (mg/L)			Isoamyl acetate (mg/L)	Ethyl caprate (mg/L)			Ethyl caprilato (mg/L)	
Sample	S. c[a]	K.m[b]	P. k[c]	P. k	S. c	K. m	P. k	S. c	P. k
B	0.015	0.115	0.035	0.009	0.009	0.007	0.004	0.006	ND
N1	**0.021***	0.091	0.034	0.010	0.009	0.006	0.004	0.008	ND
N2	0.008	0.051	0.031	0.009	0.008	0.007	0.004	0.006	ND
N3	0.006	0.221	0.051	0.009	0.008	0.006	0.004	0.006	ND
A1	0.006	0.077	0.030	0.009	0.008	0.005	0.004	0.006	ND
A2	0.006	0.072	0.036	0.005	0.007	0.002	0.002	ND	ND
A3	0.004	0.053	0.070	0.016	0.004	0.002	0.002	ND	ND
30°	0.006	0.261	0.066	0.009	0.009	0.005	0.004	0.006	ND
25° 1	0.005	0.138	0.033	0.007	0.008	0.006	0.004	0.006	ND
25° 2	0.004	0.189	0.043	0.009	0.012	**0.008***	0.006	0.008	ND
25° 3	0.002	**0.367***	0.061	0.012	0.013	0.008	0.006	0.011	ND
20° 1	0.002	0.135	0.051	0.012	0.015	0.008	0.007	0.011	ND
20° 2	ND[d]	0.323	**0.244***	**0.018***	**0.027***	0.008	**0.015***	**0.027***	**0.013***

[a] *Saccharomyces cerevisiae*, [b] *Kluyveromyces marxianus*, [c]*Pichia kluyveri*, [d]ND non detected, *highest concentration

CONCLUSION

In continuous fermentations using agave juice medium, this study confirmed that non-*Saccharomyces* yeasts produce higher concentration of acetate esters than *S. cerevisiae* and lower temperatures increased the production of these compounds. On the contrary, aeration affects negatively the esters production in both *S. cerevisiae* and non-*Saccharomyces*.

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NATIVE YEAST MIXED AS STARTER IN THE SOTOL PRODUCTION

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ABSTRACT

The SOTOL is an alcoholic regional beverage produced with only *Dasylirion* spp., or mixed with *Agave angustifolia,* both grew in Chihuahua state (North México). During the last years, the sotol production has been increased because the consumers detected a special flavor and aroma. This is an artisanal process, the fermentation is spontaneous with high microbiota diversity and it is possible that the kinetic parameters of fermentation and volatile compounds production changed between each batch process. However, the producers want to conserve the artisanal production system using native yeast during the fermentation. The aim of this work was to compare the kinetics parameters of fermentation between the spontaneous fermentation and the same process with a starter culture, using a mixture of natives yeasts. When the starter was added to the fermentation worth, the ethanol yield production increased more than 100% and the fermentation lasts only 62 h. No residual sugars were observed.

Keywords: *Dasylirion*, wild yeast, mixed yeast, sotol.

INTRODUCTION

Low intervention in the fermentation methods based on spontaneous fermentation is becoming more popular among alcoholic beverages producers and consumers are looking for this kind of process with a low intervention, then the spontaneous fermentation begin to be a technique to the alcoholic beverages processes (Chul et al. 2007). The alcoholic beverages producers have readopted spontaneous fermentation methods to generate unique attributes that differentiate their products, improve distinctive features although the variety of complex flavors increase but the gives regional procedures characterization.
Spontaneous fermentation is a complex process influenced by many factors, including the endogenous microbial flora, the raw material, climatic conditions, and the system process. The effect of the fermentation process can therefore be difficult to predict and probably change each batch process. The microbiota diversity grown and change in the worth and play a significant role during fermentation (Steensels et al. 2014). Previous studies have shown that endogenous wild yeasts principally non-*Saccharomyces* can be detected throughout the fermentation process and influence the fermentation course (Kirchmayr et al.

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2011), impacting in the beverages characteristics because of the production of volatile compounds that modify the sensory and organoleptic properties of the alcoholic beverages, introducing a variety of aromas and flavors.
The sotol is an alcoholic regional beverage produced using *Dasylirion* spp., and sometimes is mixed with *Agave angustifolia* grown in Chihuahua state in México. During the last years, the production of sotol increased because the consumers detected a special flavor and aroma. The production process is artisanal without control systems, the *Dasylirion* and agaves are cut when the maturity is reach, after are cook in a stone oven using dry wood and rocks. The *Dasylirion* and agaves cooked are crushed and put into a stone tanks using a low water level. The fermentation is spontaneous for two or three weeks. Finally the fermented wort is distilled using a pot still. The fermentation is spontaneous with a native microbiota and it is possible that the kinetic fermentation parameters change between each batch process. However, the producers would like to conserve the artisanal production using wild yeast during the fermentation.
Oxygen during the propagation of brewing yeast has a higher effect on yeast metabolism, particularity in yeast growth and viability. Oxygen is needed for the sterol and unsaturated fatty acids production, which are necessary for the synthesis of the cell membrane (Chul et al. 2007). The aim of this work was to measure the fermentation kinetics parameters during the spontaneous fermentation and compared using a mixture of native yeasts as starter growing before fermentation in aeration conditions.

METHODOLOGY

Microorganisms

In this work any commercial yeast were used and neither yeasts strains isolated previously. The spontaneous fermentations were carried out with the endogenous microbiota in the sotol factory. The yeast starter was produced using both dasylirion and agave juice at 6 brix, 30°C and with ammonium phosphate addition (1 g L^{-1}), moreover, was aerated during 18 h previous to the fermentation stage.

Dasylirion and agave cooked and milling

Dasylirion and agave pine were harvested in Cd. Madera Chihuahua. The pines were cooked in a stone oven; first in the oven were added dry and wet wood proportionally to warranty the flame during long time. When the wood was burning, stones were added to the oven. When stones turned red the dasylirion and agave pines where added. After the pines, leaves and fiber bags were added to coat the pines. Finally, the fiber bags were covered with soil. In the oven center were left a hole to add water three hours after the oven was covered and finally the hole was closed. The cook stage stopped after 5 days. The pines cooked where collected to the oven and a mill cutter was used for defibrate the pines. The pines fiber was collected directly in the stone tanks for the fermentation stage.

Fermentation conditions

The sotol production process was carried out in a sotol factory using handcraft equipment and autochthonous microbiota. Five fermentation tanks of 1000 L capacity were used; three tanks were prepared under artisanal characteristics used by producers. To the tank 1 was added agave pines 200 kg, dasylirion 500 kg and water 400 L, in the tank 2 and 3 were added agave pines 200 kg, dasylirion 400 kg and water 400L. The tanks 4 and 5 were prepared with agave pines 200 kg, dasylirion 200 kg and warm water was added to adjust the brix grades at 10ºBx, the temperature was 33°C and was added a starter of autochthonous microbiota previously grown. The fermentations were carried out during all the necessary time until the CO_2 production decreased considerably. Some samples were taken to analyze for sugar content (Miller, 1959), ethanol (Arellano et al. 2012) and cell population to determine the fermentation efficiency and productivity.

RESULTS AND DISCUSSIONS

Three spontaneous fermentation tanks and two tanks with addition of a starter were monitored. The sotol fermentation is a semisolid fermentation because juice and bagasse from dasylirion and agave cooked pines were used. The results obtained in the tank 1 (spontaneous fermentation) and tank 5 (fermentation using a yeast starter) are show in the figure 1. The spontaneous fermentation started with 200 g L^{-1} of sugars and lasted more than 94 h, after this time the sugar was not completely consumed and the ethanol produced was 30 g L^{-1}. When a yeast starter was added, the fermentation started with 55 g L^{-1} of sugars, the fermentation was complete because all the sugars were consumed and the ethanol produced was 20 g L^{-1}.

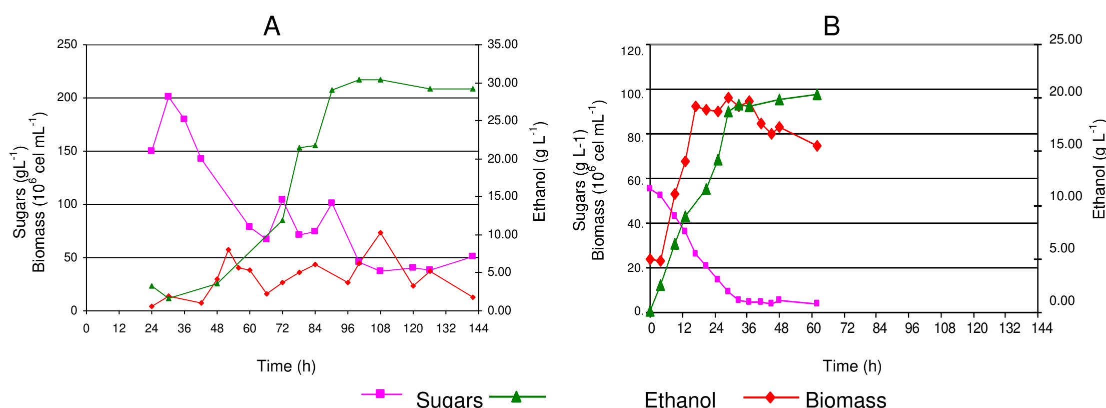

Figure 1. Sugars consumed, ethanol production and biomass cell population during spontaneous fermentation (A) and fermentation with the addition of a starter (B).

Table 1 shows the summary of results obtained. The producers prepared the tanks 1, 2 and 3 as they used to, and the tanks 4 and 5 were prepared adding a mixture of natives yeasts as starter. The fermentation times were different in all tanks but were longer in the artisanal tanks; the initial sugar could be an important factor for the fermentation time. However, the ethanol produced is not proportional to the initial sugar concentration, the efficiency of

conversion of sugar in ethanol were 40% and 34% in the tanks 1 and 2 respectively, those results shown that the fermentation is nor so efficient and could be improved. This fact is probably because the microorganism used the sugars no for ethanol production, or maybe because the ethanol was consumed by acetic acid bacteria and converted to acetic acid. The yeast population after fermentations was almost the same in all the tanks and probably the population yeast is not a factor to be considered for the efficiency in the fermentation for the sotol production. Finally, using mixed wild yeast as started increased the fermentation efficiency between 30 to 40%, this result can improve the sotol production.

Table 1. Results obtained in the fermentation sotol production.

Parameter	Tank 1	Tank 2	Tank 3	Tank 4	Tank 5
Initial sugars (g L^{-1})	200	140	110	52	58
Initial brix grades (Bx)	---	---	---	10	10
Residual sugars (g L^{-1})	50	40	20	5	4
Residual brix grades	---	---	---	3	4
Rs (g $L^{-1}h^{-1}$)	1.35	1.47	1.88	2.05	2.29
Initial ethanol (g L^{-1})	0	0	0	0	0
Final Ethanol (g L^{-1})	30	20	31	18	20
Rate production (g $L^{-1}h^{-1}$)	0.71	0.23	1.62	0.61	0.36
Yield	0.203	0.173	0.363	0.380	0.401
Efficiency (%)	40	34	71	74	79
Initial population cell mL^{-1} (10^6)	0	0	0	14	24
Final population cel mL^{-1} (10^6)	50	60	83	80	92
Fermentation time (h)	144	240	120	62	62

Finally, the addition of a yeast starter using a mixture of native yeast in the sotol wort before fermentation decreased the fermentation time and at the same time increased the fermentation efficiency, then it is suggested that before the fermentation begins prepare an inoculum in order to improve the fermentation stage

ACKNOWLEDGEMENTS.

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EXTRACTION OF SAPONINS FROM LEAVES OF AGAVES

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SUMMARY

The agave or maguey is endemic to America and Mexico is the place where you will find the largest number of species of agave. In Zacatecas, Mexico, in the south and southeast regions of the state grown Agave *tequilana* Weber (blue agave) and *A. salmiana* ssp. *crassispina* (green agave) for the main production of beverages like "aguamiel, "pulque" and "mezcal", but due to the growth of this last activity, are being generated large amounts of waste solid such as bagasse and leaves of agave, which are important to evaluate their possible use. Zacatecas generated annually at least 21000 tons being about 30 percent agave leaves and the rest agave bagasse. Currently they are not given an application to the leaves of agave and has been found to exist in these residues some secondary metabolites of interest as saponins. Agave saponins are amphipathic glycosides with triterpene or steroid skeletons whose structural diversity is related to the wide range of biological effects are source sapogenins basis for synthesis of therapeutic importance sterols. But the extraction, purification and identification of these compounds are methodological challenges. The aim of this work is to extract saponins and characterize the waste generated of the agribusiness mezcal. Characterized by optical and scanning electron microscopic bagasse fiber and infrared spectroscopy the content of organic matter, and calcium carbonate was evaluated. The analysis of the composition of the leaves indicate a high content of crude fiber but low content in fat and protein. Extracts from fresh leaves which were purified by column chromatography and analyzed by thin layer chromatography were obtained to evaluate saponins. All with the intention of seeking the possible uses of these compounds and the reduction of waste generated in the production of mezcal.

Palabras clave: Cromatography, maguey, mezcal, microscopy and sapogenins.

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INTRODUCTION

The state of Zacatecas, accounts for 3.8% of Mexico. The climate in the state varied, semiarid predominating (Medina-Garcia et al. 2003). In Zacatecas, the cultivation of Agave *tequilana* Weber and *A. salmiana* ssp. for mezcal production is concentrated in the southern and southeastern regions of the state. From the granting of the designation of origin of mezcal, the business has grown and thus the generation of waste, so it is important to evaluate their possible use. At present the greatest economic interest of this plant lies in the production of alcoholic beverages such as pulque, tequila and mezcal. From these processes large amount of by-products and wastes that can be usable as the fiber leaves and bagasse of stem agave (Caspeta et al. 2014). In the mezcal industry, the first residue generated are the leaves that correspond to 14% by weight of agave being its extremely slow degradation, but because of its sugar content, fiber, minerals and secondary metabolites could be useful (Narváez-Zapata and Sánchez-Teyer, 2009). Plants synthesize a variety of secondary metabolites, either as part of normal growth and development or in response to pathogen attack. Among the secondary metabolites of agave, of particular importance are saponins, glycosides with skeletons amphipathic triterpenes (C30 aglycone based) or steroids (aglycone C27) whose structural diversity is related to its wide range of biological effects. Furthermore saponins are a ready source of sapogenins basis for synthesis of sterols of therapeutic importance. Applications of saponins varied, as also used in the beverage, food and cosmetics as foaming agents, taste modifiers and the custodian, in the pharmaceutical industry to have anti-inflammatory, anti-fungal and take effect antitumor (Yoong-Cheok et al. 2014).

METHODOLOGY

Leaves of agave species of *A. tequilana* Weber and *A. salmiana* from the municipalities of Pinos, Zacatecas (southeast of the state) and Teúl Gonzalez Ortega, Zacatecas (south region) were used. From the agave bagasse micro-structural characterization was performed by optical microscopy, infrared spectroscopy (FTIR) and scanning electron microscopy (SEM). As the extracts which fresh leaves were cut into small pieces and air dried for one month later dried in an oven at temperatures between 50-60 °C were used. Once the samples were ground (particle size ½ mm) dehydrated. The powders were macerated in absolute methanol while maintaining constant agitation and oxygen-free atmosphere. The supernatant was filtered and concentrated under reduced pressure, after liquid-liquid extraction was performed with dichloromethane and the soluble fraction was lyophilized for storage and calculations yields. A second extract consisting of the juice of fresh stalks that obtained with a commercial juicer was prepared. The juice was repeatedly filtered and centrifuged to remove suspended solids. Finally lyophilized powder you had a liquid-liquid extraction with light petroleum for removing fats present, filtered leaving dust completely dry for storage. The compositional analysis was performed according to the Mexican Official Norms and the determination of reducing sugars was performed by the DNS method. Separation of saponins was performed on gel filtration and the compounds were monitored by thin layer chromatography with the intention of having more pure sub-fractions and in terms of their increased activity or content of saponins, fractions of interest again were separated by silica gel. To ensure the presence of saponins in work samples, an evaluation was performed on the hemolytic activity with fractions, for which bovine blood was collected, with

erythrocytes a solution of 3% v / v was made, in which were immersed loaded with fractions where have suspected presence of saponins (Sharm et al. 2013).

RESULTS AND DISCUSSION

Agaves are different in their morphological characteristics (Fig. 1). Leaves of *A. salmiana* are lanceolate, measured on average 60 cm long, their spines were subulate, color ranged from green to a grayish color. Leaves of *A. tequilana* Weber are unchanged lanceolate way with 80 cm long with apical spine, hooked and numerous, presenting a blue color. The description of these agaves corresponds to that already described by other authors.

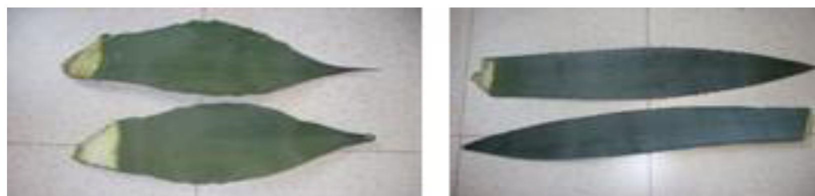

Figura No. 1. Figure No. 1 Leaves of *A. salmiana* (left) and A. *tequilana* (right).

A micro-structural level, the fibers of *A. salmiana* have a diameter ranging from 269 µm to 680 µm, in the case of **A.** *tequilana* the fiber diameters ranging from 164 microns to 363 µm (Fig. 2).

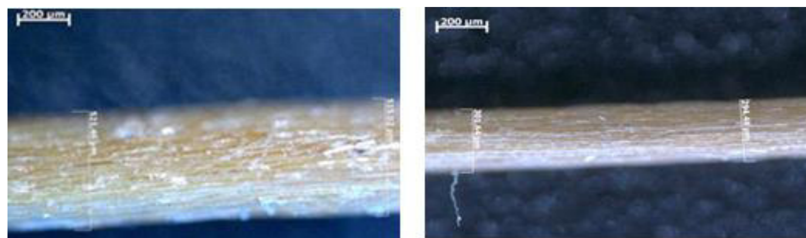

Figure No. 2. Micrographs of the fibers taken with optical microscope of bagasse of **A.** *salmiana* (left) and *A. tequilana* (right). View 5x reference bars indicate 200 microns.

The absorption spectra IR all studied samples revealed the presence of characteristic bands and by scanning electron microscope the type of fiber and the weight percent of elements for the samples were carbon, oxygen and calcium inferring the presence of calcium carbonate in the agaves. (data not shown). In the chemical composition analysis (Table No. 1) shows that the percent of ash, fiber and ether extract varies between the two species of agaves, while the percent protein is greater in the case of *A. salmiana* both fresh leaves as the extract.

Tabla 1. Composition analysis from leaves of *A. salmiana* and *A. tequilana.*

Agave	Humidity %	Ashes %	Ether extract %	Fiber %	Protein %	Reducing sugars mg/mL
A. salmiana[a]	82.40	14	0.58	20	5.1	58.47
A. salmiana[b]		14	0.08	21	7.3	57.08
A. tequilana[a]	85.30	14	0.99	21.5	5.2	64.64

A. tequilana[b]	15	0.16	22	6.4	65.98

[a] to refer to fresh leaves and [b] to the residue after extraction conducting.

Crude extracts were fractionated by column chromatography to give a total of 7 fractions E1 and E2 of 14 *A. salmiana*; and 7 fractions E1 and E2 3 *A. tequilana* Weber. In all cases, the fractions that were obtained with the mixture water: methanol 25:75 to 0: 100 were positive with anisaldehyde reagent and had erythrocyte hemolysis which indicated the presence of saponins (Figure 3). To confirm the presence saponins hemolytic activity of the extracts was assessed in erythrocytes.

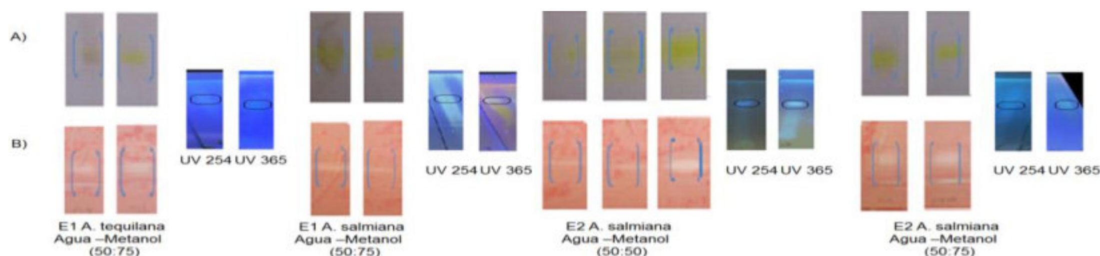

Figure 3. The photographs represent CCD plates. In the image are marked by brackets those fractions that were positive for anisaldehyde reagent (A) and showed hemolysis of erythrocytes (B) as well as the plates 254 views light and E2 have 365.

CONCLUSIONS

The phytochemical characterization showed the presence of including saponins important compounds confirmed by thin layer chromatography secondary metabolites.

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A supplement enriched with agave fructans for childhood obesity evaluated in *ex vivo* system

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ABSTRACT

Obesity is an important public health problem that national prevalence in Mexico in children between 5-11 years old is about 34.4%; however, it is significant to identify new factors involved in childhood obesity development and to create more effective intervention strategies. Studies have linked gut microbiota as a new factor involved in body weight regulation associated with obesity and its influence on metabolic host functions and immunological diseases. CIATEJ has developed techniques to extract fructans from agave with different polymerized grade and evaluated their biological and functional activity. The effect in the metabolism is according to fructans characteristics, such as prebiotic, anti adipogenic, immunomodulatory effects. A Functional Symbiotic Supplement (FSS), enriched with agave fructans, was designed as alternative against childhood obesity and evaluated *ex vivo* in ARIS (Automatic and Robotic Intestinal System) adapted to children physiological conditions.

Ten children with obesity between 8-11 years old were selected as to microbiota donors, samples were analyzed by plating. A pool of these samples was inoculated in ARIS for to evaluate nutrient availability (carbohydrates, lipids and proteins). ARIS was fed with a standard diet then it was compared with FSS.

According to the microbial analysis, this population presented dysbiosis, most of them were methanogenic and opportunistic pathogens, and the probiotics were diminished compared with previous reports for this physiological condition. This study shows amount of sugars, lipids and proteins are different when these are consumed than when they are bioavailable. Although the FSS evaluated was rich in sugars, these amounts were changed by digestive interactions. Sugars availability decreased about 50% with the FSS compared to standard feeding, also, the use of the FSS demonstrated higher protein bioavailability than standard food. Microbiological monitoring shows involution of dysbiosis, because higher levels of probiotic microorganisms were found after supplementation. It is reported that diet can modify gut microbiota and equality that affects the host energy metabolism.

Keywords: Obesity, Gut microbiota, Dysbiosis, Bioavailability, Automatic and Robotic Intestinal System.

INTRODUCTION

According to Mexican statistical reports, overweight and obesity in children between 5-11 years old had 34.4% of national prevalence in 2012. This prevalence has remained steady increase since 2006 (INSP and SSA, 2012). Despite its multiple etiologies, the cornerstone of obesity remains the relationship between consumption and energy expenditure (Ruiz et al.

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2010). It is important to identify new factors involved in childhood obesity development, and to create more effective intervention strategies. Gut microbiota is a new factor involved in body weight regulation and sickness associated to obesity and its influence on metabolic host functions and immunological diseases (Sanz et al. 2009). Gut microbiota is heterogeneously distributed in the digestive tract, about 99% are in the colon with 10^9 to 10^{11} CFU/ g, and most of them are strict anaerobes microorganisms. Fermentation processes and bacterial growth in the ascending colon produce high concentrations of volatile fatty acids with low pH. In the descending colon, fermentation processes decrease, consecutively pH increases. Studies demonstrated microbiota is an important contributing factor to body fat storage. Dysbiosis could produce elevation of glucose, insulin, leptin and triglycerides in blood (Morales et al. 2010). Studies described microbiota of obese human is associated with overweight and metabolic syndrome, an increase of reason *Firmicutes/Bacteroidetes.* Agave fructans are dietary fibers with beneficial effects on the gastrointestinal physiology by impact in selective growth of probiotics in gut microbiota that offer a promising approach for the treatment of some metabolic disorders associated with obesity (Márquez et al. 2013).

METHODS

For this study, ten obese children between 8-11 years old were selected to evaluate their nutritional status and get fecal samples. Samples were processed to obtain microbiota. A pool of mirobiota samples was inoculated in ARIS adapted to the population physiological conditions to evaluate nutrient bioavailability and microbiota behavior. ARIS was fed with a formulated diet according to the habitual feeding of this population without any modification, then, these conditions were compared with FSS.

Evaluation in ARIS

ARIS has been considered as a tool to evaluate functional food, food ingredients and active ingredients. This system contains five reactor representing five digestive tract sections (stomach, small intestine, ascending colon, transverse colon and descending colon) the last three sections are inoculated with gut microbiota and physiology adapted to the population that is represented. Each section is maintained under physiological conditions, monitoring pH, temperature, digestive processes (enzymes), motion, and microbiota. Microbiota from obese children was stabilized in ARIS for two weeks; the system was fed with a standard diet. Then ARIS was fed with standard diet plus FSS. Samples from ARIS were obtained at 0, 4 and 9 days after of feeding.

Microbiological evaluation

Donors samples were individually analyzed by plate counting to quantify probiotics (*Lactobacillus spp,* and *Bifidobacterium spp*) and opportunistic pathogens (*Salmonella spp,* and *Clostridium spp*). Also, samples from ARIS were obtained from each digestion step (stomach, small intestine, ascending colon, transverse colon and descending colon) to quantify probiotics and opportunistic pathogens to compare changes that could occur by digestion and bacterial metabolism processes.

Nutrient bioavailability

Samples obtained from ARIS were processed to quantify nutrient bioavailability, carbohydrates, lipids, protein, fiber, amino acids, in every digestion step to compare energy bioavailability and essential nutrient such as amino acids.

RESULTS AND DISCUSSION

Microbial analysis shows that this population presented dysbiosis. Children from this study presented least amount of *Bifidobacterium spp* as is shown in Figure 1. According to reports, the bifidobacterial numbers in fecal samples during infancy are higher in children remaining at normal-weight than in children becoming overweight (Kalliomâki et al. 2008). Some strains of *Bifidobacterium* typify the gut microbiota composition of the healthy child. Also, *Bifidobacterium spp* influence the total metabolic activity of the gut microbiota by interactions between mucosal, microbes and the host (Vaahtovuo et al. 2005).

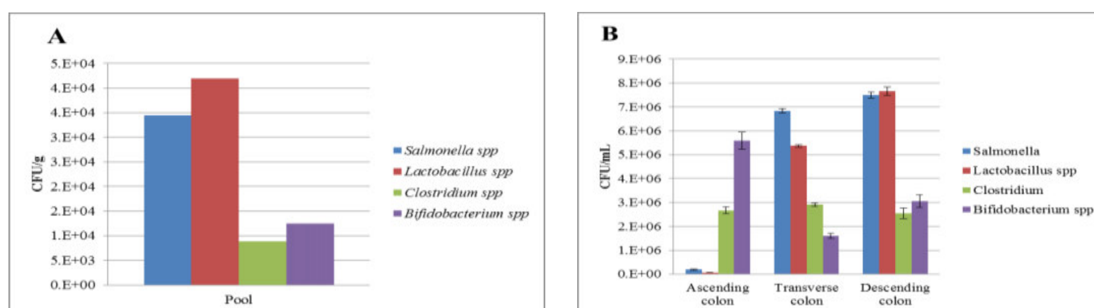

Figure 1. CFU/g of *Salmonella spp, Lactobacillus spp, Clostridium spp and Bifidobacterium spp.* A: from orginal samples of obese children. B: from ARIS samples after feeding with FSS.

Microorganism quantification from ARIS samples after feeding with FSS (Figure 1) shows amounts of *Bifidobacterium spp* were higher than original sample. This data is statistically significant ($p<.0001$). It is noted that although Salmonella genus growth with the administration of the functional food increases, come to keep the balance without exceeding the level of probiotic. *Salmonella spp, Lactobacillus spp* and *Clostridium spp* comparison in original sample and ARIS were statistically significant too ($p<.001$). Metabolites behavior shows the variability in the disposition to be absorbed. Figure 2 that initial carbohydrates availability decreased depending on the digestive process in question. The protein concentration is decreased by almost half and the total fat (refer to saturated and unsaturated lipids) showed a slight increase in the ascending colon. It should be noted that what is shown in this figure represents the conditions of digestibility in a type pediatric patient according to the characteristics of the patients included in this study; this gives us an overview of the behavior of nutrients from food commonly consumed by children in these circumstances.

Data shown in Figure 2 indicates nutrients concentrations derived from FSS digestion combined with standard feed. Initial carbohydrates concentration is almost three times the standard feed alone, but nutrients provision is decreased in dependence on FSS consumption. With regard to the protein has a slight decrease in the stomach and remains constant for the rest of the digestion, indicating that there is a balance of consumption by microorganisms.

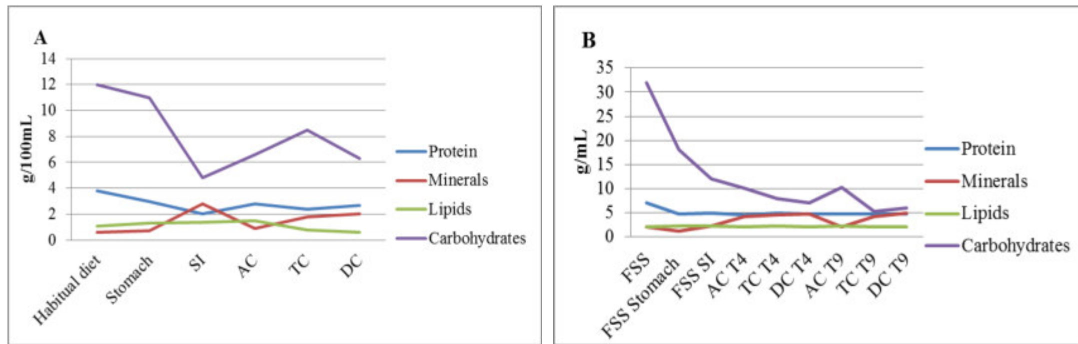

Figure 2. Nutrient bioavailability determination from ARIS. A: with standard feeding. SI: Small intestine, AC: Ascending colon, TC: Transverse colon, DC: Descending colon. B: with FSS. FSS: Functional Symbiotic Supplement, SI: Small intestine, ACT4: Ascending colon, TC: Transverse colon, DC: Descending colon, T4: Fourth feeding with FSS, T9: Ninth feeding with FSS.

CONCLUSIONS

The use of dietary strategies to modulate microbiota based on probiotics and prebiotics is likely to contribute to the control of the metabolic disorders more efficiently.

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Elaboration and characterization of encapsulated of curcumin with fructans

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ABSTRACT

Curcumin is a compound susceptible to oxidation reactions, and the encapsulation is a method used to protect bioactive compounds of these reactions. In this research, it has been shown that fructans have properties allowing its use for encapsulating biomolecules. The aim of this study was to develop and to characterize curcumin encapsulated with Agave angustifolia Haw fructans. Emulsions were prepared, using 2 different proportions of fructans of agave (25 y 35 %) and the other components (water, oil, lecithin, sucrose and turmeric oleoresin) remained unchanged. The emulsions were used to prepare the encapsulated (A and B respectively) by spray drying, which were characterized by color index (IC *) and trapping efficiency curcumin qualitatively by optical and epifluorescence microscopy, with different treatments (encapsulated: unwashed; washed with ethanol and chloroform and broken after they were washed with the solvent), and quantitatively by HPLC.

The IC * of the encapsulated A and B was similar and corresponded to a pale yellow color. Moreover, the encapsulated A retained 4887.6 µg curcumin after encapsulation process while encapsulated B retained 3981.48 µg curcumin. Meanwhile the optical microscopy showed that the particles of the encapsulated A and B were spherical with a diameter between 2 to 78 µm. They also had ability to fluoresce more than 9% of its area; in this regard a higher fluorescent area was obtained after washing with ethanol, respect to the unwashed. The encapsulated washing with chloroform not showed fluorescent area, but the encapsulated broken after they were washed with chloroform were more fluorescent area that all other treatments, approximately 49.1% for encapsulated A and 36.1% for B. In conclusion the encapsulated B retained the highest amount of curcumin and the fructans help protect the curcumin, when them are used as wall materials for the encapsulation process.

Keywords: Spray Drying, encapsulated, curcumin, fructans, microscopy.

INTRODUCTION

Some bioactive compounds such as curcumin act positively about oxidation reactions, exerting a benefit health (Cui et al. 2009). However its exposition to environmental factors such as high temperature, O_2 and pH greater than 7 may promote degradation or decreased bioactivity occur (Oetari et al. 1995). The use of an inexpensive and useful method as encapsulation by spray drying method can be useful for the protection of bioactive compounds of oxidation reactions but for successful encapsulation is necessary to consider

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the choice of wall material according to the characteristics of the material to be encapsulated (Nedovic et al. 2011).

Recently it has been considered the use of fructans as encapsulating material (Bakowska and Kolodziejczyk, 2011), because it has been observed that the bioactive compound can be maintained after the encapsulation process (Saenz et al. 2009). The purpose of this study was to develop and to characterize curcumin encapsulated with Agave angustifolia Haw fructans.

METHODS

Elaboration of encapsulated

Emulsions with two different ratios of agave fructans (25 and 35%) were prepared and the other components: water, oil, lecithin, sucrose and turmeric oleoresin at 60, 8, 1, 5, and 1% respectively, remained unchanged. Each mixture was stirred for 15 minutes at 18 000 rpm. These emulsions were used to produce the encapsulated by spray drying with a feed of 1.2 L / h, inlet temperature 180 ° C, outlet temperature of 80 ° C and pressure of 1.2 Kg / cm^2.

Color Determination

The CIELAB model was used to obtain the parameters L, a, b. and the color index *IC was calculated (Vignoni et al. 2006).

HPLC Quantification of curcumin

1 g of encapsulated was used to quantifying curcumin by HPLC with diode array detector, C18 column 15 cm x 0.25 mm. Was used as mobile phase acetonitrile: methanol: tetrahydrofuran (THF) (58: 35: 7) in an isocratic flow of 1 mL / min. Detection was performed at 440 and 450 nm. Quantification was performed through a linear calibration curve (area vs concentration) using a standard.

Encapsulated photomicrographs analysis

Observations were made at a 40 X, using an optical microscope, 50 images were captured by light and epifluorescence microscopy for each treatment with Metamorph software (V 5.0): unwashed encapsulated, encapsulated washed with 96% ethanol and / or chloroform, washed with chloroform and broken encapsulated. From de images was obtained the fluorescent area of each encapsulated was obtained and the difference between areas and the amount of curcumin with was calculated with the Image J program (1.44 p).

Statistical Analysis

Data were analyzed in the Sigma Plot software (version 11), using student t-test, one-way ANOVA and significant difference was found when the comparison method of Tukey was used.

RESULTS AND DISCUSSION

*IC was 0.54 ± 0.01 and 0.52 ± 0.01 for the encapsulated A and B respectively, there was no significant difference and both samples exhibited a pale yellow color. HPLC analysis showed a retention time of 1.52 min for the turmeric oleoresin and the encapsulated A and B were within this range and the presence of other peaks are not observed indicating not structural modification and / or the presence of isomers compounds (Patil et al. 2005).

Furthermore, considering that per g of encapsulated 4897.39 µg of curcumin was used and that after the encapsulation process 4887.69 µg of curcumin / g encapsulated A and 3803.1669 µg curcumin / g encapsulated B remained, we could say that incorporating fructans in the encapsulating matrix helps keep the bioactive compound. Due to turmeric oleoresin has the ability to fluoresce because it has double bonds and cyclic molecules (Anand et al. 2010), allowing it to absorb light in the presence of exciting radiation (Vives, 2006) in the micrograph it was observed that the encapsulated A showed 10.45% of fluorescent area, while the encapsulated B showed 9.5% of fluorescent area. With this result, it could be considered that the remaining oleoresin is dispersed in the encapsulating matrix (Gharsallaoui et al. 2007). To avoid this event, encapsulated were washed with various solvents to remove the surface of the bioactive compound and thus identify its presence within them.

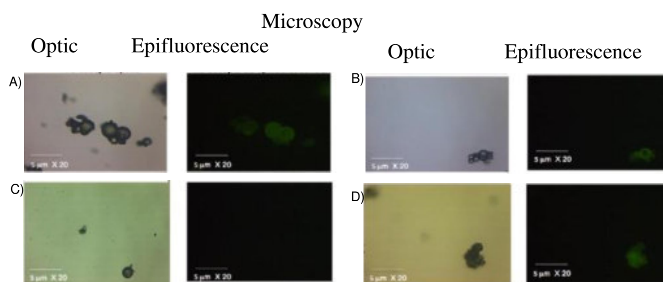

Figure 1. Micrographs to 20 x of encapsulated before and after of washes with solvents. a) unwashed; b) washed with ethanol; c) washed with chloroform; washed with chloroform and broken

In encapsulated washed with ethanol, higher fluorescent area was observed compared to encapsulated unwashed, probably due to that fructans are soluble or partially soluble in ethanol (Chacón, 2006) and possibly the solvent become soluble the capsules, releasing the oleoresin. In addition, the encapsulated washed with chloroform showed no fluorescent area, so it was considered that the non-polar nature of the solvent-soluble compounds extracted oleoresin used as the surface. On the other hand, the encapsulated washed with chloroform and then broken had fluorescent greater area than the all treatments; these were 49.1% and 36.1% of fluorescence area in the encapsulated A and B respectively.

CONCLUSIONS

The biggest content of curcumin was obtained in encapsulated A and fructans help to protect the curcumin, when it is used as wall material in the encapsulation process.

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Evaluation of agave fructans in starchy foods with and without thermal process

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ABSTRACT

Enzymatic/Spectrophotometric AOAC 999.03 fructan measurement method was modified with the objective to quantify agave fructans. Usefulness of the modified method was confirmed in pure agave fructans. Modified method result in about 25% of underestimation when fructans were mixed with food matrices. The effect over fructan losses resulted from thermal process was evaluated. Long chain fructans achieve better resistance in comparison with native fructans (8% of thermal loses) no matter the source of ingredient. In the case of native fructans, agave ingredients showed lower degradation in baked bread in comparison with chicory fructans.

Key words: agave fructans, soluble fibre, inulin measurement, AOAC 999.03, inulin thermal degradation.

INTRODUCTION

Fructans quantification is of a great concern because it is required for food labeling purposes to make a fiber claims. The choice of the analytical method has a significant influence on fructan quantification especially in heat-treated samples. Enzymatic-gravimetric method for total dietary fibre AOAC 985.29 it's not a suitable method for fructan quantification because fructans do not precipitate with alcohol in a included step. There are two other official methods for fructan measurement in foods: AOAC 999.03 and 997.08. The validation of these methods has been conducted using chicory fructans and up to now there are no information about method selectivity to agave fructans. Present study shows results from agave fructans quantification in bread with and without thermic process.

METODOLOGY

Characterization of Ingredients

Chicory ingredients for evaluation: Beneo P95, Raftiline GR and Orafti HP. Agave ingredients used were Nutriagaves and AM-101. Ingredients were characterized using ultrafiltration, TLC and HPLC and classified in: a) sugars, b)fructo-oligosaccharides (FOS) and c) long chain fructans (fructans with DP>12).

Verification of agave ingredient hydrolisis

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Enzyme kinetic experiments were conducted using endo- and exo-inulinases with the object to determine the time necessary for complete hydrolysis of agave fructans. The resulting time was introduced in the method AOAC 999.03 for further evaluations.

Bread preparation

Different types of bread were prepared for the experiments. The general bread formulation contained: 70% flour, 15% egg, 5% fresh yeast, 5% butter, 3% sugar, 1.6% milk and 0,4% salt. For fructans incorporation in BAKING experiments, flour was partially substituted by 10-12.5% of fructans. Baking was done at 200ºC for 20 min.

Types of bread:

1) White bread **without fructans** for blank and NO BAKING experiments was done using general bread formulation. After baking bread was dryed and ground and mixed with pure fructans in relation 9:1.
2) Bread with **agave** fructans in relation 9:1 was performed for BAKING experiments. Fructans incorporation was done before baking. After baking bread was dryed and ground.
3) Bread with **chicory** fructans in relation 9.1 was performed for BAKING experiments. Fructans incorporation was done before baking. After baking bread was dryed and ground.

NO BAKING experiments were prepared adding pure fructans with the powder bread no. 1 and without thermic process. This experiment was conducted to evaluate accuracy and specificity of the modified AOAC 999.03.

BAKING experiments were conducted mixing fructans with bread formulation before baking, so this fructans had a thermal process. This experiment was performed to evaluate the fructans degradation as a result of heat-treatment.

Evaluation of method for fructan measurement in bread

Method AOAC 999.03 with modifications was conducted. Specificity and accuracy were evaluated through percent of fructan recovery (%R) in dry weight basis. Fructans measurement and %R were evaluated adding fructans in relation 9:1 to bread number 1) without thermic process. The effect of the thermal process in fructan content was evaluated adding fructans as mentionted above in bread no. 2 and 3. Fructans resistance was evaluated comparing percentage of fructan recovery before and after baking.

RESULTS AND DISCUSION

Figure 1 shows the carbohydrate distribution of ingredients. Both native ingredients from chicory (Raftiline GR) and agave (Nutriagaves) were composed from a mixture about 40% FOS and 55% long fructans, with small quantity of sugars. The other three ingredients AM-101, Orafty HP and Beneo P95 meet their specification. AM-101 is an agave ingredient prepared in CIATEJ through ultrafiltration and contains only long fructans as is shown in Figure 1. Beneo P95 is a chicory ingredient containing more than 90% of FOS and Orafty HP from chicory contains mainly long chain fructans.

Initial evaluation of endo- and exo-inulinases (results not shown) allow to know that agave fructans required 2 hours instead of 30 minutes indicated at original method AOAC 999.03, to complete hydrolysis the fructans, so this modification was introduced for every fructan measurement.

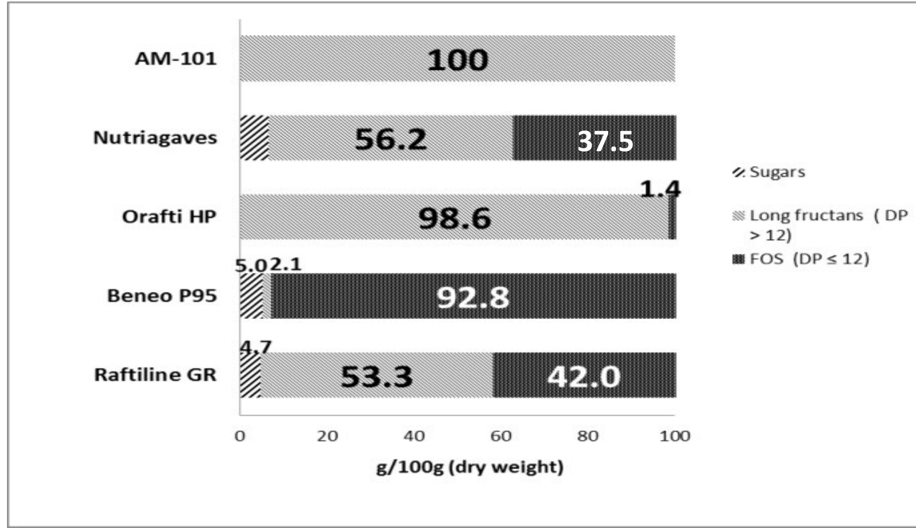

FIGURE 1. Carbohydrate distribution of four ingredients used in the experiments.

Table 1 shows results from NO BAKING experiments. The method underestimation was about 25%, with exception of FOS. Native ingredients had similar %R (mean 71%) no matter the source of fructans. Slightly better %R was obtained for long chain fructans (76.5-81.3%).
The experiment showed an underestimation of fructan content by method of about 27% in the case of native ingredient, 22% in long chain fructans, and 99.6% in FOS.
Method produced high underestimation of FOS, which has been previously reported by McCleary & Rossiter (2004). Pure agave native ingredient was evaluated resulting in 92-104 %R, showing the suitability of the method for pure ingredient evaluation.

TABLE 1. Evaluation of fructans percentage of recovery in NO BAKING experiments.

	NUTRIAGAVES Native agave	RAFTILINE GR Native chicory	AM-101 Long ch agave	ORAFTI HP Long ch chicory	BENEO P95 FOS chicory
FRUCTAN ADITION g/100g	10	10	10	10	10
FRUCTAN QUANTIFICACION g/100g	7.2 ± 0.9	7.1 ± 0.6	8.1 ± 0.5	7.7 ± 0.8	0.04 ± 0.01
PERCENTAGE OF RECOVERY	71.5 ± 9.2	70.7 ± 0.3	81.3 ± 5.0	76.5 ± 7.8	0.4 ± 0.1

The possibility to get a reduction in %R because of the percentage of FOS (40% in raw ingredients) was eliminated by experiments with long chain fructans. AM-101 and Orafti HP that didn't have FOS, resulting in 76.5-81.3%R. On the other hand, similar loses were observed when concentration ranged from 1 to 20 % of long chain fructans were added to bread without thermal process (%R between 73-86% results not shown). With this evidence

we hypothesize that loses produced by the method maybe because of interaction with other ingredients of bread formulation, since experiments made with the pure ingredient didn´t show loses.
Table 2 shows the effect of fructans reduction in heat-treated samples. The difference between NO BAKING – BAKING was used for correction of the method underestimation. . Long chain fructans achieve better resistance in comparison with native fructans (fructans reduction 8.2% vs. 16% respectively) no matter the source of ingredient. On the other hand, native agave fructans result in only 10.5% of loses after thermal process, against 21.5% from chicory ingredient.

TABLE 2. Comparisson of fructans percentage of recovery in bread from NO BAKING vs. BAKING experiments.

	AM-101 Long ch agave	ORAFTI HP Long ch chycory	NUTRIAGAVES Native agave	RAFTILINE GR Native chicory
	% of fructan recovery			
No baking	81.3%	76.5%	71.5%	70.7%
Baking	74.0%	67.5%	61.0%	49.2%
Difference by baking process	7.3%	9.0%	10.5%	21.5%

Böhm *et al*., (2005) observed significant loses on pure inulin by heat-treated experiments, e.g. they report degradation about 50% in 30 min., at 195° C, while we get 21.5% with similar ingredient. Differences could be explained because they used pure ingredient and we introduced the fructans into the bread formulation, then the other ingredients are maybe protecting them.

CONCLUSION

Usefulness of the modified method AOAC 999.03 for pure agave fructans quantitation was confirmed. The method result in about 25% of underestimation when fructans were mixed in food matrices with no heat process. Evaluation of the frcutans resistance to heat-process showed that long chain fructans achieve better resistance (8% better) in comparison with native fructans, no matter the source of ingredient. In the case of native fructans, agave ingredients showed lower degradation in baked bread in comparison with chicory fructans.

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Comparative analysis of sugars in syrups of agave, corn and honey

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ABSTRACT

The syrup of Agave is a sweet substance, produced for the hydrolysis of fructans. And know their sugars content is useful for evaluation its purity and quality. In this job, were evaluated the sugars in the syrup of *A. tequilana* elaborated in CEPROBI, compared with others syrups of agave, corn and honey. The carbohydrates in the syrup of *A. tequilana* elaborated in CEPROBI (Syrup T1) and in the commerciales syrups of *A. tequilana* (Syrup T2), corn (Syrup M) and honey of bee (Honey), were identified for TLC and FT-IR. Also were quantified their sugars by spectroscopy and ºBrix with a refractometer. The standards used were fructose, glucose, maltose and sucrose. In the syrup T1, T2 and honey were observed fructose and glucose, addition in T1 and honey, was observed sucrose; meanwhile, the syrup M had the presence of glucose, sucrose, maltose and other sugars not studied. Also through FT-IR appear bands in the region between 1408 cm-1 and 775 cm-1, particularly specific of functionals groups of carbohydrates and were obtained characteristics peaks for sucrose to 918 and 977 cm-1, glucose to 1016 cm-1 in the syrups T1, T2, M and honey. Additionally T1, T2 and honey presented fructose to 1050 cm^{-1}. Moreover the analyse of sugars content for syrup T1 in agreement to NMX-FF-110-SCFI-2008, satisfy with % of reducing sugars (90 %), fructose (82 %) glucose (17 %) required; however the syrup T2, of similar source, not satisfy with this requirements. Although, the ºBrix is in the allowed interval. Concluding than the fructose is the sugar principal of syrup *A. tequilana* elaborated in CEPROBI, followed of glucose and sucrose. Also its content is similar to the commerciales syrups and honey.

Keywords: Agave, sugars, syrup, TLC, FT-IR.

INTRODUCTION

The *Agave tequilana* Weber var. Blue is the most important crop in Mexico, mainly because it is the raw material for the production of tequila; because their main source of carbohydrate reserves (fructans), which are composed of a high content of fructose chains with lesser amounts of glucose molecules (Mancilla and López, 2006). Further according to scientific studies have shown that this crop has beneficial effects on health (Mellado and López, 2013). And An alternative use of this plant is the production of agave syrup, which can compete in the market with other commercial syrups (Rendon et al. 2007; Mellado and López, 2013). For this it is necessary to know its composition and quality assessment according to the NMX-FF-110-SCFI-2008 (Mellado and López, 2013). And also, it is

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important to implement rapid analytical techniques to detect different types of sugars such as glucose, fructose and sucrose (Kelly et al. 2005; Mellado and López, 2013).

METHODS

Thin-layer chromatography (TLC)

Were applied 3 µL of aqueous solutions of each sample (syrup T1, Syrup T2, Syrup M and Honey) (3 mg / mL) to the plates of silica gel with aluminum support. TLC plates were developed in a solvent system of butanol / propanol / water and the presence of carbohydrate was detected with an aniline / diphenylamine / acetone/ phosphoric acid (Mancilla and López, 2006).

Spectroscopy FT-IR

Was used for this analysis an equipment FT-IR with the following scan parameters: Measurement mode in% Transmittance, No of Scans: 20, resolution 4 cm^{-1} in a range of 600-4000 cm-1.

Determination of sugars total, reducing and fructose

The determinations were carried according to the method of Ting (1956). To this 2.5 mL of a diluted solution was used for each sample (syrup T1, Syrup T2, syrup M and Honey), exposed to boiling for 20 min and the sugars were quantified in a spectrophotometer at a wavelength of 515 nm, using A standard curve of fructose and glucose. However, for the determination of fructose temperature conditions and reaction time (55 ° C, 30 min) were modified.

°Brix

Brix content was determined according to standards NMX Mexicana (2008), placing a drop of each sample on a portable refractometer with scale 60-90 ºBrix.

RESULTS AND DISCUSSION

The conditions tested for TLC indicated a retention time (Rf) and a specific color for each standard: fructose and glucose with Rf of 0.79 and a brown and blue color band respectively; sucrose with Rf of 0.76 and brown band, maltose with Rf of 0.71 and blue band (Figure 1).

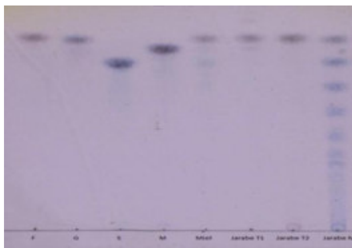

Figure 1. Thin layer chromatography of syrup and honey.

The carbohydrate identified in syrup T1were: fructose, glucose and sucrose; whereas for syrup T2 were observed the presence of fructose and glucose only. This result agrees with that reported by Rendon et al. 2007; Mellado and López, 2013; in syrup *Agave tequilana*

Weber var. Blue, where have a greater presence of fructose and concentration lower of glucose and sucrose, due to its chemical composition (Mancilla and López, 2006); moreover in honey were identified fructose, glucose and sucrose, coinciding with Mellado and López (2013) in the two first mentioned sugars, except for the presence of sucrose and other sugars absents in our sample. Regarding corn syrup was detected glucose, sucrose, maltose and other molecules with different Rf (0.62, 0.55, 0.46 and 0.38) compared to standards used. Coinciding with that reported by Mellado and López, (2013) for corn syrup, where they identified the presence of glucose and maltooligosaccharides, with the exception of the presence of sucrose.

In Figure 2, appear the spectra of the various syrups and honey. Where it was observed that all the samples have a band at 3304 cm^{-1}, due to stretching vibration of functional group OH characteristic of carbohydrates (Gallardo et al. 2009; Rios, 2010); and also, the presence of a band at 2929 cm^{-1} indicating the stretching of functional group CH. Also a small band found at 1099 cm -1 corresponding to stretching C-O of C-O-C group which is present in the glycosidic bond of saccharose (Garcia et al. 2009) and was observed bands at 977 and 918cm^{-1} indicating the C-H bending of the disaccharide sucrose; also the presence of a band at 1016 cm^{-1} which is representative of glucose. However, solely for the T1, T2 and honey syrup was observed a band at 1050 cm^{-1}, indicating the presence of fructose (Rios, 2010). These bands, coincides with that reported for the principal functional groups of carbohydrates: 775, 918, 1024, 1047, 1099, 1253, 1344, and 1408 cm^{-1} (Rios, 2010).

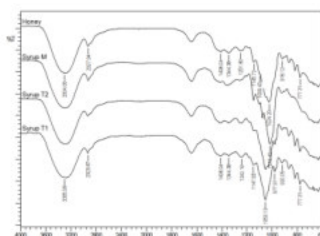

Figure 2. Spectro FT-IR of syrup and honey

In the analyses of sugars content (Table 1) in agreement to NMX-FF-110-SCFI-2008, the syrup T1 satisfies with % of reducing sugars (90 %), fructose (82 %) glucose (17 %) required. And a high fructose in syrups, is a determining factor in assessing the quality of syrups (López et al. 2003). However the syrup T2 of similar source, not satisfy with the requirements; because its content in glucose (31 %) is greater than the accepted for norm. However, the °Brix content for all the syrups is within the permitted (74 °Brix).

Table 1. Content of sugars in syrup and honey

SAMPLE	°BRIX	TOTAL SUGARS (g/25 mL sample)	REDUCING SUGARS (g/25 mL sample)	FRUCTOSE (g/25 mL sample)	GLUCOSE (g/25 mL sample)	SUCROSE (g/25 mL sample)
Honey	81.5	142.526	127.726	90.173	52.353	14.060
Syrup T_1	74.0	114.644	103.56	94.082	20.562	13.573
Syrup T_2	77.0	141.659	117.131	98.268	43.390	23.301
Syrup M	75.5	67.750	61.214	9.898	57.852	6.2092

CONCLUSIONS

The fructose is the sugar principal of syrup *A. tequilana* elaborated in CEPROBI, followed of glucose and sucrose. Also its content is similar to the commerciales syrups and honey.

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Concentration of fructans from natural *Agave salmiana* juice by nanofiltration.

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ABSTRACT

Fructans have received considerable interest as ingredients in food industry due to their functional properties as well as their health benefits. *Agave* fructans are obtained from natural juice containing significant amounts of low molecular mass impurities as glucose, fructose and sucrose, which do not contribute to the beneficial properties of fructans. In this work was studied the fructans concentration and separation of low molecular mass sugars from natural *Agave* juice using a stirred-cell nanofiltration unit operated in concentration mode. Hydrophilic cellulose membrane with MWCO of 500 and1000 Da was used to filter natural *Agave salmiana* juice of different total solid contents. The test parameters were permeate flux, solute retained fractions and separation factor. Fructans was the solute with the highest rejection between 0.74 and 0.99; the sucrose was mildly retained, while glucose and fructose rejections were greatly affected by NWCO of membrane and total solid content. A better LMW sugars removal and fructans purification was achieved with membrane of MWCO 1000 Da. Lower total solid content of juice improves the separation efficiency and has greater productivity.

Key words: fructans, nanofiltration, membrane, *Agave salmiana*, stirred cell.

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INTRODUCTION

Fructans have received considerable interest as ingredients in food industry due to their functional properties as well as their health benefits. In Mexico, fructans can be obtained from de *Agave* plants, where the process include the extraction of *Agave* juice by water diffusion or crude juice production from crushed *Agave* piña. This juice contains up to 65-80% of fructans in the fraction of carbohydrates, significant amounts of low molecular mass carbohydrates (fructose, glucose and sucrose) and some other impurities which do not contribute to the beneficial properties of fructans. These undesirable low molecular weight components can be eliminate by membrane technology, mainly in the range of nanofiltration. Others works in membrane processes (Pinelo *et al*. 2009, Moreno-Vilet e*t al*. 2013,) have noted that crucial parameters such as permeate flux and solutes rejection are influenced by several factors including type of membrane, pressure, temperature and concentration of the feed solution. In the case of *Agave* juice, the total solid concentration is highly variable, ranging from 12 to 25 °Brix, since this fluctuates as a function of plant species, region and time of harvest (Arrizon *et al.* 2010).
The aim of this work was to investigate the technical feasibility of concentrate or purify fructans from natural *Agave* juice by elimination of LMW sugars using a nanofiltration stirred cell and different MWCO membranes.

METHODOLOGY

Natural *Agave salmiana* juices of different ages (7-9 years) were provided by a local company "Productores de mieles y jarabes de maguey de Zaragoza de Solís". The juice was filtered several times using filter paper with a pore diameter of 22, 4 and 2.5 µm. To inactivate the saponins present in the juice, these were treated by heating at 80 °C for 30 min in a water bath with continuous agitation (Tarade *et al.* 2006)
A Millipore stirred cell (Model 8400, Millipore-Amicon Corporation, USA) with an effective membrane area of 4.18×10^{-3} m^2 was used in all experiments. Hydrophilic regenerated cellulose membranes with molecular weight cut-off (MWCO) of 1000 Da (Milllipore, catalogue number PLAC07610) or MWCO of 500 Da (SpectrumLabs, 887752) was inserted into the cell and afterwards the cell was filled with 50 mL of *Agave* juice, operated in concentration mode at 0.245 MPa. The experiment was stopped when a volume of 25 mL permeate was collected corresponding to a volume concentration factor (VCF) of 2. The test parameters were permeate flux, solute retained fractions SRF (Eq. 1) and separation factor SF (Eq. 2).

$$SRF = \frac{(C_F \times V_F) - (C_p \times V_P)}{(C_F \times V_F)} \quad (1) \qquad SF = \frac{SRF_{HMW}}{SRF_{LMW}} \quad (2)$$

Where C and V are the concentration and volume of feed solution (F) at initial time and permeate solution (P) at final time of the NF experiment. HWM high molecular weight component (fructans), LMW low molecular weight components (glucose, fructose and sucrose).
Sugar concentration (glucose, fructose, sucrose and inulin) were analyzed at feed and permeate streams according to the HPLC-method proposed by Michel-Cuello et al. (2012)

with Waters 600 chromatography equipment (Milford MA. USA), an Aminex HPX-87C column ion exchange (7.8 mm d.i. x 300 mm, Bio-Rad, Hercules, CA, USA), HPLC grade water with a flow of 0.6 mL/min as the mobile phase. The column temperature was kept at 75 °C. The Quick Start Empower 5.0 was used for system control and data analysis.

RESULTS

To analyze the solutes mass transfer between retentate and permeate streams, Fig. 1 shows the evolution as the filtration progressed. For this experiment a model sugar solution was used with carbohydrates concentration analogous of *Agave* juice, where the initial total feed concentration was 20°Brix. As expected, a progressive decrease-increase of solute mass was observed due to the solute transferred from retentate to the permeate flux. It is evident that there is not permeate solute mass at initial period of operation and tends toward a constant increase as permeate volume increases until reaching a VCF of 2.

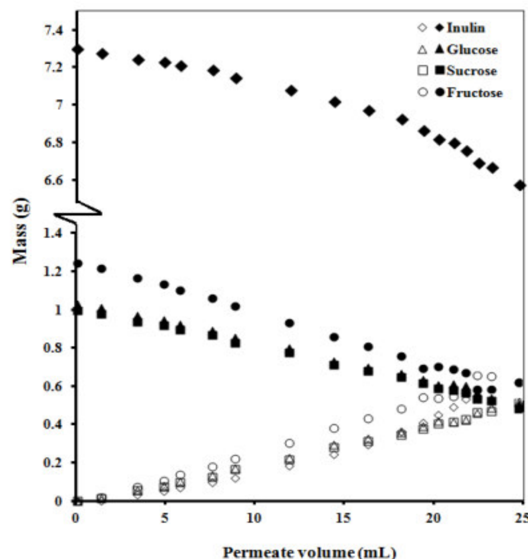

Fig. 1. Evolution of solute mass in retentate (*black figures*) and permeate (*white figures*) streams in function of permeate volume. C_F= 20 °Brix of model sugar solution and P=0.245 MPa.

Table 1 shows the experimental values of the permeate flux, and solute retained fraction for NF experiments of *Agave* juice with different total solid contents and using two MWCO membranes. Results show that the permeate flux for membrane of 1000 Da varied from 1 to 4.90 $L.h^{-1}.m^{-2}$, where the highest flux was for samples 2 and 4 which exhibit the lowest total solid content expressed as °Brix. Instead for membrane of 500 Da the flux was greatly reduced to values around of 0.3 $L.h^{-1}.m^{-2}$, which results in low productivity.

Table 1. Experimental results of permeate flux and solute retained fractions of nanofiltration of *Agave* juices operated at 0.245 MPa and VCF= 2.

Sample	*°Brix*	*Memb (Da)*	*Jp* *($L.h^{-1}m^{-2}$)*	*SRF*				*SF*
				Fructans	*Sucrose*	*Glucose*	*Fructose*	
1	14	1000	2.13	0.74	0.44	0.56	0.24	1.48
2	8.2	1000	4.98	0.84	0.72	0.53	0.48	1.58
3	20.4	1000	1.00	0.80	0.53	0.39	0.52	1.44
4	9.7	1000	4.01	0.80	0.50	0.53	0.37	1.59
5	6	500	0.37	0.99	0.69	0.73	0.76	1.36
6	11	500	0.27	0.98	0.93	0.83	0.75	1.17

Jp: permeate flux, SRF: Solute retained fraction, SF: separation factor

It can be noted that solute retained fraction (SRF) values do not exhibit a clear trend with initial solid content of *Agave* juices as was expected, but it is clear that using membrane of 500 Da bigger SRF values are obtained for all solutes. Fructans was the solute with the highest SRF between 0.74 and 0.99 the sucrose was mildly retained, whereas the SRF values for glucose and fructose were lower, ranging from 0.56 to 0.24 for membrane of 1000 Da and from 0.83 to 0.73 for membrane of 500 Da. The above indicate that membrane of 500 Da has a very small cut size to let freely pass of LMW sugars, where sucrose is even highly retained (SRF = 0.69 and 0.93) and this is not desirable for the purpose of purifying fructans. The above described, is better understood by separation factor (SF), where a higher value represent a better sugars (sucrose, glucose and fructose) removal by membrane and consequently a better fructans purification. Membrane of 1000 Da has higher SF values compared to membrane of 500 Da; besides, samples with low °Brix (samples 2 and 4) present higher SF values compared with samples with high °Brix (samples 1 and 3). This suggests that lower total solid content of juice improves the separation efficiency and has greater productivity using a membrane of 1000 Da.

CONCLUSIONS

Nanofiltration is a technically feasible membrane process for large-scale production of concentrated or purified fructans, using natural *Agave* juice as raw material. A better LMW sugars removal and fructans purification was achieved with membrane of MWCO 1000 Da. Lower total solid content of juice improves the separation efficiency and has greater productivity. The process of purification of fructans can be optimized by controlling the operation parameters such as pressure, temperature and feed concentration and by operating in diafiltration mode.

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Challenges and opportunities in the production of agave fructans and syrup

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ABSTRACT

Agave tequilana is one of the plant species with the highest content of storage carbohydrates (up to 30% w/w), which by processes of fermentation and distillation are used to produce the alcoholic beverage known as tequila. Recently, given the growing demand for alternative, healthier sweeteners and soluble fibers, the branched fructans and FOS(fructooligosaccharides) from*A. tequilana* have become an excellent raw material for the syrup and prebiotic industries. The extraction, purification and characterization processes still remain problematic, and together with the surplus/shortage cycles in the agave crop are the greatest challenges for this relatively young industry. An analysis of the different problems, from field to factory to consumer, as well as the opportunities in the production, commercialization and regulation of both the syrup and fructan will be presented.

The currently used industrial methods for the production of both products will be discussed, along with a comparative analysis of the different analytical techniques and methods for the determination of the most important parameters regarding product quality, such as moisture content, sugars, detection of foreign sugars and molecular weight/DP distribution.

Key words: Agave syrup, Agave fructan, prebiotics, Thin Layer Chromatography, Liquid Chromatography

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INTRODUCTION

There are over 210 species in the Agave genus, of which over 75% come from Mexico(Gentry, 1982). Being CAM (Crassulacean Acid Metabolism) plants(Nobel, 2003), they are particularly well adapted to the desertic and semidesertic climate of Mexico, and as such, have always played an important role in the ethnobotany of Mexico.

Since prehistory, agave has been used as a foodstuff, in some areas comprising up to 20% of the caloric intake (Leach y Sobolik, 2010). Then, as history progressed, all of its parts were utilized: the stalk as beams, the leaves as thatching material, the fibers for rope and cloth production, the thorns as needles, etc(Vela, 2014). In recent times, the fructan presentis now used as a source for alcoholic fermentation rather than a source of carbohydrates. This takes advantage of the fact that some species of agave can contain up to 30% w/w of fructan (Praznik, Löppert, y Huber, 2007). Now, as the demand for alternative sweeteners (i.e. organic certified, lower glycemic index) and fiber and prebioticsincreases, the fructans present in agave have become a very interesting source for these two products.

For the economic importance of the non-alcoholic products from agave, as an example, in 2004, the total exports of agave syrup from Mexico were 145 metric tons. For 2013, only 9 years later, that value was of15 000 metric tons; more than a 100-fold increase (Secretaría de Economía, 2014).Assuming a conservative yield of 7 kg agave/kg of syrup, the total of agave used for the production of this syrup amounted to 105 000 tons of agave. As a comparison, the tequila industry used 757 000 tons of *A. tequilana* for the production of tequila that year(Consejo Regulador del Tequila, 2014).

The aim of this work to present an analysis of the different problems, from field to factory to consumer, as well as the opportunities in the production, commercialization and regulation of both the syrup and fructan industries. Special emphasis will be placed in the currently used industrial methods for the production of both products along with a comparative analysis of the different analytical techniques and methods for the determination of the most important parameters regarding product quality, such as moisture content, sugars, detection of foreign sugars and molecular weight/DP distribution.

MATERIALS AND METHODS

For the determination of moisture, two methods were assayed: thermogravimetric loss-on-drying and Karl Fischer Titration. For the first, 20 g of sample were placed in an aluminum dish and left in a mechanical convection oven (Terlab TE-H45DM, Zapopan, México) at 105 ºC until the samples achieved constant weight. For the Karl Fischer titration, a Mettler Toledo DL-38 volumetric KF titrator(Ohio, USA) was used. Approximately 0.2 g of sample is added to the titration vessel, which already contains dry methanol and formamide (Sigma-Aldrich, Missouri, USA). To this mixture, the KF component (component 5, Sigma-Aldrich), which titrates 5 mg H2O/ml titrant is added until the titration point is reached.

The refractive properties were measured using Mettler Toledo RE40D refractometer (Ohio, USA) was used to measure both the refractive index and ºBrix of samples with different water contents. The samples were placed in the prism, and the measurement was performed when the temperature reached 20.00 ºC.

For the determination of the fructan content, methodAOAC 999.03 wasfollowed, using the fructan kit from Megazyme (Wicklow, Ireland) and the rest of the reagents by Sigma-Aldrich.

The TLC analysis is modification of the method by Praznik et al.(Praznik y col, 2013) was developed. The staining reagent used was 2% aniline, 2% diphenylamine, 15% phosphoric acid in acetone (Reiffová y Nemcová, 2006).

For the distribution of the degree of polymerizationseveral methods were compared: MALDI-TOF-MS was performed with a Voyager MS operated in the positive ion mode. The calibration was performed using a mixture of proteins. Samples were diluted 1:1 with 2,5Dihydroxybenzoic acid. Samples were desorbed from the sample plate with an N_2 laser with an intensity of approximately 2500.SEC. A chromatographic system comprised of a Superose 12 GL column plus Toyopearl HW 40S columns were used, using as eluentNaCl0.05 M + 50 mg/ml NaN_3. The eluent flow rate was 0.55 ml/min, and the detector was a differentialrefractive index.HPLC-amino. For this technique, a Carbohydrate Prevail ES 4.6 x 300 mm (Alltech associates) column was used, with the eluent being a gradient acetonitrile (A):0.4% NH4OH (B).Table 1 shows the gradient used. The detector was an ELSD detector, with a N2 flow of 1.6 lpm, nebulizer temperature 30ºC, evaporator temperature 90ºC.

Table 1. Gradient for oligosaccharide separation

% A	17	30	50	60	70	75
Time	0	20	40	60	80	70

RESULTS

A survey of the major producers of agave syrup/fructans show that all processes (with their rather different variations from plant to plant) consist of six operations: 1) carbohydrate extraction 2) removal of foreign matter 3) color removal 4) mineral removal 5) hydrolysis (in the case of agave syrup)/purification (in the case of agave fructans) and 6) removal of water. For 1), the two most common methods are the use of diffusers (large capital and operating costs, high yield) and rolling mills (low capital cost, average yield). The removal of foreign matter is usually done in two steps: a large strainer, followed by either a filter press or cartridge filter. Color and mineral removal are achieved by the use of ion exchange resins. For hydrolysis, this is usually accomplished either by the acidification of the stream by ion exchange resins or the addition of strong mineral acids. At pH 2 and 94ºC, the complete hydrolysis of the fructans present in agave takes about two hours. For the removal of water, in the case of syrup, an evaporator is used, typically a 3 effect falling film, although plate evaporators are beginning to appear. For the production of fructan powder, a spray drier is used, which lower the moisture content to below 5%.

From the description in the previous paragraph, it is clear that there are a lot of different process configurations, and yet a relatively similar product must be achieved by all producers. Up-to-date, the best attempt for the standardization of the methods and parameters in both products are the two Mexican norms that regulate these products: NMX-FF-110-SCFI-2008 for the agave syrup and NMX-F-591-SCFI-2010. These norms, however, are both of voluntary compliance and lack the technical rigor that the products demand. This has led to confusion among buyers, and a difficulty to compare the different product in the market, due to the different analytical techniques used.

Among the most important and used parameters are moisture, mono, disaccharide and fructan content and the DP distribution. For moisture,Karl Fischer titration gives the most precise values, especially in the case of syrups, since the loss-om-drying method is both unspecific and cannot evaporate all the water. For both agave syrup, and more pronouncedly

in fructan syrups, the ºBrix scale cannot be used, since differences as high as 2% in solids are shown between the ºBrix scale and the true moisture, affected by the carbohydrate composition, as well as the mineral content. For sugars determination, the enzymatic analysis are quick and accurate, however, the analyst must be very well trained to give consistent results. HPLC methods are quick and automated, making them less prone to human error. For the DP distribution, MALDI-TOF is a quick and accurate method, however, it is only semi-quantitative in the best of cases, and limitations in the ionization and detection of molecules limit the DP to about 60 units, which fall well short of the range present in many agave fructans. This is the same case for HPAEC-PAD and HPLC-amino. In both cases, it is both for technical limitations (i.e. the response decreases with increasing DP) and the lack of standards that limit the use of these methods. Thus, the best method for the DP distribution in fructan is that of SEC, although more work must be done in order to have a more robust and simple SEC column system. Figure 1 shows a comparison between a MALDI-TOF spectra and a SEC chromatogram, the latter. For TLC, a system was developed that separates very well fructose, glucose and sucrose (the main sugars in both products) as well as oligomers up to DP 10. Furthermore, the staining reagent chosen allow for the distinction of aldoses (blue) and ketoses (red). Figure 2 shows a typical analysis. This method has the advantage of being cheap, quick and does not require very specialized laboratory equipment.

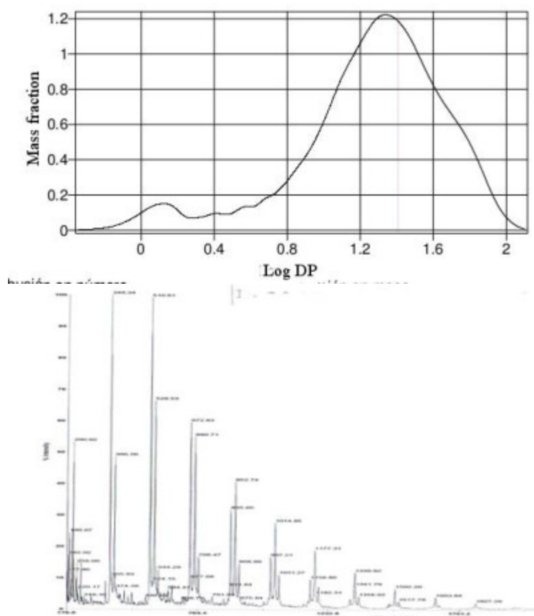

Figure 1.top. Typical SEC chromatogram for high molecular weight fraction of agave fructans. 0.5% is above 100 monomer units. Bottom. Same sample ran in an MALDI TOF, where the highest MW is 18 units long.

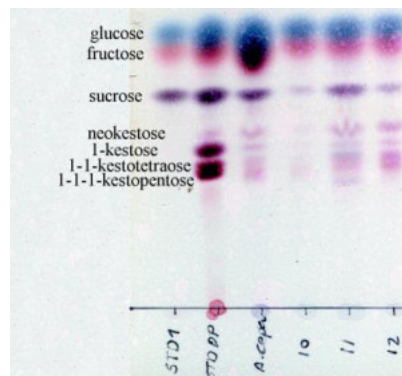

Figure 2.TLC of carbohydrate standards (shown in figure). Lane A. cepa, onion extract. Lane 10, *Agave inequidens* leaves extract, lane 11 and 12, *Agave salmiana* leaves extracts, 11 outer part, 12 inside part.

CONCLUSIONS

The best methods for the determination of moisture, sugar content, fructan content and DP distribution were selected, and a description of the most common processing methods for the production of agave fructans and syrup was presented. A TLC method was developed which allows the determination of sugars and the oligosaccharide profile cheaply and quickly.

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Optimization of the hydrolysis of *Agave tequilana* bagasse and methane production from hydrolysates

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ABSTRACT

The *Agave tequilana* bagasse is a lignocellulosic residue from the tequila industry that has great potential for biofuels production. Therefore, first part of this study aimed to optimize the conditions of acid hydrolysis for sugar recovery from two types of *Agave tequilana* bagasse, which were obtained from two different processes of tequila production, the traditional, in which the *Agave tequilana* heads are cooked, and the alternative, in which the *Agave tequilana* heads are uncooked. Meanwhile, second part of this work aimed to evaluate the feasibility of methane production in batch reactors from the acid hydrolysates under two conditions, with and without nutrient addition. Results showed that at optimal conditions for acid concentration, temperature and reaction time, the hydrolysis over the cooked bagasse was more effective for sugar recovery compared to the hydrolysis over the uncooked bagasse. Sugar concentrations in the cooked and uncooked bagasse hydrolysates were 27.9 g/L and 18.7 g/L, respectively. However, the presence of 5-hydroxymethylfurfural was detected in the cooked bagasse hydrolysate, and therefore, the uncooked bagasse hydrolysate was selected as a more suitable substrate for methane production. Interestingly, the anaerobic digestion results showed that the batch reactor operated without nutrient addition obtained a higher methane yield (0.26 L CH4/g COD) than the batch reactor operated with nutrient addition (0.18 L CH4/g COD), proving that it is feasible to produce methane from hydrolysates of *Agave tequilana* bagasse and suggesting that addition of nutrients is not needed when this type of substrate is used.

Key words: Acid hydrolysis, anaerobic digestion, lignocellulosic biomass, nutrient effect.

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INTRODUCTION

The lignocellulosic biomass is recognized as an environmental friendly feedstock for biofuel production; moreover, due to the fact that some types of lignocellulosic biomass can be obtained as wastes from industrial processes (e.g. the *Agave tequilana* bagasse from the tequila industry), biofuel production from these type of substrates is also economically attractive (Caspeta et al., 2014; Kumar et al., 2009; Saucedo-Luna et al., 2010). Regarding the *Agave tequilana* bagasse, in 2008 the total consumption of *Agave tequilana* for tequila production was estimated at $1.12 * 10^6$ tons, from which, approximately 40% corresponded to bagasse (Saucedo-Luna et al., 2011). Thus, the *Agave tequilana* bagasse has great potential for biofuels production.

In order to enable the production of biofuels from lignocellulosic biomass, such as the *Agave tequilana* bagasse, hydrolysis of its main polysaccharides (cellulose and hemicellulose) is required. In this regard, dilute acid hydrolysis is the most common hydrolytic method because of its effectiveness and low cost (Kumar et al., 2009; Saucedo-Luna et al., 2010). Typically, during dilute acid hydrolysis most of the hemicellulose fraction is recovered as monosaccharides and oligosaccharides; however, depending on the severity of the hydrolysis conditions (acid concentration, temperature and reaction time), toxic products formation may also occur (Saucedo-Luna et al., 2010). Thus, in order to increase the sugar yield and maintaining a low generation of toxic products, optimization of the acid hydrolysis is required prior to biofuel production. On the other hand, due to the fact that main sugars from lignocellulosic biomasses are hexoses (glucose, mannose and galactose) and pentoses (xylose and arabinose), bioethanol production from hydrolysates of *Agave tequilana* bagasse is a limited option, since pentoses are barely consumed by yeast (Hahn-Hägerdal et al., 2007; Young et al., 2010). Therefore, anaerobic digestion emerges as an excellent alternative for energy recovery from lignocellulosic hydrolysates, since microbial consortiums involved in this process are able to consume both, hexoses and pentoses (Gomez-Tovar et al., 2012).

Therefore, this work aimed to evaluate the feasibility of using acid hydrolysates from *Agave tequilana* bagasse for methane production. First, the optimization of the hydrolysis conditions was carried out on two types of *Agave tequilana* bagasse by means of the response surface methodology. Then, the suitability of using acid hydrolysates for methane production in batch reactors was evaluated under two conditions, with and without nutrient addition.

METHODOLOGY

Optimization of the acid hydrolysis of *Agave Tequilana* bagasse

Agave tequilana bagasse and hydrolysis procedure

The *Agave tequilana* bagasse was obtained from two different processes of tequila production at Casa Herradura distillery (Amatitan, Jalisco, Mexico), the traditional process in which the *Agave tequilana* heads are cooked (cooked bagasse) and an alternative process in which the *agave* heads are uncooked (uncooked bagasse). Prior to the hydrolysis process, both types of bagasse were dried at room temperature and fibers size was reduced to an average length of 1 cm. Then, acid hydrolysis was performed in an oven at controlled

temperature by using bagasse at 5% (w/v) in a dilute HCl solution. Different temperatures, HCl percentages and reaction times were evaluated according to the experimental design described in the following section. At the end of the treatment, the hydrolysate was filtered through a 0.45 µm membrane for its posterior analysis.

Experimental design and optimization

A central composite experimental design was employed to evaluate the effect of the temperature, HCl concentration and reaction time over the agave bagasse-acid hydrolysis. Two levels of each response variable were assigned according to previously reported conditions that showed high effectiveness for sugar recovery (Gomez-Tovar et al., 2012). Thus, central points for temperature, HCl percentage and reaction time were set at 90 °C, 2 % HCl and 2 h, while the step change values were set at 20 °C, 0.5 % HCl and 1 h, respectively. The central composite experimental design was completed with six axial and five central points. Experimental results obtained with the central composite experimental design were analyzed by the response surface methodology. During analysis of the response surface methodology, the relationship between independent (temperature, HCl percentage and reaction time) and response variables (total sugar concentration) for both bagasse types was described by quadratic polynomial equations, which in turn were used to generate response surface plots to predict optimal values. It is important to point out that all experiments were run in triplicate and that reliability of the polynomial equations were evaluated by correlation coefficients R^2. The analysis of the data, the generation of the response surface plots and the statistical significance analysis (by ANOVA analysis) were carried out by using the software Statgraphics centurion XV (Statpoint, Technologies, USA).

Anaerobic digestion experiments

Inoculum, substrate and mineral medium

Anaerobic granular sludge from a full-scale up-flow anaerobic sludge blanket (UASB) reactor was used as inoculum. The UASB treats effluents of a local brewery at Guadalajara, Jalisco, Mexico. The hydrolysates from *Agave tequilana* bagasse were used as substrate for methane production under two conditions, without and with nutrient addition. For the latter condition, minerals reported by Gomez-Tovar et al. (2012) were added to hydrolysates.

Batch reactors operation

In order to evaluate the feasibility of using the acid hydrolysates as substrate for methane production, with and without nutrient addition, two independent batch experiments were run. Both experiments were carried out at the same conditions where the only difference was due to nutrient addition. For this purpose, a lab-scale reactor made of polyvinyl chloride (PVC) with a working volume of 3.6 L was employed. The temperature of the reactor was controlled at 32 °C by using a water jacket while the pH was regulated at 7.5 (± 0.3) by adding 2N NaOH. At the beginning of both experiments, the reactors were inoculated with 5.8 g VSS/L of anaerobic sludge and then fed with hydrolysates at 5 g COD/L.

Analytical methods

The *Agave tequilana* bagasse was characterized in terms of soluble compounds, hemicellulose, cellulose and lignin by using a semiautomatic fiber analyzer (ANKOM Technology, Macedon, NY, USA). On the other hand, the hydrolysates were characterized

in terms of: total sugars by using the method reported by Dubois et al. (1956); furan and phenolic compounds (5-hydroxymethylfurfural (HMF), furfural, syringaldehyde and vanillin) by using HPLC analysis as previously described (Arreola-Vargas et al., 2013); and COD, Nitrogen and Phosphorus concentration by using colorimetric methods with a HACH digester DRB200 and a HACH spectrophotometer DR2800. During anaerobic digestion experiments, the measurement of variables such as temperature, pH, pressure and biogas flow rate was carried out online. For this purpose, a National Instruments cRIO9004 device equipped with analogical and digital cards was used in the acquisition, treatment and storage of the data. In addition to the online readings, offline measurements were also carried out. COD and total sugar concentrations were measured as mentioned above, whereas the volatile fatty acids (VFA) concentrations and biogas composition were measured as previously described by using an HPLC and a GC-TCD, respectively (Méndez-Acosta et al., 2013).

RESULTS AND DISCUSSION

Lignocellulosic composition of the different types of *Agave tequilana* bagasse

The *Agave tequilana* bagasse is produced in either, the traditional or the alternative tequila production process. Regarding the traditional process, it consists of four main steps: cooking of the *Agave tequilana* heads, sugar extraction by grinding (where the bagasse is generated), fermentation and distillation. In contrast, the alternative process consists on the following four steps: syrup extraction of the *Agave tequilana* heads by grinding (where the bagasse is generated), hydrolysis of the syrup, fermentation and distillation. Because of the differences on the tequila production process, both types of *Agave tequilana* bagasse (cooked and uncooked) may present different lignocellulosic compositions, which would influence the sugar recovery yield and the biofuel production potential from this lignocellulosic material. Therefore, the lignocellulosic composition of both types of *Agave tequilana* bagasse was determined in the present study. The cooked bagasse composition was the following: 11% hemicellulose, 31% cellulose, 11% lignin and 47% extractives; in contrast, the uncooked bagasse presented the following composition: 23% hemicellulose, 47% cellulose, 11% lignin and 19% extractives. Lignocellulosic composition of the uncooked bagasse was similar to composition reported by Saucedo-Luna et al. (2011) (20% hemicellulose, 42% cellulose and 15% lignin); nonetheless, the difference on lignocellulosic composition between both types of *Agave tequilana* bagasse is for first time reported.

Acid hydrolysis optimization

A central composite experimental design was employed in order to evaluate the effect of temperature, HCl percentage and reaction time over the hydrolysis of both types of *Agave tequilana* bagasse. Even though several studies have reported the use of H_2SO_4 for the acid hydrolysis of different types of lignocellulosic materials (Kumar et al., 2009), including a hydrolysis optimization on *Agave tequilana* bagasse (Saucedo-Luna et al., 2010), the presence of sulfate in the anaerobic digestion process could promote the growth of sulfate-reducing bacteria, which are electron donor competitors of methanogens (Isa et al., 1986). Therefore, during the present study the presence of sulfate in the hydrolysates was avoided by using HCl instead of H_2SO_4. The responses obtained for the hydrolysis applied to both

types of *Agave tequilana* bagasse were analyzed by the response surface methodology and the following response surfaces were obtained (Figure 1).

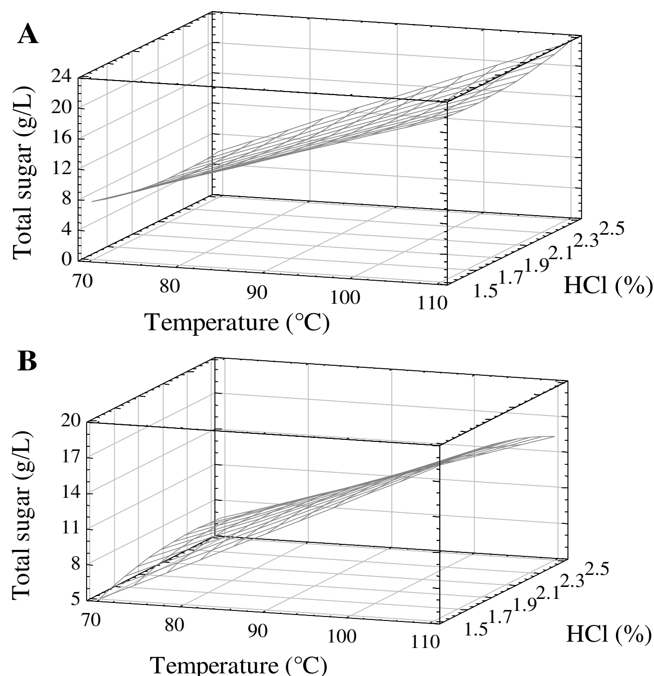

Figure 1. Response surfaces obtained for hydrolysis of the cooked (A) and uncooked (B) *Agave tequilana* bagasse.

Figure 1 shows that temperature has the strongest effect over the sugar recovery from both types of bagasse. Moreover, the ANOVA analysis demonstrated that this variable was the only factor that showed statistical significance ($p=0.0001$ for both cases). On the other hand, Table 1 shows that optimal predicted values for HCl percentage and reaction time were already included within the studied range (1.2-2.8% for HCl and 0.3-3.7 h for reaction time). However, the optimal value for temperature corresponds to the upper limit of the studied range (123.6 °C), which means that it might be possible to increase the sugar concentration in both hydrolysates by increasing the temperature beyond the original range. Nonetheless, an increase on the temperature beyond the original range might negatively impact the process by both decreasing the final energy balance and increasing the generation of toxic byproducts (Kumar et al., 2009). Therefore, it was decided to maintain the predicted conditions as the optimal for producing the acid hydrolysates from *Agave tequilana* bagasse.

Table 1. Optimal values obtained for the hydrolysis of the different types of *Agave tequilana* bagasse at studied conditions

Factor	Cooked bagasse	Uncooked bagasse
Temperature (°C)	123.6	123.6
HCl (%)	2.7	1.4

Reaction time (h)	1.3	2.1
Predicted sugar concentration (g/L)	33.4	22.4
Experimental sugar concentration (g/L)	27.9	18.7

Regarding the experimental responses obtained at optimal conditions, the cooked bagasse hydrolysate contained a higher sugar concentration than the uncooked bagasse hydrolysate, 27.9 g/L vs. 18.7 g/L, respectively (Table 1). These values are close to the predicted ones and correspond to sugar recovery yields of 120% for the cooked bagasse and 48% for the uncooked bagasse. The sugar recovery yield from the uncooked bagasse is very similar to a previous report, in which a yield of 48.5% was achieved by applying a two-stage acid hydrolysis over *Agave tequilana* bagasse (Saucedo-Luna et al., 2010). Furthermore, this yield is higher than reported yields for acid hydrolysis applied to different lignocellulosic materials, such as sugarcane bagasse or oat straw (Gomez-Tovar et al., 2012; Hernández-Salas et al., 2009). However, the sugar recovery yield from the cooked bagasse suggests that the lignocellulosic composition analysis underestimated the hollocellulose content of this bagasse. A possible explanation for this issue is found in the different content of extractives between the cooked and uncooked bagasse, 47% and 19% respectively. This wide difference suggest that part of the holocellulose fraction could have been destabilized during the cooking of the *Agave tequilana* heads, and therefore, it was more easily extracted during the lignocellulosic composition analysis and accounted as extractives. Moreover and reinforcing the former hypothesis, unlike the uncooked bagasse hydrolysates the presence of HMF was detected in the cooked bagasse hydrolysates at concentrations up to 1200 mg/L, suggesting that part of the sugars from the cooked bagasse were more easily extracted during the acid hydrolysis and therefore were more susceptible for being dehydratated to HMF.

Overall, even though the cooked bagasse hydrolysate contained a higher sugar concentration than the uncooked bagasse hydrolysate, the latter was selected as a more adequate substrate for the anaerobic digestion experiments, since the presence of HMF in the cooked bagasse hydrolysate could affect the methane production rate and/or yield as previously reported (Monlau et al., 2014).

Methane production from hydrolysates

Feasibility of methane production from the uncooked bagasse hydrolysates (obtained at optimal conditions) was evaluated in batch reactors under two conditions, with and without nutrient addition. The evaluation of the nutrient addition effect is relevant because of the potential presence of nutrients in the lignocellulosic hydrolysates (Kumar et al., 2009; Salgado et al., 2012). Indeed, the presence of Nitrogen and Phosphorus was detected in the uncooked bagasse hydrolysates at concentration of 294 mg/L and 99 mg/L, respectively.

Figure 2 shows that both batch assays, with and without nutrient addition, produced methane as a result of the degradation of the organic fraction of the hydrolysates. However, Figure 2 also shows that the batch assay operated without nutrient addition produced higher amounts of methane (2.6 L) compared to the batch assay with nutrient addition (1.3 L). Furthermore, also a similar pattern was observed in COD removals. It is intriguing that the batch assay without nutrient addition achieved a better performance than the batch assay supplemented with nutrients. Thus, possible causes for the negative effect observed in the batch assay supplemented with nutrients could be an excess of ammonia and/or an excess of heavy metals, as it has been previously reported (Hickey et al., 1989; Liu & Sung, 2002).

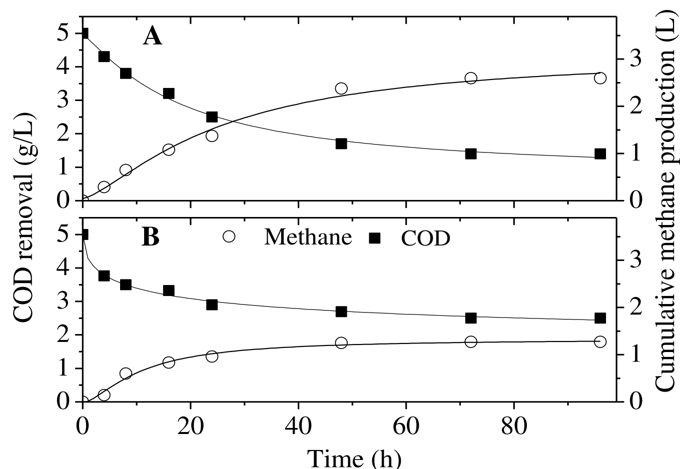

Figure 2. Profiles of COD removal and cumulative methane production in the batch assays. A: without nutrient addition; B: with nutrient addition.

On the other hand, methane yields for batch assays without and with nutrient addition were 0.26 L CH_4/g COD and 0.18 L CH_4/g COD, respectively. The highest methane yield obtained without addition of nutrients in the present study is very similar to the value reported by Kaparaju et al. (2009), in which a different lignocellulosic hydrolysate was used and a methane yield of 0.27 L CH_4/g COD was reached. However, the highest methane yields from lignocellulosic hydrolysates have been achieved by using UASB systems (Gomez-Tovar et al., 2012; Kaparaju et al., 2009), which is understandable due to the high concentrations of biomass that can be achieved in attached biomass systems. Therefore, further studies are encouraged in order to evaluate the effect of the reactor configuration over the methane production from acid hydrolysates of *Agave tequilana* bagasse.

Figure 3 shows the detected byproducts at the end of the batch experiments. In the batch assay without nutrient addition, low concentrations of acetate and formate were detected, 77 and 33 mg/L respectively. These byproducts at similar concentrations have been previously reported in reactors with high methane yield (Gomez-Tovar et al., 2012), agreeing with the present study. In contrast, in the batch assay with nutrient addition, high concentrations of propionate and acetate were detected, 203 and 424 mg/L. This result along with the low COD removal (figure 2) is well correlated with the low production of methane in this experiment.

Finally, it is worth noticing that even though the total concentration of VFAs in the batch assay operated with the addition of nutrients was below the concentration of 4000 mg/L reported as inhibitory for methane production (Siegert & Banks, 2005), the production of propionate (which may be linked to a change in the metabolic pathway promoted by the excess of some nutrients), could in somehow affected the methanogen population and hence inducing the accumulation of VFAs in the system.

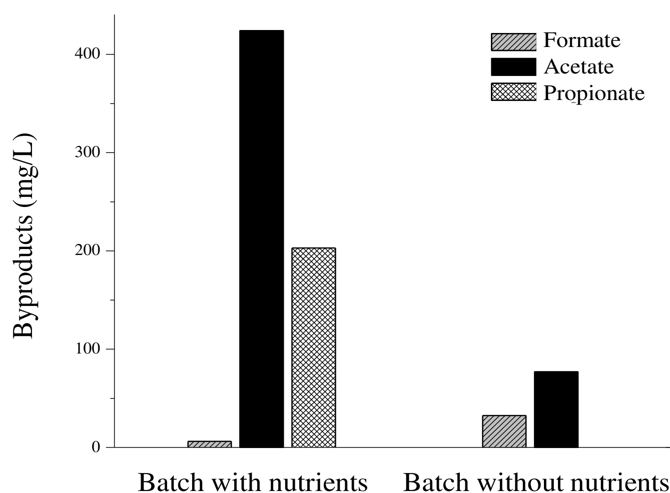

Figure 3. Production of VFAs during batch experiments with and without addition of nutrients.

CONCLUSION

Overall, this study demonstrates the feasibility of producing methane from acid hydrolysates of *Agave tequilana* bagasse. The optimization of the acid hydrolysis evidenced that the cooked bagasse is a more suitable biomass for sugar recovery compared to the uncooked bagasse. However, due to the detected presence of HMF in the cocked bagasse hydrolysate, the uncooked bagasse hydrolysate was selected as a more suitable substrate for methane production. Furthermore, feasibility of methane production from this hydrolysate was proved in batch reactors; nonetheless, it was observed that addition of nutrients negatively affected the methane production. Thus, a probable hypothesis for the latter behavior is that some of the nutrients favored metabolic pathways that produced propionate, which in turn negatively affected the production of methane.

ACKNOWLEDGEMENTS

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MECHANICAL AND THERMAL PROPERTIES OF GREEN COMPOSITES OF POLYLACTIC ACID AND AGAVE BAGASSE FIBERS

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ABSTRACT

In this work fiber obtained from Agave tequilana bagasse (FATB), residue of the industrial production of Tequila, was used as a reinforcing agent for the production of green (biodegradable) composites using PLA as matrix. PLA/FATB composites were obtained into sheets form (10 cm wide) using a twin screw extruder equipped with a flat die. The effect of the content and particle size of fiber on the mechanical and thermal properties of the composites was evaluated. It was observed that an increase in the fiber content resulted in an increase in elastic modulus, but a decrease in tensile strength of the composite due to poor interaction between fiber and matrix. The impact strength, with respect to the matrix of PLA, show a 100% increase with 40% fiber content due to the dissipation of impact energy by fiber-release matrix. By differential scanning calorimetry (DSC) was observed as the sample crystallized during heating at about 147 °C with immediate melting of the crystals at 157 °C, with a very slight decrease in this latter was observed in the composite due to the presence of fiber.

Keywords: agave bagasse, lignocellulose fiber, polylactic acid, green composites, mechanical properties.

INTRODUCTION

The pollution caused by plastic waste, especially those used by packaging has increased in recent years and has become a problem of environmental contamination. Because of this, it has been an impulse to research on biodegradable materials to replace the traditional materials used for these applications (Ludvik et al, 2007; Hu et al, 2012). Since the 1990s, it has studied the developing biodegradable polymers such as polylactic acid (PLA), synthetic aliphatic polyester derived from renewable agricultural products, comparable to petroleum-based plastics and readily biodegradable (Kim et al, 2012; Way et al, 2012). This material can be used in different fields, from pharmaceutical and medical supplies to industrial packaging; however, its use is limited due to their low heat distortion temperature and high production cost (Kim et al, 2012; Cameron et al, 2012). One strategy to solve this problem is to obtain mixtures or composites of PLA with other biodegradable materials cheap and that are from renewable sources such as starch, cellulose fibers, lignocellulosic fibers, etc., thus

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generating an interest in marketing and reducing addition polymers consumption of petroleum (Sawpan et al, 2011; Shih and Huang, 2012). In Mexico, the agave bagasse, from the production of spirits such as Tequila and Mezcal, emerges as source of natural fibers (Canché-Escamilla, 2012), which could be used to obtain green composites, which after its useful life may biodegrade to be incorporated in the municipal solid waste stream.

In this work, composite of PLA and agave bagasse fibers (FATB) were obtained by extrusion and their mechanical and thermal properties were determinate.

METHODOLOGY

Materials

Polylactic acid (PLA) Ingeo 2003D was obtained from Nature Works ™. Fibers were obtained from Agave tequilana bagasse after cooking the pineapple agave and the separation of non-fiber fraction. The fibers were provided by the company Sol y Agave in Arandas Jalisco, Mexico. The PLA and FBAT were milled in a Brabender blade mill with sieves of 1 mm and 2 mm, respectively. Fiber size fractions of 40-60 and 60-bottom mesh were used to obtain the composites.

Composites Preparation

The materials were dried at 80 °C for 24 hours, mixed in the desired ratios and homogenized in a Brabender mixer for powders of 5 kg capacity, at a speed of 50 rpm for 5 minutes. Blends of PLA with FBAT were dried again before the extrusion process, for 4 hours at 80 ° C. Te materials were extruded using a twin screw Brabender extruder mark 4 equipped with four heating zones (180, 190, 200 and 190 ° C) and a ribbon die of 10 cm wide and 1 mm aperture. PLA composites with 0, 10, 20, 30 and 40% FBAT were prepared.

Thermal and Mechanical Characterization

The specimens for tensile and bending tests were cut directly from the ribbons using a vacuoruter according to ASTM D638 and ASTM D790, respectively. The tensile and flexural properties were determined using a Universal machine (Instron 5500R Model 1125) with a 500 kg load cell at a speed of 5 mm/min and 0.0672 mm/min, respectively. The specimens for impact tests were obtained by compression with temperature and pressure (rectangular sections 12 cm long by 1 wide) using a Carver hydraulic press. Impact tests were performed under the conditions of ASTM D256 standard using a CEAST impact pendulum with a hammer of 1 J. The DSC thermograms were obtained using a calorimeter (Perkin Elmer model Diamond), with sweeps temperature 55 to 200 ° C at 10 ° C/min under nitrogen atmosphere.

RESULTS AND DISCUSION

Mechanical properties of composites

Table 1 shows the mechanical properties of composite with PLA as matrix and FBAT as filler. An increase in tensile modulus was observed to yield more rigid materials having a tensile modulus of 1428 MPa to composites with 40% fiber. On the other hand, a sudden decrease of almost 50% of the tensile strength of the matrix was obtained with low content of 10% FBAT and resistance strength of 26 MPa was obtained for composite with 40% FATB. This reduction in tensile strength is due to the poor interfacial interaction between fiber and matrix as well as agglomeration of the fibers, which can result in points of failure of the specimen. This behavior has been reported by other authors (Kim et al, 2012) for PLA composite with pineapple fiber. In flexion tests a similar behavior to tensile testing was

obtained. It can be seen that the impact resistance of the composites increased almost linearly with the content FBAT, obtaining a 100% increase of the impact resistance with respect to the matrix of PLA, when content FBAT 40% was used. This behavior may be due to dissipation of the impact energy by the friction required to disengage the matrix fibers (pull-out) which increases with increasing fiber content (Cameron et al, 2012). Similar behavior has been reported in the literature for composites with PLA and abaca fiber (Bledzki, 2009).

Table1. Mechanical properties of composites PLA/FATB.

Mesh	Relation PLA/FBAT	Tensile		Flexure		Impact Strength (kJ/m^2)
		Modulus (MPa)	Strength (MPa)	Módulus (MPa)	Strength (MPa)	
60-40	**100 / 0**	1311 ± 97	62.7 ± 1.8	4793 ± 232	105.1 ± 3.3	2.19 ± 0.48
	90 / 10	1290 ± 37	33.1 ± 1.5	2529 ± 322	43.79 ± 9.8	3.23 ± 0.15
	80 / 20	1333 ± 51	35.7 ± 0.9	3367 ± 234	26.91 ± 3.5	3.43 ± 0.15
	70 / 30	1366 ± 39	30.8 ± 2.3	3158 ± 159	11.25 ± 0.7	3.97 ± 0.37
	60 / 40	1428 ± 53	26.2 ± 1.6	3233 ± 174	8.48 ± 2.0	4.62 ± 0.42
60-bottom	**80 / 20**	1520 ± 11	50.3 ± 1.4	--	--	--
	70 / 30	1605 ± 98	46.4 ± 4.8	--	--	--

Thermal properties of composite

Figure 2 shows DSC thermograms of PLA matrix and composite materials obtained with different fiber content. It can be observed over the range studied, the PLA virgin not provide thermal transitions although the literature has reported a PLA crystallization temperature (Tc) and a melting point (Tm) at 93 °C and 164 °C, respectively (Kim et al, 2012), these transitions are observed when the material is processed to obtain composite materials. This is a characteristic behavior of PLA; it crystallizes during heating between 111 and 147 ° C, with a peak at 127 ° C. Then a melting peak with maximum temperature Tm = 157 ° is observed (Cao, 2003). The thermograms of composites show the crystallization peak and melting of the PLA matrix, which are better defined and slightly shifted to lower temperatures in comparison with virgin PLA. Composites PLA/FBAT presented two adjacent melting peaks (Tm_1 and Tm_2) which were more apparent with increasing the fiber content. This behavior can be attributed to both; the formation of crystals due the fibers can act as nucleating agents and the lamellar arrangements which cause imperfect crystals during processing and PLA hydrolysis during processing (Cameron et al, 2013).

CONCLUSIONS

Sheets of matrix composites and FBAT PLA were obtained by extrusion to content of 40% fiber. The tensile and flexural decreased with increasing fiber content while the elastic modulus of the composite was greater at higher fiber contents. The best dispersion of smaller particles resulted in higher mechanical properties compared to the larger fibers. The poor fiber-matrix adhesion promotes the dissipation of impact energy by fiber-release matrix. The fibers act as nucleating agents and increase the crystallization rate of the PLA array.

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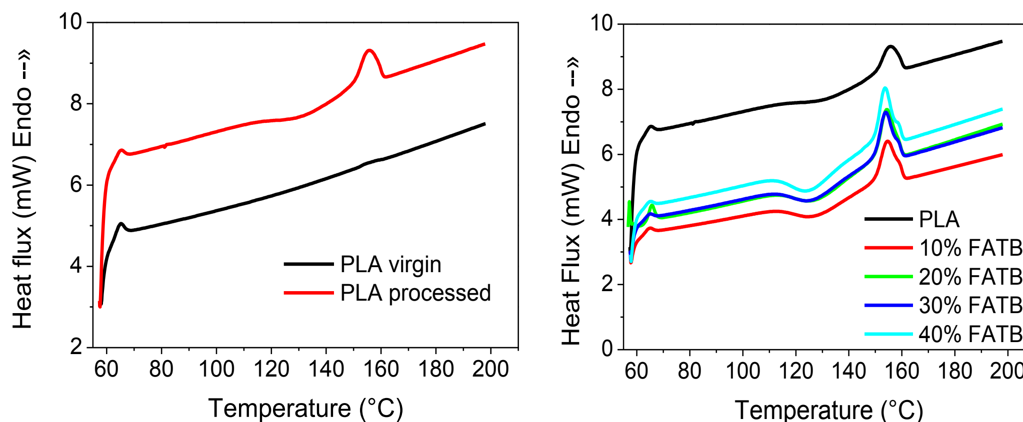

Figura 2. DSC Thermograms of PLA composites with FATB.

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Accelerated vermicomposting with *Bjerkandera adusta* pre-treatment of agave bagasse

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ABSTRACT

The *Agave tequilana* Weber is used to generate products such as tequila and fructans, in which agave bagasse is generated as solid waste. The principal use of bagasse is to produce composts in a traditional process of 7-month average. The aim of this study was to improve the agave bagasse degradation with an accelerated vermicomposting during 90 days, which consisted of a pre-treatment of fractionated or whole bagasse with *Bjerkandera adusta* UAMH 8258 or native fungi followed by a vermicomposting with *Eisenia fetida*. Also, the stability and maturity of obtained vermicomposts were evaluated. Results indicated that the fractionated bagasse favoured degradation either with native fungi or with *B. adusta*. The pre-treatment accelerated the degradation of bagasse when it was subjected to a vermicomposting process. Degradation of hemicellulose, cellulose and lignin in pre-treatment (with native or *B. adusta* fungi) were greater than in vermicomposting. The better indexes of stability and maturity were obtained with the vermicompost from bagasse fractionated, indicating that a minor particle size favoured the degradation. In this work, with an accelerated vermicomposting it was possible to reduce degradation time to 3 months.

Keywords: *Eisenia fetida*, lignin, lignocellulosic enzymes, white rot fungi

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INTRODUCTION

High quantities of blue agave crops are used almost exclusively for tequila production, due to the existence of the appellation of origin (AO). However, blue agave is cultivated in other states, outside the AO such as Zacatecas, Sinaloa and Durango among others. Recently, these crops outside the AO have been used to generate other products such as fructans and inulin to diversify the utilization of *A. tequilana* Weber (Waleckx et al., 2008). In 2013, 226.5 million litters of tequila were produced from 776.9 thousand tons of *A. tequilana*, which generated a large amount of bagasse and vinasses as waste from tequila production process (CRT, 2014). Iñiguez et al. (2001) estimated that bagasse represents 40% of the total weight of the agave heads (wet weight). An estimation with this base indicated that 310.76 thousand tons of bagasse were generated in 2013 by tequila production. Other estimations indicated that in the fructans production process 73% of agave heads (wet weight) are generated as bagasse (Personal communication). Some tequila factories use the agave bagasse to generate compost in windrow systems where they irrigate the vinasses. However, the degradation process is slow with a process time from 6 to 8 months due to the lignocellulosic composition of the bagasse (Iñiguez et al., 2011). The high generation of bagasse and the long composting process time makes insufficient treatment and causes environmental issues due to improper disposition. The main objective of this study was to accelerate the degradation of the agave bagasse using a pre-treatment with *Bjerkandera adusta* fungus followed by vermicomposting with *Eisenia fetida*. At the end of the process the stability and maturity of obtained vermicomposts were evaluated.

MATERIALS AND METHODS

We collected samples of fractional (< 4 mm) and whole bagasse (6-8 cm) of an agave from the state of Zacatecas and derived from fructans production. The bagasse was pre-treatment in a solid-state fermentation with the fungus *B. adusta* and/or with native fungi, during 45 days in different treatments (Table 1). We measured the degradation of hemicellulose, cellulose, lignin (Van Soest and Wine, 1967) and total carbohydrate (Dubois et al., 1956) at 0, 30 and 45 days. In addition, we evaluated the enzymatic activity of lignin peroxidase (LnP), manganese peroxidase (MnP) and Laccase (Lac) (Leonowicz and K, 1981). After the pre-treatment, we carried out a vermicomposting process with *E. fetida* complementing the input of nitrogen with a sewage sludge obtained from a plant of treatment of domestic wastewater, which was free of pathogens and with an heavy metals content below to permissible maximum limits of NOM-004-SEMARNAT-2000 and the USEPA, with 146 mg kg^{-1} of inorganic N and 1525 mg kg^{-1} available phosphorus. At 0, 30 and 45 days were evaluated the content of hemicellulose, cellulose, lignin. The final vermicomposts were determined for inorganic nitrogen (NO_3^-, NO_2^-, NH_4^+), germination index (Mathur et al., 1993), breathing index (Bartha and Pramer, 1965), humic and fulvic acids, and the content of *Salmonella spp*., and faecal coliforms according to NMX-AA-042-1987. All parameters evaluated were subject to an analysis of variance (ANOVA) using a PROC GLM with the SAS statistical program (2009) to analyse the significant differences among treatments in the pre-treatment with *B. adusta* and in the vermicomposting process with Tukey's test and $P < 0.05$.

Table 1 Combinations of treatments with whole (W) and fractionated (F) bagasse for the pre-treatment with and without *B. adusta* UAMH 8258 fungi (B) under sterile (s) and non-sterilized (ns) conditions.

Treatments in pre-treatment [a]	Bagasse Whole	Bagasse Fractionated
B. adusta with Native fungi, (non-sterile)	BNWns	BNFns
B. adusta (sterile)	BWs	BFs
Native fungi, (non-sterile)	NWns	NFns
Biotic controls	CWs	CFs
Treatments in vermicomposting [a]	**Whole**	**Fractionated**
B. adusta with Native fungi, (non-sterile) plus earthworm	BNWns+E	BNFns+E
B. adusta (sterile) plus earthworm	BWs+E	BWs+E
Native fungi (non-sterile) plus earthworm	NWns+E	NFns+E
Controls plus earthworm	CWs+E	CFs+E
B. adusta (sterile) without earthworm	BWs-E	BFs-E
Native fungi (non-sterile) without earthworm	NWns-E	NFns-E

[a] All treatments were in triplicated (n = 3).

RESULTS AND DISCUSSION

The native fungi of bagasse and *B. adusta* consumed the residual sugars (carbohydrates totals) from bagasse in 30 days, without significant differences between the treatments (Figure 1).

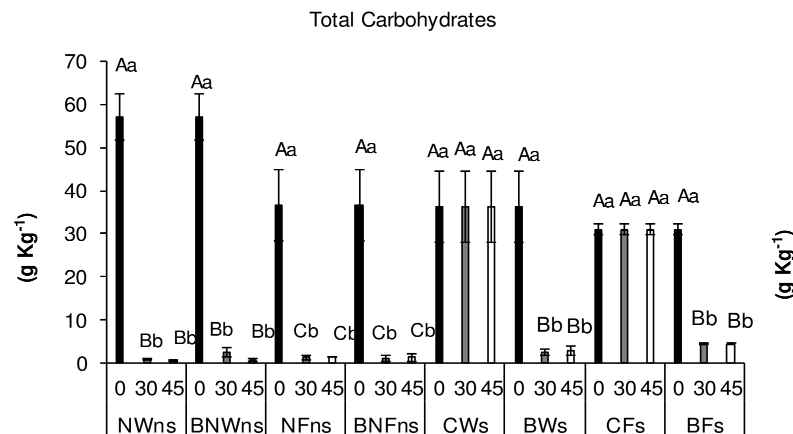

Figure. 1. Degradation of total carbohydrates, during pre-treatment of fractionated bagasse (F) or whole (W) with *B. adusta* (B) or native fungi (N) in sterilized (s) or non-sterilized (ns) conditions.

The bagasse fractional favoured the degradation of lignin by the mixture with fungi native, plus *B. adusta* and *B. adusta* only (NFNs, BFNs and BFs), where the degradation of lignin was BFE > CFNe > BFNe without significant differences between them ($P > 0.05$) (Table 2). Similar degradation have been found by other authors using bagasse with fungi as *Lenzites betulin*, *Daedalea elegans*, *Polyporus giganteus* on sugarcane with a degradation of lignin of the 47%, 59% and 65% respectively in 90 days (Oluseyi and Isola, 2009). The treatments of the native fungi and *B. adusta* presented activities of MnP and Lac to 30 days. The treatment in bagasse whole with native fungi plus *B. adusta* (BWNs) showed enzymatic activities for the day 30 of MnP (7.65×10^{-6} U g^{-1}) and Lac (44×10^{-6} U g^{-1}), while in fractional bagasse (BFNs) only showed activity of MnP of 155.5×10^{-6} g^{-1}. Treatments with *B. adusta* in whole bagasse (BWs) only had activity of Lac (34.5×10^{-6} U g^{-1}) on 30 days and for the day 45 showed both MnP (12.4×10^{-6} U g^{-1}) and Lac (84.5×10^{-6} U g^{-1}). The same treatment with fractional bagasse (BFs) presented only activity of MnP (812.1×10^{-6} U g^{-1}) at 30 day with a 72% of lignin degradation, and for the day 45 presented the two activities of MnP (77.5×10^{-6} U g^{-1}) and Lac (60.5×10^{-6} U g^{-1}) with a lignin degradation 74%.

Table 2. Total degradation of lignocellulosic components in the different stage of process.

Treatment	Pre-treatment degradation (%)			Vermicomposting degradation (%)[a]			Total degradation (%)		
	H*	C*	L*	H	C	L	H	C	L
BWs+E	54	32	62	40	60	12	94	92	74
BNWns+E	51	58	61	43	34	12	94	92	73
BFs+E	65	49	72	0	0	0	65	49	72
BNFns+E	71	43	71	22	50	5	93	93	76
CWs+E	0	0	0	21	29	15	21	29	15
NWns+E	64	51	56	15	37	0	79	88	56
CFs+E	0	0	0	0	29	24	0	29	24
NFns+E	67	43	74	27	43	17	94	86	91
BWs-E	54	32	62	17	13	4	71	45	66
BFs-E	65	49	72	3	0	1	68	49	73
NWns-E	40	0	33	12	24	14	52	24	47
NFns-E	41	0	29	5	26	25	46	26	54

[a] The percentage of degradation in the vermicomposting was calculated by difference of final degradation minus the degradation in the pre-treatment; * H = hemicellulose C = cellulose L = lignin.

This showed that the presence of *B. adusta* favoured the production of these enzymes to get the breaking of lignin as biological pre-treatment to degrade the bagasse agave. Also, it was observed that the enzyme activity and the degradation of lignin were greater in the bagasse fractionated than whole. García-Torres y Torres-Sáe (2003), found in sugarcane bagasse (0.08 cm particle size) with the fungi *Trametes versicolor* and *Pleurotus floridae* a Lac concentration of 0.11 U g^{-1} and 0.05 U g^{-1}; MnP concentration of 0.03 U g^{-1}, 0.05 U g^{-1} and 0.01 U g^{-1} respectively, at 21 days.

On the other hand, degradation was greater in the vermicomposting of fractional bagasse and pre-treated with *B. adusta* + fungi native (BFNs) (hemicellulose 93%, cellulose 93% and lignin 76%) than native fungi (NFNs) (hemicellulose 94 %, cellulose 86% and lignin 91%). The treatments with whole bagasse (NWNs, NWs, BWNs, BWs) had a lower degradation that the fractional bagasse (NWNs, NWs, BWNs, BWs) (Table 2). This suggested that particle size has an unimportant role in the colonization of the fungus and that a smaller particle size favoured the degradation of the bagasse in an accelerated vermicomposting with a pre-treatment with fungi of the white rot. In a similar study, Kumar et al. (2010) reported degradation of 69% lignin, 32% hemicellulose and 62% cellulose in sugarcane bagasse pre-treated with a mixture of fungi (*Pleurotus sajor-caju, Asperigillus niger* and *Trichoderma viridae Asperigillus*) and followed by vermicomposting with earthworms *Drawida wills* during 40 days.

The vermicompost obtained from the treatment of *B. adusta* + fungi native (BWNs) had characteristics of maturity and stability very close to or within the indices reported by other authors, such as 200 mg NH_4^+ kg^{-1}, 8 mg NO_2^- kg^{-1}, 175 mg NO_3^- kg_{-1}, 1.3 NH_4^+ /NO_3^-, 1.4 HA/FA and 175 mg CO_2 kg^{-1} h^{-1}. Followed by the NFNs treatments and BFNs that had values of these indices adequate. All treatments had values >100% of germination index which indicated that they were not phytotoxic (Table 3).

Table 3 Maturity and stability indices for vermicomposts obtained with accelerated vermicomposting process.

Tratamiento	N-NH4 (mg kg-1)	N-NO3 (mg kg-1)	N-NO2 (mg kg-1)	NH4/NO3	HA/FA	CO2 (mg kg-1 h-1)
NWNs+E	45 ± 49	1322 ± 676	16 ± 0	0.033 ±0.05	1.24 ±0.14	143.3 ± 14.1
BWNs+E	200 ± 99	175 ± 42	8 ± 0	1.300 ± 0.9	1.37 ± 0.08	57.60 ± 8.98
NFNs+E	186 ± 0	772 ± 0	24 ± 0	0.241 ± 0.1	0.41 ±0.00	15.56 ± 0.00
BFNs+E	40 ± 0	532 ± 0	8 ± 0	0.075 ± 0.04	1.07 ±0.28	65.02 ± 0.00
NWs+E	30 ± 14	312 ± 66	10 ± 8	0.093 ± 0.05	0.53 ±0.27	45.28 ± 17.57
BWs+E	40 ± 0	347 ± 211	15 ± 2	0.163 ± 0.1	0.88 ±0.17	57.06 ± 6.24
NFs+E	50 ± 0	1936 ± 0	8 ± 0	0.026 ± 0.01	0.53 ±0.0	15.21 ± 0.00
Indices	75-5002	>402	<52	0.5-32	>1.91	≤1203

[1] (Raj y Antil, 2011); [2] (Wichuk y McCartney, 2010); [3] (Hue y Liu, 1995)

CONCLUSIONS

The agave bagasse pre-treatment either with *B. adusta* UAMH 8258 or native fungi accelerated degradation when it was subjected to a vermicomposting process, which was higher in fractionated than in whole bagasse. Degradation of hemicellulose, cellulose and lignin in pre-treatment were greater than those attained in the vermicomposting process. An accelerated vermicomposting (pre-treated with *B. adusta* plus native fungi) could reduce the degradation time of agave bagasse to 3 months compared to a traditional composting performed in the tequila factories by 7 months. The vermicomposts obtained were stable and mature according to most of the standards and limits established to composts.

ACKNOWLEDGMENTS

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Removal of fibers, change in the crystallinity of cellulose and formation of inhibitors for hydrogen production during cooking process of agave heads and during enzymatic hydrolysis of agave bagasse

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ABSTRACT

Previous studies demonstrated that the hydrogen production is feasible from enzymatic hydrolysates from agave bagasse, derived from the cooking process of agave heads for the elaboration of tequila. However, the yields and rates are low compared to models substrates such as glucose or xylose. This study sought to find the causes for the low hydrogen production. For this, the next parameters were assessed: changes in the content of lignin, hemicellulose and amorphous and crystalline cellulose, in agave head, bagasse and bagasse hydrolysate, before and after of cooking process and enzymatic hydrolysis. Also the presence and concentration of inhibitors of hydrogen production were evaluated. For this, furfural, hydroxymethylfurfural, vanillin and syringaldehyde formed during the cooking of the agave heads and enzymatic hydrolysis of the agave bagasse, were measured in the washing water of bagasse and in the enzymatic hydrolysates. Our results showed that the fibers content do not show important changes during enzymatic hydrolysis, indicating the need for pre-treatment or optimization of enzymatic hydrolysis. The crystalline index obtained by X-ray diffraction of the fibers, suggest the enrichment of crystallinity due to inulin removal (during agave heads cooking) and hemicellulose and cellulose removal during enzymatic hydrolysis. While the concentration of phenolic compounds and furan derivatives measured by HPLC in bagasse washing water and in enzymatic hydrolysate of bagasse were below detectable limits, the results suggest the presence of phenols different to vanillin and syringaldehyde, as indicated by the Folin-Ciocalteu method.

Keywords: agave head, agave bagasse, bagasse hydrolysate, enzymatic hydrolysis, crystalline cellulose.

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INTRODUCTION

The cooking process of heads of *Agave tequilana* Weber Var. azul is an important step in the production of tequila, as it produces soluble fermentable sugars and agave bagasse. However, some of the soluble sugars can be dehydrated, generating furan derivatives due to the length of cooking time and temperature (Lamas *et al.* 2004). The bagasse is a lignocellulosic waste, which can be used as a substrate for hydrogen production after hydrolysis treatment. However, due to cellulose crystallinity and the formation of furan derivatives as well as phenolic compounds, which act as inhibitors depending on their concentrations, low hydrogen production yields and rates are observed (Cao *et al*. 2010; Mussatto and Roberto, 2004).

Cellulases can rapidly digest the 'easy and amorphous' cellulose material (Park *et al.* 2010). However, cellulose accessibility is affected by crystallinity, and several other parameters, such as lignin and hemicellulose contents and distribution, porosity, and particle size.

The aim of this study was to evaluate the changes in the content of lignin, hemicellulose and amorphous and crystalline cellulose and the level of inhibitors of hydrogen production due to the cooking of the agave heads and enzymatic hydrolysis of the agave bagasse.

METHODS

The heads and bagasse of A*gave tequilana* Weber var. azul collected from a tequila factory, “La Herradura”, were air dried. The bagasse was washed with distilled water and dried in oven.

Agave bagasse was exposed to enzymatic hydrolysis using a commercial cellulose mixture, Celluclast 1.5L from *Trichoderma reesei*, using a concentration of 1g of enzyme per gram of bagasse, suspended at 4% (w/v) in 50 mM citrate buffer solution to pH 4.5. The hydrolysis was performed at 45 °C for 10 hours.

The determination of neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) in the fibers of agave heads, agave bagasse, and bagasse hydrolysate was measured by a batch sequential procedure (ANKOM), based on the method of Van Soest. This method allows determination of the constituents of plant cell walls (cellulose, hemicellulose and lignin).

X-ray diffraction (XRD) was made with monochromatic Cu Kα radiation (λ= 1.5818 Å), at operating conditions 35 KeV and 25 mA to determine the crystallinity of the fiber following regular diffraction. The amorphous and crystalline fraction in the fibers was calculated at 2ɵ values between 5° and 70°, using the XRD deconvolution method, through MagicPlot software to separate amorphous and crystalline contributions to the diffraction spectrum using a curve-fitting process, assuming Gausssian functions for each peak. The crystallinity index was calculated using the equation: $CI(\%) = Ic/(Ic + Ia) \times 100$ where Ic is the scattering of the crystallinity portion and Ia is the scattering of the amorphous part (Park *et al.* 2010).

A search for furfural, hydroxymethyl furfural, vanillin and syringaldehyde in bagasse washing water and enzymatic hydrolysates of bagasse was performed by high performance liquid chromatography (HPLC) system (Zorbax Eclipse XDB-C18, Agilent Technologies) operating at 40 °C with a mixture of acetonitrile and deionized water (ratio 92:8) as the mobile phase at a flow rate of 0.8 ml/min. Total phenols were measured by the Folin-Ciocalteu method using gallic acid as standard (Singleton *et al.* 1999).

RESULTS AND DISCUSSION

The agave head showed a higher percentage of the soluble fraction (87.8%) due to the presence of inulin (Fig. 1a). Hemicellulose, cellulose and lignin percentage increased in bagasse compared to agave heads (Fig. 1b) due to the removal of inulin by saccharification during the agave heads cooking process. Removal of hemicellulose (14.3%) and cellulose (10.4%) due to enzymatic hydrolysis was low (data not shown), indicating the need for pre-treatment or optimization of enzymatic hydrolysis.

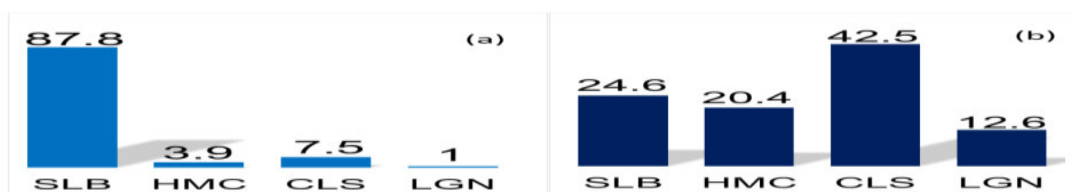

Fig. 1. Fiber content in percentage of agave head (a) and agave bagasse (b); soluble (SLB), hemicellulose (HMC), cellulose (CLS) and lignin fraction (LGN).

X-ray diffraction patterns of fibers shown several sharp peaks, which may not be associated with the crystallinity of agave fibers (indicated with arrows in Fig. 2a). This sharp peaks may correspond to calcium oxalate which has been reported as a component of the agave head. The crystallinity index obtained for agave heads was 0.8% (Fig. 2a), while for agave bagasse and agave bagasse hydrolysate were 51.2% and 57.2% respectively, considering only the peaks corresponding to the crystalline cellulose (see arrows in Fig. 2b and 2c). These results suggest the enrichment of crystallinity due to inulin removal (during agave heads cooking) and hemicellulose and cellulose removal during enzymatic hydrolysis.

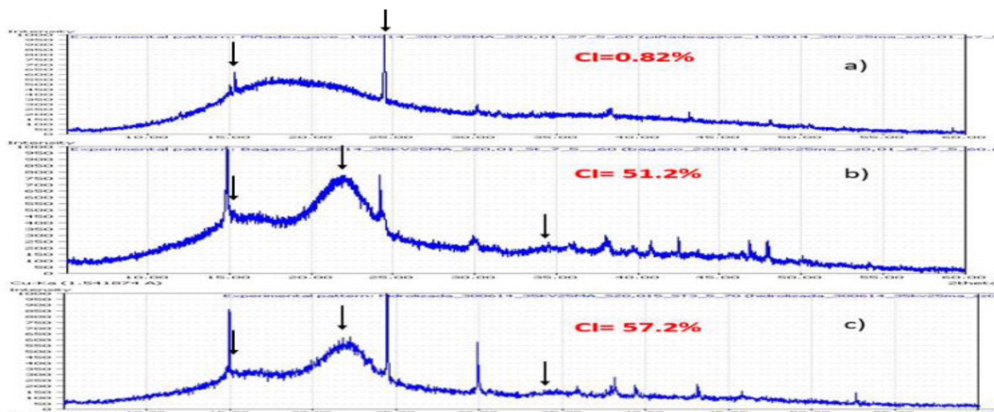

Fig. 2. X-ray diffractogram of *Agave tequilana* Weber var. azul: a) agave head, b) bagasse, and c) bagasse after hydrolysis.

The concentration of phenolic and furan derivatives measured by HPLC in bagasse washing water and in enzymatic hydrolysate of bagasse were below detectable limits. However, a concentration of 80-130 ppm of total phenols, as gallic acid, was detected in the bagasse washing water.

CONCLUSIONS

1. Crystallinity seems to be unaffected by the agave head cooking process, nor the hydrolysis treatment of agave bagasse.
2. X-ray diffraction analysis of agave fibers suggest the presence of other crystalline compounds, which do not correspond to the crystalline cellulose.
3. It is necessary to optimize the enzymatic treatment of bagasse to increase the saccharification of hemicellulose and cellulose.
4. Total phenols detected in the bagasse washing water suggest the presence of phenols different to vanillin and syringaldehyde.

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MECHANICAL PROPERTIES OF "WOOD PLASTIC COMPOSITE" FROM POLYOLEFINS AND AGAVE BAGASSE FIBERS

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RESUMEN (TIMES NEW ROMAN, NEGRITAS, TAMAÑO DE LA FUENTE 12, ALINEADO A LA IZQUIERDA, MAYUSCULAS)

In this work, lignocellulosic fiber from agave tequilana bagasse was used as reinforcement of virgin polypropylene (PP) to obtain a wood plastic composite (WPC). Extrusion process was used to obtain ribbon of the WPC (20 cm wide) with 5% w/w coupling agent, and 3 w/w of process aid. The ribbons were thermoforming to corrugated sheets using a undulate press. The effect of fiber concentration on mechanical properties of flat sheets (tensile and impact) and corrugated sheets (bending) were studied. The composite showed higher tensile modulus than the matrix and this was attributed that the fiber have a higher Young´s modulus that the PP virgin. The mechanical properties of the flat sheets increased with fiber content and at 40% fiber content the modulus values and tensile strength were 900 MPa and 19 MPa, respectively. The corrugated sheet had a flexural load up to at 30% fiber content and they maintained their integrity during the blending test.

Keywords: Agave bagasse fibers, wood plastic composites, recycled, mechanical properties, corrugated sheet

INTRODUCTION

In Mexico, the agave bagasse, generated during the production of spirits such as Tequila and Mezcal, is considered a waste, causing contamination problems in the accumulation places. This bagasse can be a lignocellulosic fiber source (Canché-Escamilla et al, 2012). A form to reduce the contamination by this materials (besides obtaining products of added value), is the elaboration of "wood plastic composite" or WPC. This material can be obtained from virgin or recycled thermoplastic and lignocellulosic fibers (Ye et al, 2009; Fabiyi and McDonald, 2010; Ayrilmis et al, 2011). The use of polyolefines such as polyethylene (PE) and polypropylene (PP) mixed with fibers to manufacture WPC materials, it has become in a new field of the investigations related with the timber industry (Min and Shuai, 2007). The WPC have certain advantages against others materials such as dimensional stability, maleability, tensile and impact strength (Hamel et al, 2013; Migneault, 2014), are renewable, have a low maintaining cost, and durability. One of the biggest challenges of this material is to optimize the relation fiber/polymer trying to decrease costs and to compete with the commercial products (Kumari et al, 2007; Yang et al, 2007). Nevertheless, the materials

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composed of lignocellulosic fibers and polyolefin need to be made compatible to improve the poor adhesion fiber-polyolefin and improve its mechanical properties. Use of coupling agents as the polyolefin-g-maleic anhydride improves the fiber-polyolefin adhesion (Kumari et al, 2012).

The purpose of this work is obtaining a wood plastic composite (WPC) using polypropylene and fiber obtained from agave tequilana bagasse (FATB). Corrugates sheet of WPC were obtained and the mechanical properties were evaluated.

METHODOLOGY

Polypropylene (PP) (HP423M) of INDELPRO with a density of 0.9 g/cm^3 and a melt index of 3.8 dg/min extrusion grade was used as matrix. Epolene E-43 43 (with acid number (mg KOH / g) 45 and molecular weight of 9100 daltons) from Eastman was used as coupling agent. This material have groups of maleic anhydride (MA) grafted onto the polymer chain. As processing aids Struktol TPW113 from Struktol Company was used. The polymers and additives were ground using a knife mill from Brabender fitted with a 1 mm mesh to separate the ground material. The fiber was obtained from agave tequilana bagasse which is a residue of the tequila production. The fibers were reduced in size and the fraction recovered at 40-80 was used to obtain the composites.

The WPCs were obtained in the form of continuous ribbons (20 cm wide and 2.5 mm thick) using a twin screw extruder from Brabender, with a speed of 40 rpm and temperatures of 180 °C in the extruder and 190° C on the die. The fiber content was varied of 30 to 50 wt%. The amount of coupling agent (copolymer PP-g-MA) and the processing aid agent (fatty acids stearate) used to obtain the composites with agave fibers, were 5% and or 6% by weight, respectively. An undulate press was used to obtain the corrugate sheets.

The tensile properties of WPC ribbons and corrugate sheets were conducted using an Instron universal machine with load cell of 500 N. The Type I specimens, specimens were conditioned for 48 h prior to the test at 23 °C. Micrographs were obtained from the rupture surface of the composites with scanning electron microscopy from JEOL (JSM 6360 LV model), the samples were coated with a layer of gold prior the SEM observation.

RESULTS AND DISCUSION

Figure 1 show the mechanical properties of WPC with different fiber content. It is noted that when the load is applied parallel to the direction of extrusion of the composites, the module increases with fiber content. A modulus value of 899 and 706 MPa for 40% and 25% fiber content, respectively, was obtained. The tensile strength showed a slight increase at a concentration of 30% fiber and remains constant at higher concentrations, due to improved fiber-matrix adhesion as has been reported by the literature (Serrano et al, 2013).Use of coupling agent (Figure 1, right) improves the interaction between the fiber and the polymer matrix and the fibers are embedded in the matrix and a polymer residue is observed on the surface of the pull out fibers (Figure 1c and 1 d). Different behavior is observed when the load is applied perpendicular to the direction of extrusion of the sheets (data not shown therein), since the composite materials show an increase in modulus and tensile strength with increasing content of fiber, reaching a maximum at a content of 30% and higher fiber concentrations decrease. Maximum values of modulus and tensile strength of 657 MPa and 13.2 MPa, respectively, were obtained. This behavior is characteristic of an anisotropic material, as during the extrusion process, the fibers tend to be oriented in the direction of

flow, and a material in which the properties are not equal in the axial direction and in the transverse direction is obtained.

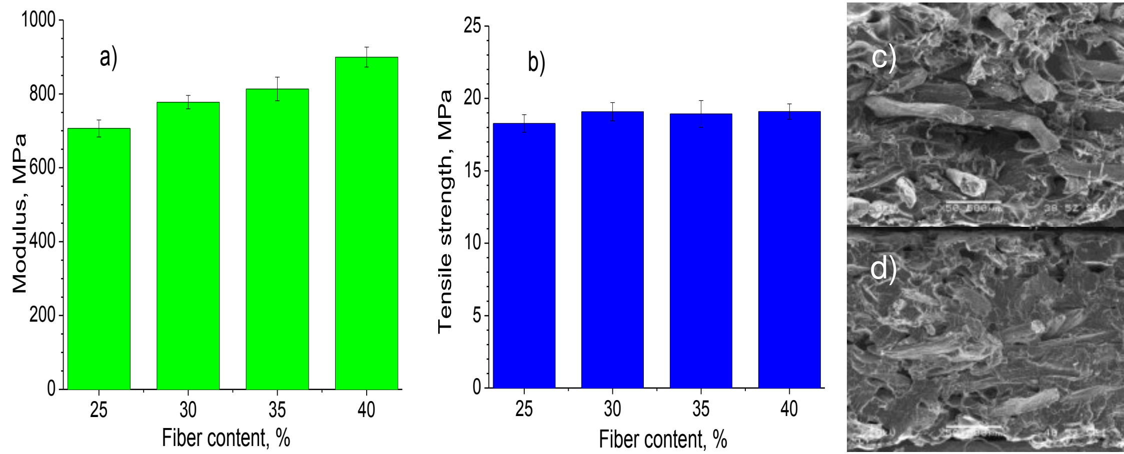

Figure 1. Effect of fiber content on mechanical properties of WPC: a) Modulus; b) Tensile strength. SEM microphotopraphs of fracture zona of WPC: c) with out and d) with coupling agent.

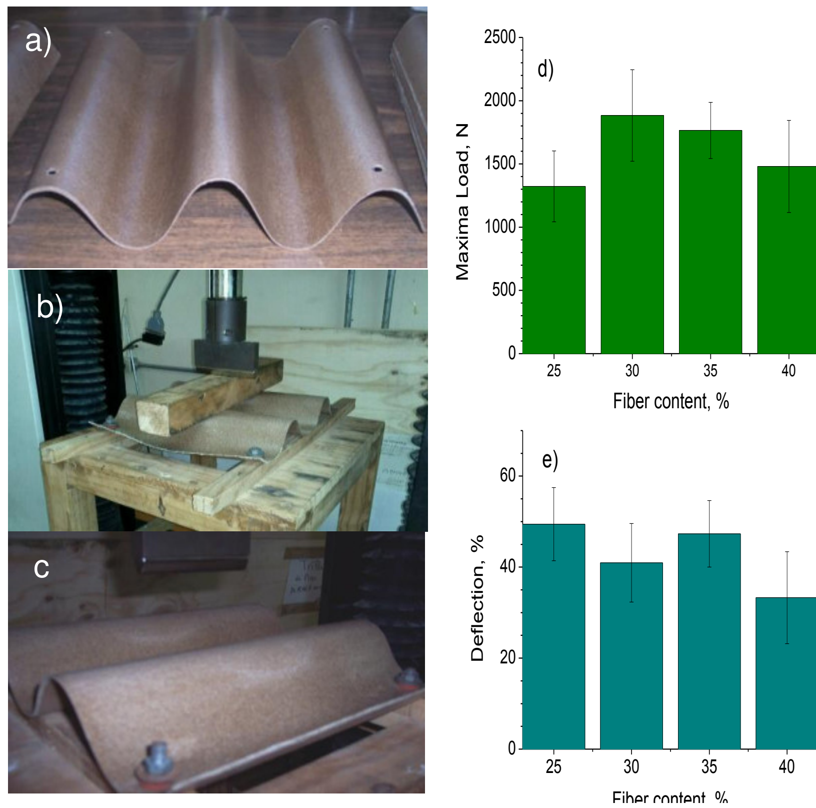

Figure 2. Corrugated sheet of PLA/FATB composite before the compression test (a); during the test (b) and after the test (c). Mechanical properties: Maxima Load (d) and Deflection (e).

Figure 2 show the corrugated sheets obtained by compression molding flat sheets and the mechanical properties of the bending test of this sheets. It was noted during the trials that sheet recovering its shape irrespective of the fiber content, but a higher fiber content (> 35%) the fracture formation was observed (Figure 2c). This behavior could be due to a high content of the composite fiber becomes more rigid and thus fracture could be generated during the test. The corrugated sheets have a maximum load value 1882 N containing 30% fiber, and then decrease as the fiber content in the material is increased, obtaining a value of 1480 N to a blade content of 40% fiber.

CONCLUSION

WPC with polyolefin and agave bagasse fiber were obtained. The use of coupling agent improved the compatibility between fiber and matrix increasing the mechanical properties of the material. Corrugate sheet obtained from WPC ribbons maintained their integrity during the blending test. The bests results were obtained using WPC with 35% w/w fiber content.

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TOMATO PLANT GROWTH IN "MAGUEY MEZCALERO" BAGASSE COMPOST WITH APLICATION OF TWO NUTRIENT SOLUTIONS

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ABSTRACT

The "mezcal" industry in Oaxaca, Mexico, produce a great volume of residues, mainly vinasses and bagasse, which with a process of composting may have use in agriculture as soil amendment and substrate for soilless cropping. The aim of the work was to record the effects of using composted mezcal bagasse as substrate, irrigated with two nutrient solutions, in the development of tomato plants. Growth of plantlets of indeterminate saladette tomato Cv. Ramses were used as indicators of the effects of three substrates (composted mezcal bagasse –BM-, peat –P-, and mixture 50:50 BM-P) and two nutrient solutions (Hoagland and Steiner universal solutions) in a complete two-factor experimental design. None of the recorded variables showed statistically significant difference in response to nutrient solutions. The BM and BM-T substrates had higher values of plant height, stem diameter, branch and leave number. The BM-T treatment recorded the highest number of set fruits per plant. The stabilized mezcal bagasse compost, alone or mixed with peat (50:50) can be used in the cropping of tomato as a substitute for peat.

Key words: Agave baggase compost, nutrient solution, soilless culture

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INTRODUCTION

Many factors, such as the global need to reduce pollution and decreasing input costs, have led to the search for sustainable production systems, incorporating organic practices in crops or experimenting protected agriculture with composted agricultural and agro industrial wastes as substrates (Cruz et al., 2012).

The “mezcal” production has boomed in Oaxaca, Mexico. The development of this agribusiness brings the generation of a great volume of the residues bagasse and vinasses, susceptible to stabilization by composting. These residues may be used in agricultural production, such as agave bagasse compost from “tequila” agroindustry in tomato production (Iñiguez et al., 2011).

The work records the effects of using composted mezcal bagasse as substrate, irrigated with two nutrient solutions, in the development of tomato saladette type.

METHODS

The experiment was conducted in Santa Cruz Xoxocotlan (Oaxaca, Mexico) in a greenhouse, during two months, June-July 2014. Saladette indeterminate type tomato, cv. Ramses F1 was used. Plantlets were transplanted 35 days after seeding to black bags of 4.63 L. The topological arrangement was conducted in three parallel rows, 30 cm between pots and 100 cm between rows. A complete two-factor experimental design with five replications was used. The factors were: substrate and nutrient solution; the substrate were bagasse compost 100% (BM), peat 100% (T), and bagasse compost - peat (BM-T) in 50:50 rate. The bagasse compost was sieved in of 8 mm sieve, and Canadian peat was used as it comes. The Table 1 shows the characteristics of bagasse compost used.
The universal nutrient solutions Hoagland (H) and Steiner (S) were evaluated. It were applied to the 60 days after transplant, at a rate of 200 mL per plant. The variables measured were: plant height (ph), stem diameter (sd), number of branches (bn), leaves (ln), clusters (nfc), flowers (fln) and set fruits per plant (nfr). Root dry weight (rdw) and shoot dry weight (sdw) also were recorded.

Table 1. Physical and chemical characteristics of mezcal bagasse compost used in this work.

Parameter	
pH	8.35
Electric Conductivity (dS m^{-1})	2.05
Organic matter (%)	64.55
C/N rate	18.15
Field density (Kg m^{-3})	146.40
Bulk density (g cm^{-3})	0.252
Total pore space (%)	85.58
Water retention capacity (ml L^{-1})	36.685

RESULTS AND DISCUSSIONS

None of the recorded variables showed statistically significant difference in response to nutrient solutions. A similar result was recorded in an experiment on lettuce (http://www.agroparlamento.com/agroparlamento/notas.asp?n=0072). With respect to factor substrates, Table 2 shows that, until the day 41 of the evaluation, the BM-T treatment showed the highest values in ph, sd, bn, ln. Substrates BM and BM-T promoted, on average, significantly higher values in all the measured variables. The BM-T treatment recorded the highest number of set fruits per plant, which could mean increased production and crop yield, similar to that reported by Iniguez et al. (2011) using composted tequila bagasse and comparing with commercial substrate of coconut fiber.

Table 2 Values of the variables measured in tomato plants at 41 and 71 days after transplantation.

	day 41				day 71		
Variable	BM	BM-T	T		BM	BM-T	T
ph	98.79 b	127.85 a	94.41 b		203.17 a	176.07 b	178.6 b
st	5.64 b	7.09 a	5.88 b		8.22 b	9.32 a	8.18 b
nb	12.80 b	14 .00 a	11.60 c		15.80 a	14.40 b	13.60 c
nl	121.10 b	143.40 a	117.40 b		226.30 a	192.40 b	151.30 c
nfc	2.00 a	1.10 b	1.90 a		5.00 a	3.30 c	4.00 b
nfl	14.40 a	8.40 b	13.50 a		30.00 b	39.60 a	27.20 b
nfr					11.4 ab	14.7 a	11.4 ab

Same letters in the same row, (same column group) presented no significant estatistical differences.

The interaction of the factors shows the same trend until the day 41, the BM-T-S y BM-T-H treatments have the highest values in the variables ph, st, nl and nb, changing the trend at the end of the experiment, maintaining the values above T-S and T-H. At the end of the evaluation, the BM-T-H treatment showed the highest values of the variables st, nfl and nfr. The sdw was significantly higher in BM-TS and BM-T-H treatment, the latter being the highest.

CONCLUSIONS

The values of the measured variables of tomato plants growing in compost of mezcal bagasse compost showed higher values compared to plants grown in peat. We conclude that mezcal bagasse compost, alone or mixed with peat (50:50) can be used in the cropping of tomato as a substitute for peat.

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COMPARATIVE ANALYSIS BETWEEN SEVERAL SOURCES OF INSOLUBLE FIBER FOR FOODS RESPECT TO LIGNOCELLULOSIC FRACTIONS OF AGAVE

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ABSTRACT

Infrared spectroscopy with attenuated total reflectance has revolutionized instrumental analyzes on a variety of materials, including those of biological origin. The applications are focused on identification of chemical compounds, detecting changes at the molecular level and contrast the authenticity of a product. In the present study the aim was to use infrared spectroscopy by Fourier transform (FT-IR) to compare fractions of lignocellulosic agave regarding the different functional groups present in cereals. The agave bagasse several absorption bands centered at 1599, 1502, 1423 and 1232 cm-1 denoting the presence of lignin fractions were observed. In the spectrum of agave a band at 1726 cm -1 ascribed to C = O, ester extension vibrations carbonyl group, whereas cereals occurs lso 1740 cm-1 is observed. The spectra showed bands cereals 1643 cm-1 assigned to the vibration of C = O type extends to the protein amide I, 1535 cm-1 the NH group in the flexion and amide II NH 1238 cm-1 in bending the amide III. In samples bands were detected around 3330 cm-1 by the presence of the OH group from the phenols and alcohols. In turn the vibration of the CH group is presented to a wavelength of 2924 and 2852 cm-1. The bands located at 1741 and 1631 cm-1 denoted the presence of pectin samples amaranth and oats, 894 cm-1 shows the deformation of the glycosidic bond β (1-4) present in the spectrum of agave and amaranth

Key words: Agave, Cereals, Fiber.

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INTRODUCTION

The FT-IR spectroscopy is a nondestructive analytical technique that provides rapid qualitative information to elucidate molecular structures. The infrared spectrum of a molecule is based on molecular vibrations which depends on the mass and the length of link, so that a complete infrared spectrum of an organic compound gives a unique fingerprint which is distinct from the pattern of infrared absorption of drugs, including isomers. Previous studies have demonstrated the effectiveness of this technique to detect the chemical composition of lignocellulosic materials. Cereals are essential in the diet be a major source of carbohydrates, protein, vitamins, and soluble and insoluble fiber which is a beneficial nutrient which reacts in an organism through the metabolism which is associated with the reduction of obesity, diabetes, cardiovascular disease, ulcerative colitis, constipation, inflammatory bowel disease, gastrointestinal disorders, and colon cancer (Schneeman, 1999). The main sources of fiber in the diet are cereals like amaranth (Amaranthus sp) Oats (Avena sp) Wheat (Triticum sp), however bagasse Agave (Agave tequilana) for their chemical components (cellulose hemicellulose and lignin) could be used as an alternative source of fiber. Therefore the aim of this study was to compare the lignocellulosic fractions agave Infrared Spectroscopy by Fourier transform (FT-IR), with respect to the different functional groups present in cereals.

METHODOLOGY

The samples were analyzed by infrared Fourier transform spectroscopy (FT-IR). A Shimadzu, Model IRAffinity-1, which has an accessory ATR (Attenuated Total Reflection) with zinc selenide crystal allowing the analysis of samples in the mid-infrared (MIR) was used. With the following scan parameters. Mode: % Transmittance, sweeps No: 20, 4 cm^{-1} resolution in the range of 600-4000 cm^{-1}.

RESULTS AND DISCUSSION

According to analysis by FT-IR spectroscopy (Figure 1), in all samples of cereal as sample Agave bagasse showed a band at 3335 cm-1 stretch feature of all hydroxyl groups which may be molecules of phenols, alcohols, water or proteins present in the samples, also can be observed bands wavelength of 2924 and 2852 cm-1 which represents stretching vibration of aliphatic methyl, methylene groups and compounds, is observed a band around 1740 cm-1 in the spectra of oats, wheat bran and amaranth, and 1726 cm-1 in the sample of agave some authors attribute to the stretching vibration of free aldehyde carbonyl group , ketone and carboxylic acids. (Bouanda y col. 2002). In cereals identified bands 1643, 1535, 1238 cm-1, characteristic of the stretching of C = O, CN and NH bending for the amide I, NH flexion and stretch to the amide II CN and NH flexion and CH stretching in the amide III (Carbonaro y col. 2008, Aït y col. 2008); bands that were not present in the spectrum of the sample Agave bagasse, however bands were obtained 1599, 1502, 1423 and 1232 cm-1, which came from the vibration of the aromatic backbone of lignin and lignocellulosic fractions (Xu y col, 2006, Bouanda y col. 2002). The bands around 1149, 1076, 1010 cm-1 were assigned to the stretching of CO and CC in the molecules of starch grains, (Buranov y col. 2010). The band that appears near 1022 cm-1, represents the deformation of the CH bonds in aromatic substructures guayacil, complex vibrations associated with CO, lignin or cellulose, for the case of agave, and about 1010 cm-1, CC stretching and C-OH bending to

the polysaccharides in samples of amaranth, oats, wheat germ and bran. (Naumann y col. 2010). The bands around 1149, 1076, 1010 cm-1 were assigned to the stretching of CO and CC in the molecules of starch grains, (Naumann y col. 2010). Bands were also observed near 1741 and 1631 cm-1 which carbonyl groups denoted by the presence of pectin samples amaranth, oats and wheat bran not the case in Agave bagasse and wheat germ. (Kacŭráková and Wilson 2001) Finally, the band at 894 cm-1 shows the presence of the deformation of the glycosidic bond β (1-4) links between glucose and cellulose, band observed in the spectrum of the sample and agave amaranth (Wu y col. 2011, Sakdaronnarong and Jonglertjunya. 2012, Hozová y col. 2007).

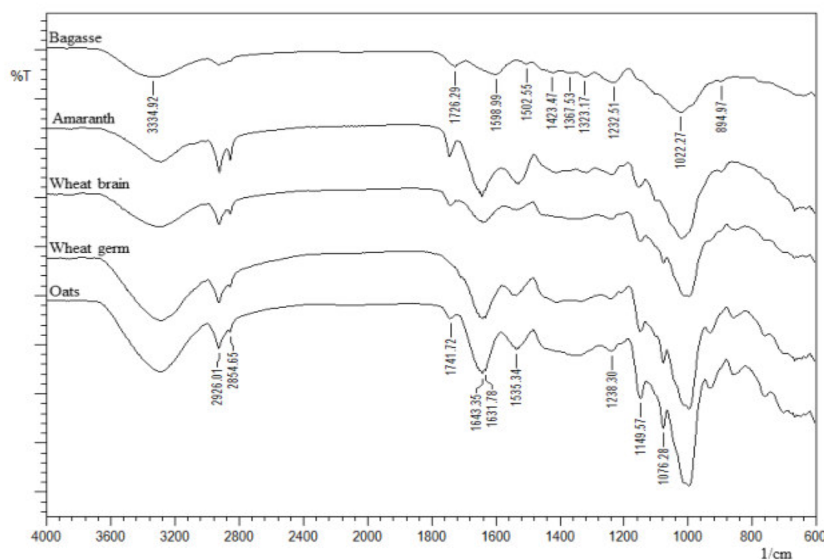

Figure 1: FT-IR spectrum of agave bagasse, Amaranth, Oats, Wheat Germ and Bran.

CONCLUSION

Functional groups of Agave bagasse and some cereal components were identified for ATR-FTIR, by this technique was possible to identify qualitatively in Agave bagasse the presence of several absorption bands from lignin fractions, in contrast to the amide I, II , III groups, identified in the cereals and the other bands related with the content of soluble and insoluble fiber.

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