OPEN ACCESS

African Journal of **Biotechnology**

26 June 2019
ISSN 1684-5315
DOI: 10.5897/AJB
www.academicjournals.org

ACADEMIC JOURNALS
expand your knowledge

About AJB

The African Journal of Biotechnology (AJB) is a peer reviewed journal which commenced publication in 2002. AJB publishes articles from all areas of biotechnology including medical and pharmaceutical biotechnology, molecular diagnostics, applied biochemistry, industrial microbiology, molecular biology, bioinformatics, genomics and proteomics, transcriptomics and genome editing, food and agricultural technologies, and metabolic engineering. Manuscripts on economic and ethical issues relating to biotechnology research are also considered.

Indexing

CAB Abstracts, CABI's Global Health Database, Chemical Abstracts (CAS Source Index) Dimensions Database, Google Scholar, Matrix of Information for The Analysis of Journals (MIAR), Microsoft Academic, Research Gate

Open Access Policy

Open Access is a publication model that enables the dissemination of research articles to the global community without restriction through the internet. All articles published under open access can be accessed by anyone with internet connection.

The African Journals of Biotechnology is an Open Access journal. Abstracts and full texts of all articles published in this journal are freely accessible to everyone immediately after publication without any form of restriction.

Article License

All articles published by African Journal of Biotechnology are licensed under the Creative Commons Attribution 4.0 International License. This permits anyone to copy, redistribute, remix, transmit and adapt the work provided the original work and source is appropriately cited. Citation should include the article DOI. The article license is displayed on the abstract page the following statement:

This article is published under the terms of the Creative Commons Attribution License 4.0

Article Copyright

Self-Archiving Policy

The African Journal of Biotechnology is a RoMEO green journal. This permits authors to archive any version of their article they find most suitable, including the published version on their institutional repository and any other suitable website.
Please see http://www.sherpa.ac.uk/romeo/search.php?issn=1684-5315

Digital Archiving Policy

The African Journal of Biotechnology is committed to the long-term preservation of its content. All articles published by the journal are preserved by Portico. In addition, the journal encourages authors to archive the published version of their articles on their institutional repositories and as well as other appropriate websites.
https://www.portico.org/publishers/ajournals/

Metadata Harvesting

The African Journal of Biotechnology encourages metadata harvesting of all its content. The journal fully supports and implement the OAI version 2.0, which comes in a standard XML format. See Harvesting Parameter

Memberships and Standards

Academic Journals strongly supports the Open Access initiative. Abstracts and full texts of all articles published by Academic Journals are freely accessible to everyone immediately after publication.

All articles published by Academic Journals are licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0). This permits anyone to copy, redistribute, remix, transmit and adapt the work provided the original work and source is appropriately cited.

Crossref is an association of scholarly publishers that developed Digital Object Identification (DOI) system for the unique identification published materials. Academic Journals is a member of Crossref and uses the DOI system. All articles published by Academic Journals are issued DOI.

Similarity Check powered by iThenticate is an initiative started by CrossRef to help its members actively engage in efforts to prevent scholarly and professional plagiarism. Academic Journals is a member of Similarity Check.

CrossRef Cited-by Linking (formerly Forward Linking) is a service that allows you to discover how your publications are being cited and to incorporate that information into your online publication platform. Academic Journals is a member of CrossRef Cited-by.

Academic Journals is a member of the International Digital Publishing Forum (IDPF). The IDPF is the global trade and standards organization dedicated to the development and promotion of electronic publishing and content consumption.

Contact

Editorial Office: ajb@academicjournals.org

Help Desk: helpdesk@academicjournals.org

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

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

Academic Journals
73023 Victoria Island, Lagos, Nigeria
ICEA Building, 17th Floor,
Kenyatta Avenue, Nairobi, Kenya.

Editor-in-Chief

Editorial Board Members

Table of Content

Vol. 18(26), pp. 548-563, 26 June, 2019
DOI: 10.5897/AJB2019.16826
Article Number: 7118D0361330
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

A comparative assessment of the performance of a stay-green sorghum (*Sorghum bicolor* (L) Moench) introgression line developed by marker-assisted selection and its parental lines

Isaac K. Galyuon[1,2*], Allan P. Gay[1], Charles T. Hash[3], Fran R. Bidinger[4] and Catherine J. Howarth[1]

[1]Institute of Biological, Environmental and Rural Sciences, Aberystwyth, SY23 3EB, UK.
[2]Department of Molecular Biology and Biotechnology, University of Cape Coast, Cape Coast, Ghana.
[3]International Crops Research Institute for the Semi-Arid Tropics - Sadore, BP12404, Niamey, Niger.
[4]International Crops Research Institute for the Semi-Arid Tropics - ICRISAT, Patancheru, 502324, India.

Received 6 April 2019; Accepted 10 June 2019

A glasshouse experiment was conducted to compare the performance of a stay-green QTL introgression line (RSG 03123) and its parental lines (B35, a stay-green and R16, a high yielding cultivar with rapid senescence) after flowering, when water-limited (WL) or well-watered (WW). Flowering time and plant height were similar in RSG 03123 and the recurrent parent, R16. Under WW conditions, retention of green leaf area (GLA) and chlorophyll was similar in RSG 03123 and R16, whereas under WL conditions RSG 03123 retained significantly ($P<0.05$) more GLA. The rate of senescence was also lower in RSG 03123 as compared to R16 under WL conditions. B35 displayed a delayed onset of senescence under both WW and WL conditions while both R16 and RSG 01323 displayed an earlier onset of senescence under WL conditions. Photochemical efficiency of photosystem II (ΦPSII), gas exchange characteristics, and total soluble carbohydrates contents, in both leaf and stem, were higher in RSG 03123 than in R16 under WL conditions. These parameters were enhanced in RSG 03123 due to higher retention of chloroplast proteins, such as light-harvesting chlorophyll binding protein complex of PSII (LHCPII), oxygen-evolving complex 33 kDa (OEC33), phospho*enol*pyruvate carboxylase (PEPC) and the large subunit of ribulose bisphosphate carboxylase-oxygenase (Rubisco). The stay-green characteristics of RSG 03123 were intermediate between R16 and B35; nonetheless, the results show that stay-green QTL are functional during senescence and improve tolerance to water limitation after flowering.

Key words: Stay-green QTL, introgression, drought, photosynthesis.

INTRODUCTION

Feeding the increasing population is a challenge to all nations (Foley et al., 2011), especially those in the drought-prone agro-ecological regions. Sorghum is an important crop grown for food, feed and fibre as well as fuel (Paterson et al., 2009a, b; Boyles et al., 2019) and is able to withstand drought conditions and give a good yield (Borrell et al., 2014a; Lasky et al., 2015). However, post-flowering drought stress results in rapid senescence and premature death, stem collapse, lodging and rot which results in reduced grain yield in senescent cultivars (Xu et

al., 2000b; Badigannavar et al., 2018).

Stay-green (SG) genotypes, on the other hand, do not display this drought-induced senescence and retain their green leaves longer resulting in improved grain yield under stress (Rosenow, 1994; Duvick et al., 2004; Jagadish et al., 2015; Badigannavar et al., 2018). Senescence is characterised by chlorophyll loss and a progressive decline in photosynthesis. Hence, early onset of senescence, consequently, affects assimilation and grain-filling (Xu et al., 2000b). Stay-green genotypes are either functional or cosmetic (Thomas and Howarth, 2000). Functional stay-green genotypes possess an increased duration of both green leaf area and of photosynthesis, whereas cosmetic stay-greens remain green due to defective chlorophyll breakdown but photosynthetic competence is lost (Thomas and Howarth, 2000).

Therefore, stay green can be viewed at a cell (Johnson et al., 2015), leaf, whole plant, crop and system level (Borrel et al., 2014a, b; Blümmel et al., 2015; Badigannavar et al., 2018). Sorghum genotypes possessing the SG trait retain green stems and upper leaves for longer periods than those not possessing the trait; thus, they are able to fill their grain normally under post-flowering drought conditions (Subudhi et al., 2000; Tao et al., 2000; Borrell et al., 2001). Since SG leaves are maintained after physiological maturity (Sanchez et al., 2002), the need for translocation of photosynthates from the stem during grain filling is reduced (Van Oosterom et al., 1996). Therefore, grain yield in sorghum under post-flowering drought stress correlates positively with green leaf area at mid-grain-filling (Borrell et al., 1999) and green leaf area at maturity (Borrell et al., 2000). However, some SG genotypes efficiently remobilize assimilates during grain filling, which results in maintenance of grain weight, quality and nutrient efficiency (Jagadish et al., 2015).

The longevity of leaves in the stay-green sorghum might be promoted by a combination of several biochemical factors, which interact to regulate nitrogen (N) remobilisation (Borrell and Hammer, 2000) and chlorophyll turnover, maintain the integrity of the photosynthetic apparatus as well as enzyme activity (Oh et al., 2003), particularly those involved in carbon and N assimilation (Hortensteiner and Feller, 2002).

Both grain and stover yields are dependent on photosynthesis, which, in turn, is influenced by green leaf area (GLA) and green leaf area duration (GLAD), especially under drought stress. Photosynthesis is also affected by stomatal conductance (gs), nutrient availability and enzyme activity. The efficient use of water for carbon assimilation, which is affected again by gs, transpiration and the capacity for CO_2 assimilation, might be related to yield maintenance and drought resistance. Hence, analyses of GLA, GLAD, leaf chlorophyll retention rate, leaf gas exchange and their effects on grain yield would evaluate the functioning of the SG QTL in maintaining yield under drought stress. Such findings, in the long-term, would enhance the selection and breeding for improved genotypes.

Retention of chloroplast proteins are maintained in sorghum containing the KS19 source of SG (de Villiers et al., 1993), which explains, in part, the ability of these plants to maintain photosynthesis for longer periods than their senescent counterparts. However, these observations were made under non-drought stress conditions, whereas in sorghum the trait is fully apparent under severe drought stress. Therefore, investigating changes in chloroplast proteins and leaf N under both well-watered and water-limiting conditions should enhance understanding of the biochemical basis of the trait. In addition, assessment of changes in the carbohydrate metabolism in the leaf and stem, in SG genotypes, would enhance our understanding of the different mechanisms by which yield is maintained under drought stress.

B35 is a dwarf stay-green BC1 selection from IS12555 *durra* sorghum, a land race from Ethiopia (Rosenow et al., 1983) and has been released as BTx642 (Rosenow et al., 2002). B35 is the stay-green parent used in several quantitative loci (QTL) mapping studies of drought tolerance in sorghum (Walulu et al., 1994; Tuinstra et al., 1996, 1997, 1998; Crasta et al., 1999; Xu et al., 2000b; Tao et al., 2000; Subudhi et al., 2000; Sanchez et al., 2002). R16 is a released variety that is high yielding but highly senescent under post-flowering drought stress conditions (Blümmel et al., 2015). Four major QTL associated with stay-green (SG), namely: *Stg1* on chromosome SBI-03 (LG 03), *Stg2* on chromosome SBI-03 (LG 03), *Stg3* on chromosome SBI-01 (LG 01) and *Stg4* on chromosome SBI-05 (LG05) have been identified to be consistent in a range of genetic backgrounds and environments. These QTL account for up to 53.5% of the phenotypic variance and co-locate with the QTL for chlorophyll content at physiological maturity (Xu et al., 2000b; Sanchez et al., 2002). *Stg2* was identified as the most important in contributing to the SG phenotype, followed by *Stg1*, *Stg3* and *Stg4* (Xu et al., 2000b).

The SG trait has been used extensively in the breeding of sorghum for regions where post-flowering drought is prevalent because Sg genotypes are tolerant to both drought and heat (Pinto et al., 2016). A number of breeding programmes are using marker-assisted selection

*Corresponding author. E-mail: igalyuon@ucc.edu.gh.

to incorporate these SG QTL in advanced breeding lines (Hash et al., 2003; Harris et al., 2007; Kassahun et al., 2010). Individual QTL have been assessed and found to influence canopy development and, consequently, crop water use efficiency and grain yield (Borrell et al., 2014a). However, some SG QTL (e.g., *Stg*B) have been found not to have any concomitant improvement in stover and grain yield in R16 background, indicating the functioning of such QTL might be background-dependent (Blümmel et al., 2015). Since the genetic background of SG QTL can influence their expression, it is important to examine the functioning of SG QTL in a senescent background in comparison to the parental lines, such as R16 and B35. Findings from such studies should provide the basis for recommending appropriate SG introgression lines for farmers' use. The objective of this study was to evaluate the physiological functioning of RSG 03123, a marker-assisted backcross (MABC) derived stay-green QTL introgression line along with the donor parent (B35) and recurrent parent (R16) under well-watered and water-limiting conditions. Specifically, green leaf area retention, chlorophyll fluorescence, photosynthesis, carbohydrate, nitrogen and protein metabolism were evaluated.

MATERIALS AND METHODS

Plant culture and treatment

Seeds of B35, R16 and RSG 03123 were obtained from ICRISAT, India. RSG 03123 is a BC1F5 selection developed after marker-assisted backcrossing using markers flanking *Stg1*, *Stg2*, *Stg3* and *Stg4* from B35 as described in Kassahun et al. (2010). The recurrent parent, R16, is a high-yielding cultivar from Maharashtra, India, which has a very rapid rate of leaf senescence (Van Oosterom et al., 1996). Confirmation of the genotype of RSG 03123 is described in Galyuon et al. (2016). This indicated that *Stg1*, *Stg3* and *Stg4* had been successfully transferred from B35 but that *Stg2* was not present in RSG 03123.

Twenty (20) plants per genotype were raised in plastic pots with perforated bottoms filled with a mixture of black soil, peat, grit and perlite in a ratio of 3:3:3:1, respectively. They were grown in a glasshouse in Aberystwyth with daily maximum and minimum temperature of 35°C (day) and 18°C (night). Plants were exposed to a 12-h supplementary light to simulate the conditions in sorghum growing areas in Africa and Asia.

At flowering, when at least 50% of the plants for each genotype had visible anthers at the top section of the panicle, half of the plants remained well-watered (WW), by adding 1000 ml of water daily. The other 50% were exposed to water-limited (WL) conditions by supplying them with 250 ml of water daily. For the stay-green trait to be expressed sufficiently to be used for selection, a prolonged drought period is required during grain-filling enough to accelerate senescence but not sufficient to cause premature death of plants (Mahalakshmi and Bidinger, 2002). Preliminary studies indicated that the drought treatment used in this study enabled the trait to be expressed and prevented premature plant death.

Measurement of plant height

Plant height was measured at flowering from the soil surface to the collar of the flag leaf, and, at physiological maturity, it was measured from the soil surface to the tip of the panicle.

Determination of green leaf area (GLA)

Green leaf area (GLA) per plant was determined weekly as described by Wolfe et al. (1988). The length of each leaf (from the collar to the tip) along the midrib was measured and the width determined at the middle, the widest part of ensiform leaves (Doggett, 1988). At flowering, leaves were harvested from four plants of each cultivar and leaf area per plant measured using a Delta-T Area Meter (MK II). The linear relationship between the product of the length and width and the size measured using the area meter was determined. The relationship between the product of the length × width and the size measured using the area meter from 180 leaves was determined as $y = 0.95 + 0.75x$ with an R^2 = 94.7% ($P<0.001$), where y is the area measured using the area meter and x the product of the measured length and width for each leaf. This relationship was then used to determine the area for each leaf. The area of each leaf was corrected for senescence by subtracting the area of the lamina lost to senescence defined by visible yellowing using a percent score visually.

Determination of chlorophyll levels

A chlorophyll meter (Minolta Chlorophyll Meter SPAD-502, Minolta Camera Co., Ltd., Japan) was used to measure leaf chlorophyll levels weekly beginning at flowering. Each leaf was divided into three sections, base, middle and top. Within each section, six readings were taken, three on either side of the midrib and averaged.

Measurement of leaf gas exchange

Gas exchange was measured weekly beginning at flowering on the middle section of the fourth leaf, the flag leaf. CO_2 assimilation rates (*A*), leaf conductance (g_L) and transpiration rates (*E*) were measured on 5.6 cm^2 of leaf lamina using an open portable gas exchange system (CIRAS-1, PP Systems, Hitchin Herts, UK). Cuvettes were maintained at 30°C, with an ambient CO_2 concentration of 350 $\mu L.L^{-1}$ and exposed to photosynthetically active radiation (PAR) of 1100 $\mu mol\ m^{-2}\ s^{-1}$ from a LED lamp fitted and connected to light meter. Measurements were taken between 8:00 and 13.00 h. Photosynthetic water use efficiency (WUEL) was calculated by dividing CO_2 assimilation rate (*A*) by the transpiration rate (*E*) for each leaf (that is, *A*/*E*).

Measurement of chlorophyll fluorescence

Chlorophyll fluorescence was measured weekly on the same leaves used for gas exchange. An EARS Plant Photosynthetic Measurement (PPM) System (EARS Earth Environment Monitoring B.V. Kanaalweg 1, 2628EB Delft, The Netherlands) was used to measure the quantum yield of photosynthetic electron transport of photosystem II (ΦPSII) as described by Maxwell and Johnson (2000) in light-adapted leaves. Three readings were taken between the collar and tip of the leaf at equal spacing and averaged.

Analysis of basal soluble carbohydrates (SC)

At flowering and physiological maturity, samples were taken from the middle section of the leaf, and fresh weight, length and width were recorded. Stem samples were taken from the fourth internode

above the soil surface. Sugars were extracted using 80% ethanol according to a method described by Cairns et al. (2002). Total SC (TSC) extracts for both leaf and stem samples were freeze-dried for 48 h, re-dissolved in 500 µl sorbitol (40 µg/µl; internal standard) sucrose, glucose and fructose contents in the samples were analysed using an HPLC system with an Autosampler (DIONEX ASI-100), a carbohydrate analysis column [Bio-Rad, Aminex HPX87C column (125-0095)], a guard column (Bio-Rad, MicroGuard Carbo-C) and an Alltech (UK) Rheodyne Filter (3 mm). The system was run by DIONEX Chromeleon software (version 6.10) at a flow rate of 0.6 ml/min for 25 min for each sample using membrane-filtered [Nitrocellulose (0.42 µm), Whatman Filters] distilled water (82°C) as eluant.

Analysis of leaf nitrogen (N) content

Leaf samples were taken from the top four leaves of each plant, at flowering and physiological maturity, and ground into fine powder using a Retsch MM300 Mixer mill. The percentage of N in a known weight of the ground sample was determined by mass spectrometry. The amount of N (µg) per mg of dry weight (DW) of leaf sample was calculated along with the specific leaf nitrogen which is the quantity of N per unit leaf area.

Protein extraction, analysis of chloroplast proteins and photosynthetic enzymes

Total protein was extracted as described by Mae et al. (1993) at flowering and physiological maturity. Tissue was homogenised with ice-cold buffer (50 mM lithium phosphate pH 7.2 containing 120 mM 2-mercaptoethanol, 1 mM sodium monoiodoacetate, 1 mM phenylmethylsulfonyl-fluoride and 5% v/v glycerol) in the proportion of 5 ml g^{-1} fresh weight using a pestle and mortar. Lithium dodecyl sulphate was added to a final concentration of 2% (w/v) and the mixture incubated for 5 min at 100°C. The extract was centrifuged for 10 min at 13000 g and aliquots of the supernatant were stored at -80°C until analysed by electrophoresis.

One-dimensional sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using a 12.5% running gel in a Bio-Rad Mini-protean system. Gels were loaded with equal volumes of homogenate, equivalent to equal fresh weights of tissue. Protein standards ranging from 113 to 20.9 kDa (Bio-Rad) were run alongside the samples on each gel.

Western analysis was conducted following the electrotransfer of proteins onto nitrocellulose paper and specific proteins detected using antibodies. The blots were then developed chemiluminescently using BM Chemiluminescence Blotting Substrate (POD) (Roche Applied Science, Germany) and visualised on x-ray film. The following publications give the sources of antibodies used in this study: Hilditch et al. (1989), Bachmann et al. (1994) and Borland et al. (1998).

Data analysis

The data were checked by calculating the means and standard errors and then subjected to analysis of variance (ANOVA) using MINITAB (Release 13) statistical software. Data for all cultivars were combined and analysed to find out if there were any interactions between genotypes, drought stress and duration of drought stress. Further, differences between means of treatment combinations were analysed for each sampling date. Differences between means were separated by Fisher's least significance difference (LSD) at 5% level of significance.

RESULTS

Plant height and flowering

RSG 03123 plants were 198.1 and 192.8 cm tall at physiological maturity under WW and WL conditions, respectively. Similarly, R16 plants were 172.4 and 178.6 cm tall, while B35 pants were 118.8 and 92.4 cm tall at physiological maturity under WW and WL conditions. Within genotypes there were no significant ($P>0.05$) differences between the WW and WL plants. The differences between RSG 03123 and R16 plants were also not significant ($P>0.05$). However, compared to B35, both RSG 03123 and R16 were significantly ($P<0.05$) taller under WW and WL indicating that the introgression of the SG QTL from B35 did not change plant height. Days to flowering were the same in R16 and RSG (67 days after seedling emergence) whereas flowering was eight days later in B35.

GLA retention

Water limitation reduced GLA compared to the WW plants in all genotypes with the greatest reduction in R16 and the smallest reduction in B35 (Figure 1A). The difference between the WW and WL plants was not significant ($P>0.05$) for either B35 or RSG 03123. In R16, GLA was significantly ($P<0.05$) reduced in the WL plants compared to the WW from 14 DAF. GLA retention (GLA as percent of the total leaf area at flowering per plant or %GLA) was also lower under WL conditions. B35 plants had the highest %GLA under both WW and WL conditions throughout the experiment (Figure 1B). The stay-green trait was clearly expressed in B35 with GLA declining after 21 DAF although the rate of loss was slightly greater in WL plants. By physiological maturity GLA in the WL B35 plants was 91% of that in the WW. The WW plants of R16 and RSG retained similar GLA. However, under WL conditions, retention of GLA was significantly ($P<0.05$) reduced in the R16 plants compared with the WW. At physiological maturity, the WL R16 plants retained 29% GLA compared to the WW. In the RSG plants, even though %GLA was lower under WL conditions, the differences were not significant ($P>0.05$) compared with the WW and at physiological maturity GLA in the WL was 60% of that in the WW.

Differences were also apparent in the onset and rate of senescence both between genotypes and watering regimes. A split linear regression was conducted on the %GLA data and from this the duration to 95% GLA (onset of senescence) and the rate of senescence were calculated. Under WW conditions, days to 95% GLA were 29.60, 9.50 and 6.33, respectively, in B35, R16 and RSG 03123 indicating that for both R16 and RSG, onset of senescence was much earlier than in B35. The values for the WL plants were 27.10 for B35, 2.47 for R16 and 3.99 for RSG 03123, indicating that in all 3 genotypes WL

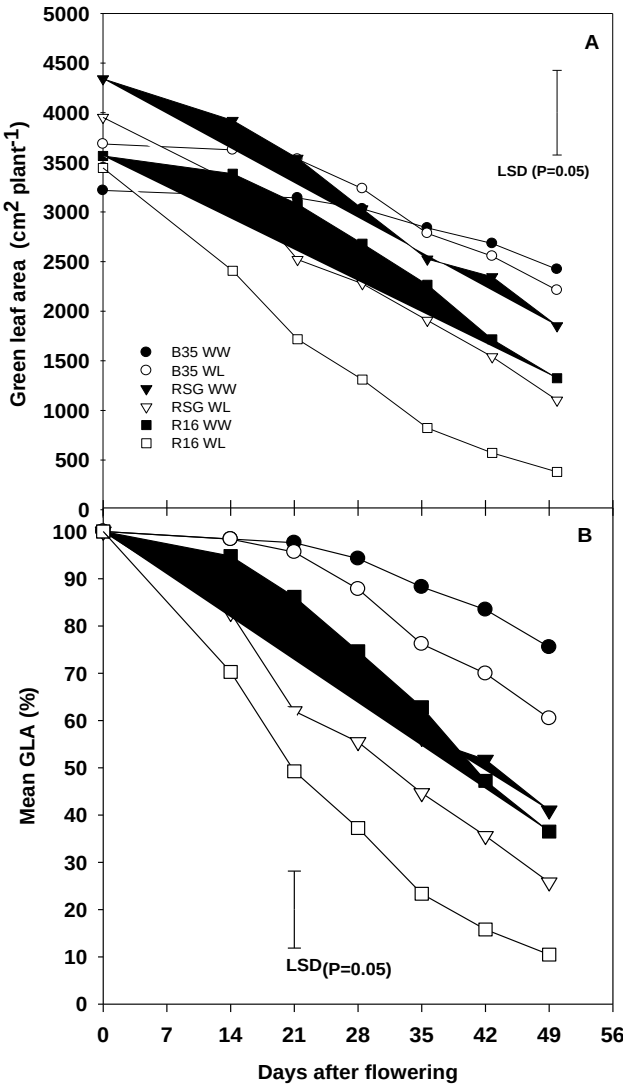

Figure 1. Green leaf area (GLA) retention per plant from flowering to physiological maturity (A) and %GLA per plant relative to GLA at flowering (B) in well-watered (WW) and water-limited (WL) plants. Values are means of 4 replicates.

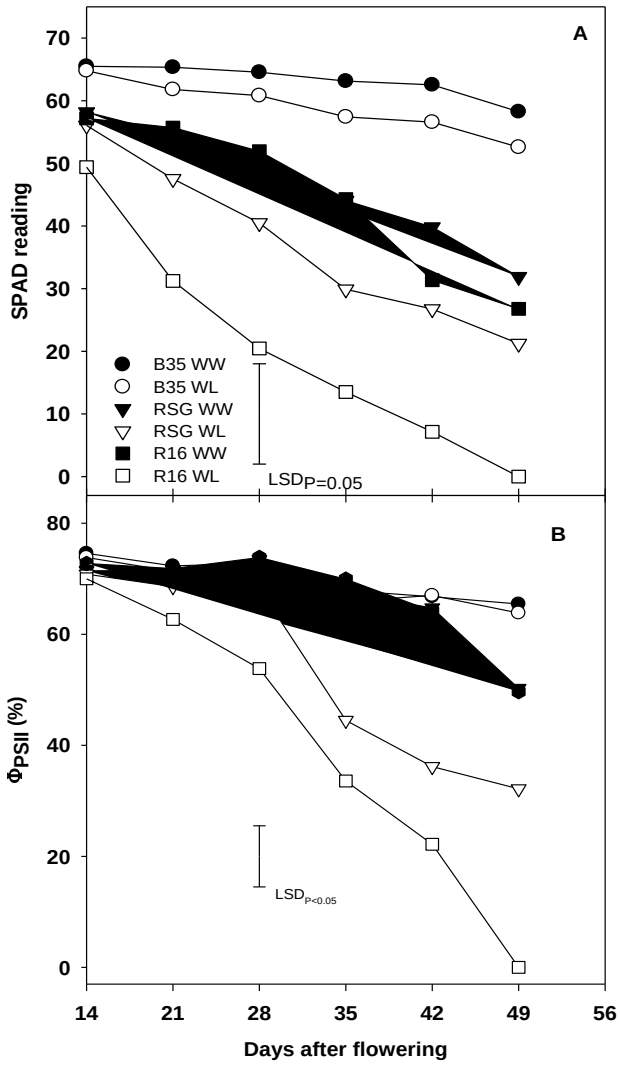

Figure 2. Chlorophyll levels determined by SPAD readings (A) and photochemical efficiency of photosystem II (ΦPSII) on whole leaf (A) from 14 to 56 DAF in well-watered (WW) and water-limited (WL) plants. Values are means of 4 replicates.

conditions resulted in an earlier onset of senescence. The onset of senescence was similar in RSG and R16. The rates of senescence per day were 0.87, 1.71 and 1.39 for B35, R16 and RSG 03123, respectively, under WW conditions, while under WL conditions the corresponding values were 1.26, 2.22 and 1.51. Thus, once senescence had started, the rate of senescence was greater in all three genotypes under WL conditions but the rate of leaf loss was slower in RSG 03123 than in R16.

Chlorophyll retention

Chlorophyll retention in the fourth leaf from the top, as measured by SPAD, was highest in B35 followed by RSG 03123 and the lowest in R16 under WL conditions (Figure 2A). Chlorophyll levels were significantly ($P<0.05$) higher in B35 compared with RSG 03123 or R16 under both WW and WL conditions. RSG 03123 also had significantly ($P<0.05$) higher chlorophyll levels than R16 from 21 DAF under stress conditions, whereas there were no differences between RSG 03123 and R16 when well-watered. In addition, chlorophyll levels in R16 were significantly ($P<0.05$) reduced by drought stress whereas in both B35 and RSG 03123, the differences between the WW and WL plants were not significant ($P>0.05$). Under WL conditions, the rate of chlorophyll loss also was more gradual in B35 and RSG 03123 compared to R16.

Quantum efficiency of photosystem II (ΦPSII)

ΦPSII was reduced in the WL plants of R16 and RSG 03123 (Figure 2B). The onset was earlier and magnitude of reduction greater in the WL R16 plants, where ΦPSII continually fell from 14 DAF and the reduction was significant (P<0.05) compared to the WW. In the WL RSG 03123 plants, ΦPSII began reducing from 28 DAF and by 35 DAF the reduction (33%) was significant (P<0.05) compared with the WW plants. In contrast, in B35 there were no differences between the WL and WW plants. ΦPSII in the B35 plants was higher than in either RSG or R16 under WL conditions. Under WW conditions, however, there were no differences between genotypes, except at physiological maturity when ΦPSII was significantly (P<0.05) higher in B35. Hence, for both absolute values and in comparison with the WW plants, ΦPSII in B35 was the highest under both WW and WL conditions, RSG 03123 was intermediate and R16 was worst, particularly, under WL conditions.

Leaf gas exchange

Carbon dioxide (CO_2) assimilation rate

CO_2 assimilation rate (*A*) was reduced in all WL plants and the lowest in R16 (Figure 3A). Cultivar × treatment, and cultivar differences were significant (P<0.05). Overall, *A* in the WL R16 plants was reduced by 70% compared to the WW, whereas in the WL RSG 03123 and B35 plants the reductions in *A* were 32% and 19%, respectively. *A* was significantly (P<0.05) reduced in the WL R16 plants compared to the WW from 14 to 49 DAF. Indeed by 42 DAF *A* in R16 was reduced to zero. The reduction of *A* in the WL plants of both RSG 03123 and B35 was not significant (P>0.05) compared to their respective WW ones. For R16 *A* was significantly (P<0.05) reduced in the WL plants compared to the WW. Among the WW plants, *A* was similar, except at 14 and 49 DAF. The higher *A* in both B35 and RSG 03123 plants is consistent with the higher ΦPSII observed in these plants than in R16 under WL conditions. This confirms that under WL conditions photosynthesis was improved in the RSG 03123 plants as compared to R16.

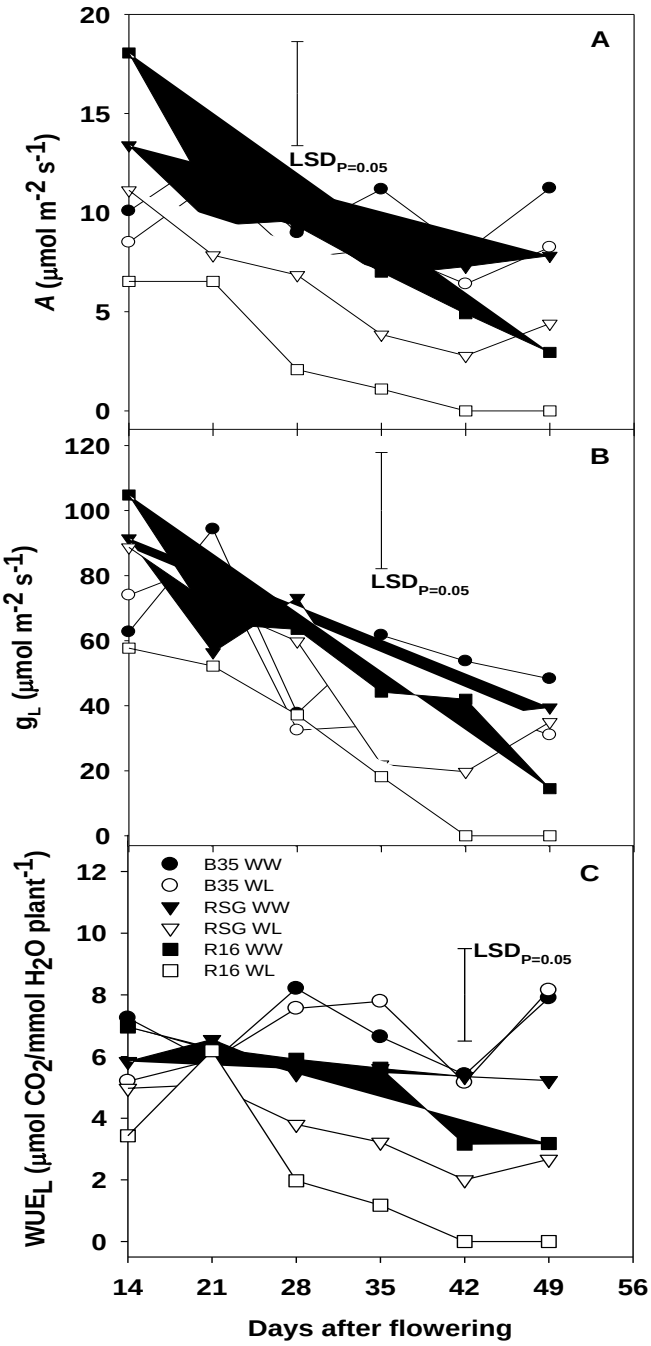

Figure 3. Carbon dioxide assimilation rate (A), stomatal conductance (B) and photosynthetic water use efficiency (C) from 14 to 56 DAF in well-watered (WW) and water-limited (WL) plants. Values are means of 4 replicates.

Leaf conductance (gL)

Overall, gL was reduced by 50, 14 and 15% in the WL plants of R16, RSG 03123 and B35 compared with the WW. In the WL R16 plants gL continually declined from 14 to 42 DAF when it became zero (Figure 3B). In RSG 03123, the rate of decline was similar to R16, but gL stayed higher and the decline stopped at 35 DAF. For most sampling dates, gL in B35 and RSG 03123 were higher than in R16 under drought stress and this increased conductance could partially be responsible for the higher *A* in RSG 03123 and B35. However, the differences were not significant (P>0.05) when compared with the WW in all genotypes. Overall differences between genotypes were significant (P<0.05) with the highest g_L in B35 followed by RSG 03123 and the lowest

in R16. This indicates that small differences in gL can result in large differences in *A*, particularly under WL conditions.

Transpiration water use efficiency (WUE_L)

WUE_L, computed as *A*/*E*, was reduced by drought stress, except in B35 and the reduction was greatest in the WL R16 plants (Figure 3C). It was significantly (P<0.05) reduced in the WL R16 plants compared to the WW from 28 DAF. For B35, WUE_Lin both WW and WL plants were similar at all sampling dates. Even though WUE_L in RSG 03123 was lower in the WL plants than in the WW, the differences were not significant (P>0.05). It was also higher in both RSG 03123 and B35 than in R16 under WL conditions.

These findings also indicate that WUE_L was improved in the RSG 03123 plants compared to R16 under WL conditions. Also, for both B35 and RSG 03123, WUEL was high due to a much higher *A* values over *E* rather than reduced *E* caused by reduced g_L. In R16, however, reductions in both *A* and *E* and, consequently, WUEL followed a reduction in g_L. Changes of WUE_L as those in the WL RSG 03123 and B35 plants should have beneficial effects for grain yield under drought stress, since water would be economically used and photosynthates made available for grain filling.

Changes in leaf and stem total soluble carbohydrates (TSC)

At flowering and physiological maturity, leaf TSC was highest in B35 followed by RSG 03123 and then R16 under both WW and WL conditions (Figure 4A). At physiological maturity, leaf TSC was reduced by 36% in R16 when grown under WL conditions as compared to WW, whereas in RSG 03123 and B35 the reduction was just 12% and 4%, respectively, between the WW and WL plants.

TSC in the stem increased in all genotypes between flowering and physiological maturity (Figure 4B). This increase was greatest in B35 under both WW and WL conditions. The amounts of TSC in the stem were unaffected by limitation of water supply in both B35 and RSG 03123, whereas in R16 they were reduced by 34% in the WL plants compared to WW.

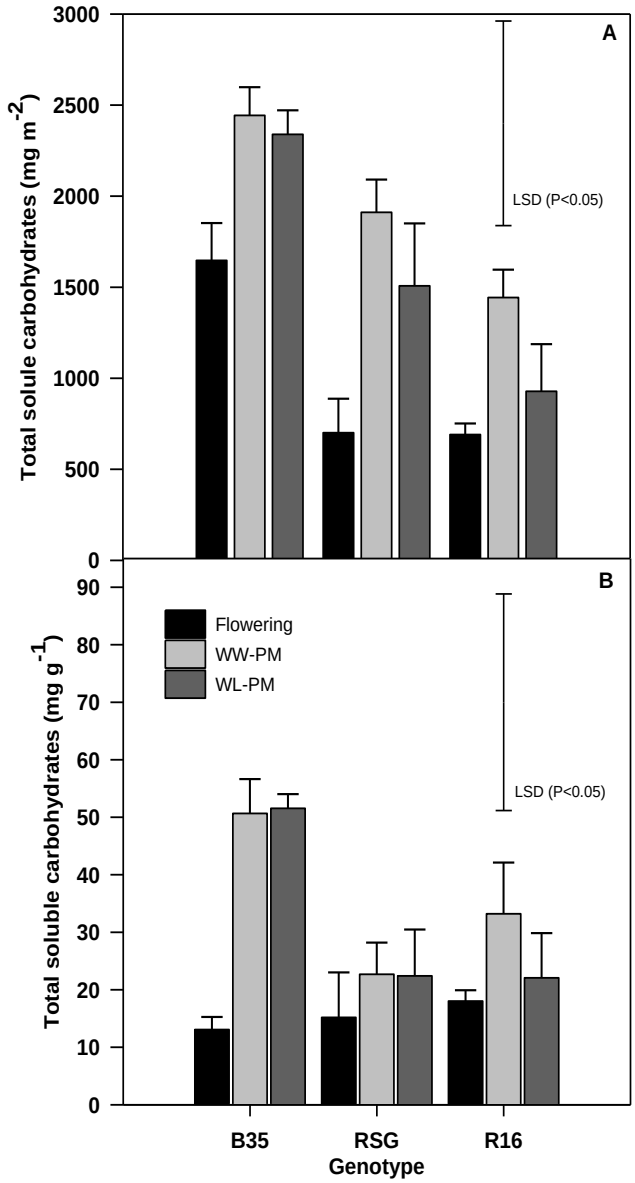

Figure 4. Total soluble carbohydrates in the leaf (A) and stem (B) at flowering and physiological maturity under well-watered (WL) and water-limited conditions. Values are means 4 replicates ± SEM.

Specific leaf nitrogen (SLN)

SLN was higher in both RSG 03123 and R16 compared to B35 at flowering (Figure 5). At physiological maturity under both WW and WL conditions, B35 maintained a much higher SLN than both RSG and R16. Under WW conditions, both R16 and RSG had a SLN of 45% at flowering. However, under WL conditions, SLN was greatly reduced in R16 (61% as compared to that in WW conditions), whereas in both B35 and RSG 03123, SLN was 88% of the WW value.

Changes in photosynthetic proteins

For most of the proteins examined, all four replicates had similar detectable amounts in any given genotype at flowering (Figure 6). There were considerable differences between genotypes at physiological maturity. The light-harvesting chlorophyll-binding proteins of photosystem II

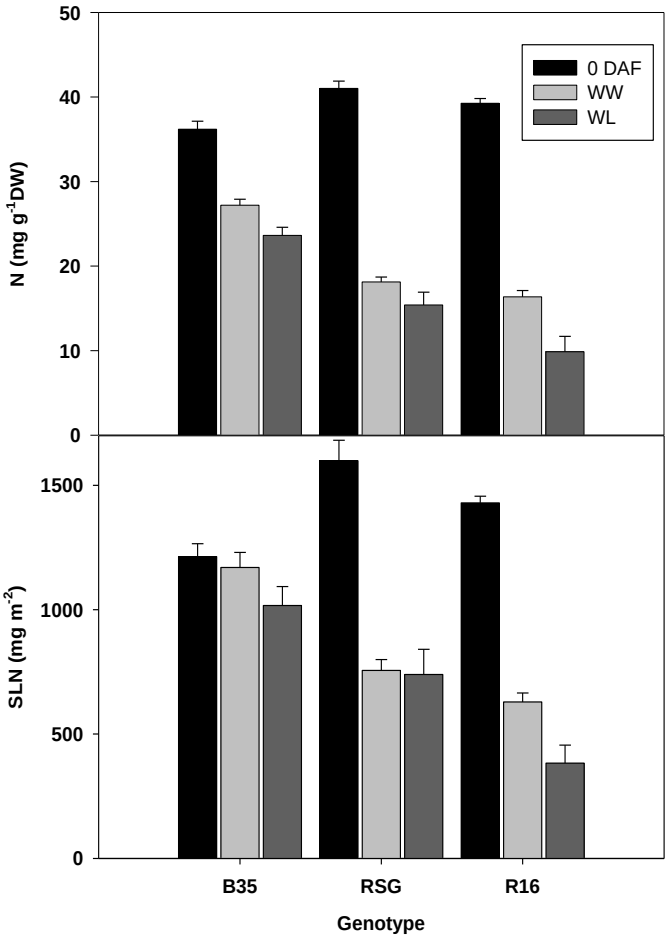

Figure 5. Mean leaf nitrogen content (A) and specific leaf nitrogen (SLN; B) of the top four leaves at flowering (0 DAF) and at physiological maturity in well-watered (WW) and water-limited (WL) plants of B35, RSG30123 (RSG) and R16.

(LHCPII) did not differ much in the amounts at flowering and physiological maturity in B35 for all 4 leaves in the WW and WL plants. A similar result was found for RSG 03123. In R16, LHCPII abundance was reduced at physiological maturity and the reduction was greater in the WL plants, especially in leaf 4.

The amounts of the 33 kDa oxygen-evolving complex of PSII (OEC33) at flowering were greater in B35 and RSG 03123 than in R16. At physiological maturity the amounts were reduced in all genotypes but the reductions were greater in R16 under either WW or WL conditions. Under WW conditions the amounts of OEC33 were higher in all leaves of RSG 03123 compared to B35, while there were no differences between them under WL conditions. For R16, the reductions were much greater in the WL plants with barely detectable amounts in leaves 3 and 4.

B35 had the highest amounts of phospho*enol*pyruvate carboxylase (PEPC) at flowering and physiological maturity followed by RSG 03123. PEPC did not change

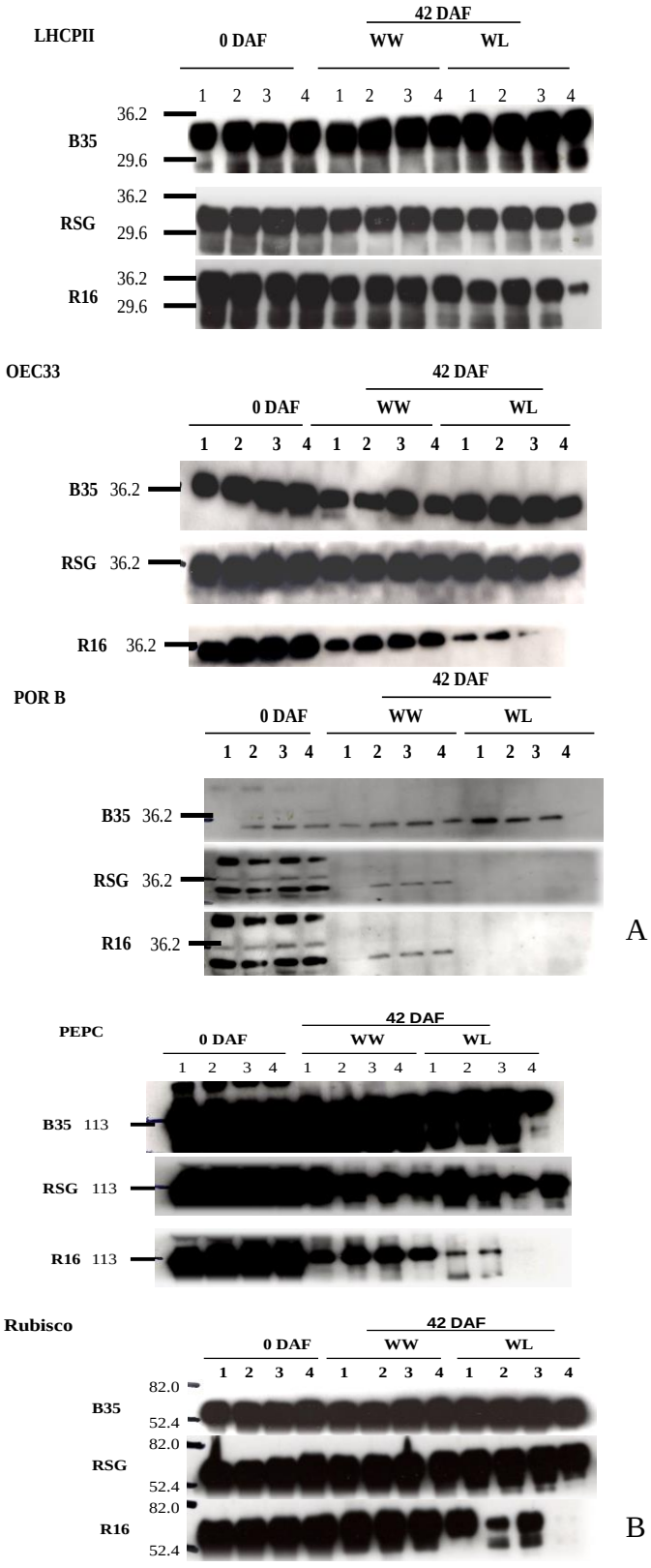

Figure 6. (A) Abundance of LHCPII, OEC33 and POR B in the top four leaves of well-watered (WW) and water-limited (WL) plant at flowering (0) and 42 DAF. (B) Abundance of PEPC and Rubisco in the top four leaves of well-watered (WW) and water-limited (WL) plants at flowering (0 DAF) and 42 DAF.

much between flowering and physiological maturity in B35, except in leaf 4 of the WL plants. At physiological maturity the bands of PEPC in B35 were about two times as large as in RSG 03123 for the same leaves under both WW and WL conditions. Similarly, RSG 03123 plants had about two times the amounts in R16 under WW conditions and much more than R16 under WL conditions. Leaves 3 and 4 of the WL R16 plants had no detectable bands and the intensity of the bands for leaves 1 and 2 was very low compared to those for RSG 03123.

The amounts of the large subunit of Rubisco did not change much between flowering and physiological maturity in both B35 and RSG 03123 in either WW or WL conditions or for any leaf sampled with very little differences between them (Figure 6B). R16 plants had similar amounts of Rubisco as in RSG 03123 at flowering; however, at physiological maturity, the amount in the WL plants of R16 was highly reduced compared to the WW. Indeed, in leaves 3 and 4 of the WL plants of R16 there were no bands for Rubisco. Thus, the introgressed QTL from B35 improved the retention of PEPC and Rubisco also in RSG 03123. This could, partially, explain the maintenance of higher CO_2 assimilation rates in B35 and RSG 03123 compared to R16 as stated earlier.

The amounts of protochlorophyllide-oxidoreductase B (POR B) at flowering were similar in R16 and RSG 03123 in all 4 leaves. However, at physiological maturity the level of POR B was reduced in all leaves of RSG 03123 and R16 while it was increased in B35. In B35 the WL plants had the higher amounts, except for leaf 4, whilst in RSG 03123 and R16 the amounts were greater in the WW plants. Since POR B is involved in chlorophyll synthesis, these findings thus confirm the higher chlorophyll levels in B35 and RSG 03123 than R16.

DISCUSSION

Plant height and flowering

Slight changes in the genetic code, which include simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs), are directly linked to phenotypic differences (Barth et al., 2016). It is important that introgression of SG QTL does not have adverse effects on other aspects of the phenotype. The height and flowering time of RSG 03123 plants were not significantly different from the recurrent R16 parent, which is much taller than B35 at physiological maturity. Thus, in regions where stover is as important economically as the grain, introgression of SG QTL from a dwarf genotype, such as B35, could enhance survival in a tall genotype without affecting stover production.

Green leaf area duration and chlorophyll retention

Senescence is an endogenously controlled degenerative biochemical and physiological set of events comprising the final stages of leaf development from the mature fully expanded state to death (Verma et al., 2003). Drought stress during grain-filling hastens leaf senescence leading to premature death (Rosenow and Clark, 1981). However, stay-green sorghum genotypes retain more green leaf area than do genotypes not possessing this trait, and they also continue to fill grain normally under drought conditions (Rosenow et al., 1983; Borrell et al., 2014a,b). It has been argued that the extent of the effect of SG QTL on canopy development varies with environmental and management conditions experience by the crop (Borrell et al., 2014b). Green leaf area (GLA) at physiological maturity has proved to be an excellent indicator of stay-green, and has been successfully used to select drought-resistant sorghum genotypes in the USA (Rosenow et al., 1983) and in Australia (Henzell et al., 1992). Key components determining GLA at physiological maturity are: (i) maximum green leaf area at flowering, (ii) the timing of the onset of senescence, and (iii) rate of leaf senescence (Borrell et al., 2000). Genetic variation in the inheritance of all three components was reported (Van Oosterom et al., 1996; Badigannavar et al., 2018; Boyles et al., 2019). Van Oosterom et al. (1996) for instance concluded that the inheritance of the onset of senescence was additive, whereas the inheritance of the rate of senescence was completely dominant for a slow rate. Therefore, the relative green leaf area duration, being the sum of an additively and a dominantly inherited trait, displayed partial dominance for a large green leaf area duration.

The current results confirm the stay-green nature of B35 and the senescent nature of the recurrent parent, R16 (Blümmel et al., 2015). In the absence of stress, RSG 03123 displayed a similar senescence pattern to R16. However, under WL conditions, RSG 03123 retained significantly more GLA than the R16 parental line. This confirms that marker-assisted transfer of QTL identified for delayed-leaf-senescence from B35 to R16 was able to improve the retention of GLA in a senescent background. Thomas and Howarth (2000) have described how a stay-green phenotype could arise in a number of fundamentally distinct ways. In type A stay-greens, senescence is initiated late but then proceeds at a normal rate. Type B stay-greens initiate senescence on schedule but thereafter senesce comparatively slowly. This could arise from different genes being involved in the onset of senescence and in the regulation of its rate of progress. B35 displayed a delayed onset of senescence (Type A stay-green) under both WW and WL conditions. The onset of senescence in RSG 03123, however, was much earlier than in B35 under both WW and WL conditions suggesting that either the region(s) of the genome controlling this aspect of senescence was not introgressed from B35 or that it was not functional for some reason in RSG 03123. The onset of senescence in RSG 03123 was more similar to the recurrent parent,

R16, but the rate of senescence was slower than R16 suggesting that RSG 03123 behaved like a type B stay-green. The differences in GLA discussed earlier were partly due to differences in chlorophyll loss. Hence, genotypes with reduced chlorophyll loss also had high GLA and/or retained higher proportion of GLA. This also confirms the improvement of GLA retention in RSG 03123 over R16. The findings of this study complements that of Harris et al. (2007) in which the retention of GLA under post-flowering drought conditions in the field was determined using near isogenic lines (NILs) developed by marker-assisted introgression of B35 SG QTL into the senescent RTx7000 background. Harris et al. (2007) found that *Stg1, Stg2, Stg3* or *Stg4* individually contributed to the SG phenotype but that NILs containing B35 DNA corresponding to *Stg2* retained more green leaf area at maturity under terminal drought stress conditions than the recurrent parent RTx7000 of the other RTx7000 NILs.

The stay-green parent, B35 was not included in the study of Harris et al. (2007); but, in a previous study, it was shown that B35 exhibited delayed onset of senescence (Borrell et al., 2000). Xu et al. (2000a) also reported that under post-flowering drought stress conditions, B35 showed a much higher retention of chlorophyll content than that of the senescent parent Tx7000 at physiological maturity. A similar study carried out by Kassahun et al. (2010) found majority of the introgression lines with stay-green QTL from B35 having higher levels of leaf chlorophyll, which is a distinctive trait of the donor parent B35, than the senescent parent, R16. They also observed that the introgression lines also had a greater percentage GLA during the part of grain-filling than did the R16 recurrent parent. The present findings further confirm the findings of Kassahun et al. (2010) that none of the QTL introgression lines achieved the same level of stay-green as B35.

Visual scoring though easy and quick, particularly, when dealing with large numbers of plants in the field, is subject to individual biases and differences in ratings among observers (Rosenow, 1984). A visual rating of stay-green is limited by an inability to distinguish among the various mechanisms that ultimately determine the phenotype. Loss of chlorophyll is a prominent feature in post-flowering drought-induced senescence in leaves and stalks of sorghum. Thus, measurement of chlorophyll levels with time is an alternative method of indicating the onset and rate of senescence in leaves. SPAD values for sorghum have been found to be highly correlated with total leaf chlorophyll determined by spectrophotometry (Xu et al., 2000a). Apart from being non-destructive, SPAD measurements are easy and quick, especially for assessing large number of plants in the field. Chlorophyll concentration is also correlated with leaf nitrogen, which, in turn, is correlated with leaf longevity as well as grain yield (Blackmer and Schepers, 1995; Borrell et al., 2001). Hence, a SPAD meter can be used in the field for easy quantification of stay-green. Therefore, the present results also indicate that, not only is green leaf area duration extended in the RSG 03123, leaf nitrogen remobilization was also delayed compared to the R16 parent.

Chlorophyll fluorescence

Stay-green genotypes continue to fill grain normally under drought conditions (Rosenow et al., 1983). This suggests that they also maintain more photosynthetically active leaves under post-flowering drought stress and this is the first direct confirmation that the stay-green photosynthetic advantage can be transferred to a highly productive but senescent (R16) background. Chlorophyll fluorescence can be used as a tool for assessing the effect of stress on leaf photosynthesis. The reduction of Fv/Fm and ΦPSII indicates a reduction in photochemical efficiency of photosystem II (Maxwell and Johnson, 2000). ΦPSII in the RSG 03123 line was higher than in R16, but lower than in B35, for all sections of the leaf under drought stress, while there were no differences between RSG 03123 and R16 under WW conditions. Efficiency of the PSII antennae (Fv'/Fm') (Lu and Zhang, 1999) and ΦPSII (Lu and Zhang, 1999; Martinez et al., 2003) were reduced in drought-stressed wheat plants, whereas Fv/Fm was unaffected (Lu and Zhang, 1999). They also found that the reductions in ΦPSII and Fv'/Fm' were greater under severe drought stress. Furthermore, the decline of ΦPSII sets in earlier and is faster in the non-stay-green genotypes of wheat than in the stay-green genotypes (Spano et al., 2003). Similar findings were observed in the WL R16 in this study, where ΦPSII declined at a faster rate than in either RSG 03123 or B35.

Improved photosynthesis leads to better drought tolerance (Vadez et al., 2013). Photosynthesis depends on the functioning of light-harvesting and electron transport systems of the chloroplast, which is indicated by the photochemical efficiency of ΦPSII. Loss of efficiency of PSII can be attributed to the breakdown of proteins of PSII and photosynthetic enzymes and the destruction of membranes by lipid degradation (Thomas, 1987). Thus, in plants such as B35 and RSG 03123, photosynthetic efficiency was retained longer than in R16. This is indicative of higher CO_2 assimilation in RSG 03123 and B35 compared to R16 plants under WL conditions.

Leaf gas exchange

Thomas and Howarth (2000) have described five types of the stay-green trait, three being cosmetic and two being functional. Maintenance of green leaf area at physiological maturity in sorghum correlates positively with grain yield and negatively with senescence (Borrell et al., 2000, Kassahun et al., 2010). Such findings

indicate that the trait in sorghum is functional; however, actual CO_2 assimilation rates have not previously been reported. Drought stress causes drastic reduction in photosynthesis and growth due to stomatal closure and associated changes in carbon and nitrogen mobilization (Badigannavar et al., 2018). CO_2 assimilation rate (*A*) declines at moderate drought stress or even before leaf water status is changed in response to a reduction in soil water potential (Gollan et al., 1986). The limitation of leaf conductance (gL) to *A* depends on the severity of drought stress (Yordanov et al., 2003). Reduction of gL is the primary regulator of *A* under mild drought stress (Cornic and Briantais, 1991). *A* and gL were generally lower in RSG 03123 compared to B35 under both WW and WL conditions, however, the differences were not significant ($P>0.05$). Furthermore, just as in B35, the differences between the WW and WL plants were not significant ($P<0.05$) in RSG 03123 either. Both *A* and gL were also higher in RSG 03123 than in R16 and the rate of decline was slower in RSG 03123 under WL conditions, but under WW conditions there were no differences between them. The reductions in *A* were partially due to reductions in gL, as well as leaf senescence or chlorophyll loss since *A* was reduced with a decline in g_L and an increase in senescence. The current findings are similar to those reported for wheat mutants with the stay-green trait, which had longer photosynthetic activity than the non-stay-green wild types (Spano et al., 2003).

Increased water use efficiency is of great interest to growers so that yields are maximised for available water supply in each growing season (Sinclair and Muchow, 2001). In water-limited environments, such as the arid and semi-arid regions, plant productivity is determined by the amount of water available and efficiency of its use by the plant (Xu and Hsiao, 2004). A reduction in WUE_L with stomatal closure indicates that carbon metabolism and stomata limit photosynthesis as has been observed in sweet sorghum (Massacci et al., 1996). WUE_L in RSG 03123 and R16 did not differ under WW conditions, but under WL conditions it was higher in RSG 03123. In these plants WUE_L also declined with reductions in stomatal conductance and transpiration rate indicating that both carbon metabolism and stomata limited *A*. WUE_L is higher in drought tolerant plants than in susceptible ones (Yordanov et al., 2001), indicating that B35 was drought tolerant. Similarly, RSG 03123 was more tolerant to WL conditions than R16. Differential gene expressions could account for the differences in tolerance to water-limited conditions. For instance, delta-1-proline-5-carboxylate synthase 2 (P5CS2) is highly expressed in B35 compared to R16, which correlated with high proline level in the SG line (Johnson et al., 2015). This may be responsible for a better tolerance to drought by SG genotypes than the senescent one. The enhanced water use efficiency conferred by SG QTL may be due to roots exploiting a larger volume of soil, which could enhance better uptake of water (Manschadi et al., 2006). Sorghum, maize and rice plants can reduce water loss by enhancing water uptake through profuse root proliferation (Badigannavar et al., 2018). Indeed, evidence exists that, in particular, the *Stg4* SG QTL in sorghum co-locates with qRA1_5, a QTL for nodal root angle, indicating that root architecture can be a component of increased water use as found in stay-green near-isogenic lines (Mace et al., 2012; Borrell et al., 2014b). The current study did not look at root characteristics since root growth is restricted in pot experiments. Changes in WUE_L reflect changes in stomatal conductance as well as the internal capacity for CO_2 fixation, which is affected by enzyme activity (von Caemmerer et al., 1997) and nutrient status (Payne et al., 1992). In pearl millet, WUE_L was found to increase with high soil phosphorus (P), but did not change with low P under drought stress (Brück et al., 2000). In this study, however, growing plants in the same growth medium eliminated differences in soil nutrients, even though differences in nutrient requirements and extraction could have led to differences in nutrient depletion in the growth medium. The differences in WUE_L observed in this study appeared to have been influenced by differences in stomatal movements, rate of senescence and, maybe, enzyme activity (discussed later). The results show clearly that RSG 03123 was intermediate between B35 and R16 with improved performance compared to R16.

Metabolism of total soluble carbohydrates (TSC) in the leaf

Moderate drought stress can lead to increased concentration of TSC in the leaf, while severe drought stress can result in a constant concentration of SC in leaves, in spite of low *A*, because of the concomitant limitation of growth and export (Pinheiro et al., 2001). TSC in the leaf was reduced by more than a third in R16, whereas the reduction in RSG 03123 was one-eighth due to limitation of water availability. In B35 there was no difference between the WL and WW plants. These results indicate that in R16, the reduction in *A* due to limited water availability also resulted in a decrease in TSC. Similarly, TSC in RSG 03123 was lower than in B35 which had a higher *A* under WL conditions. TSC in the leaves of the plants grown under WL conditions reflected the reduction in photosynthesis in those leaves.

TSC metabolism in the stem

The principal non-structural carbohydrate stored by sorghum in the stem is sucrose, which accumulates after anthesis (McBee and Miller, 1982). Non-senescent sorghum cultivars accumulate more non-structural carbohydrates in the stem than senescent cultivars after anthesis (McBee et al., 1983; Vietor et al., 1990; McBee

and Miller, 1993). The reduction in current assimilation due to leaf senescence during grain-filling in senescent cultivars has the potential to induce a greater stem reserve mobilization to, and utilization by, the grain; thus, one might expect higher non-structural carbohydrates in the stem of stay-green cultivars than in senescent ones. In the current study, TSC in the stem increased between flowering and physiological maturity. In B35 and RSG 03123, there was no significant difference between the amounts of TSC at physiological maturity in plants grown under WW or WL conditions. However, in R16 plants grown under WL conditions, TSC was reduced by 34% compared to the WW. This finding corroborates the earlier observations reported earlier. The introgression of the stay-green QTL from B35 into R16 thus reduced the necessity of remobilisation of stem reserves for grain-filling as observed in RSG 03123. The reduction in R16 was mainly due to a reduction in sucrose (results not shown), indicating that there was remobilisation of stem reserves. Stem reserves are increasingly recognized as an important source for grain filling when current photosynthesis is inhibited by stress.

Nitrogen

The longevity of a leaf is intimately related to its nitrogen (N) status (Thomas and Rogers, 1990). Stay-green trait is characterised by delayed-leaf-senescence and thus can also be viewed as a consequence of a balance between supply and demand for N during grain-filling (Borrell and Hammer, 2000). Under conditions of abiotic stress or N deficiency, remobilisation from vegetative parts becomes important for grain development (Ta and Weiland, 1992). Delayed remobilisation of N from leaves maintains photosynthetic activity for longer periods and can result in higher grain yield (Borrell and Hammer, 2000). Borrell and Hammer (2000) found that stay-green genotypes had more N than senescent genotypes under drought stress. N retention in RSG 03123 and R16 were similar under WW conditions, whereas under WL conditions the retention was better in RSG 03123. However, B35 retained more leaf N content (LNC) and specific leaf N (SLN) than RSG 03123 under both WW and WL conditions. High SLN allows more carbon and N to be allocated to roots of stay-green hybrids during grain-filling, thus maintaining a greater capacity to extract N from the soil compared to senescent genotypes (Borrell et al., 2001). As shown earlier, photosynthesis was prolonged in RSG 03123, just as in B35, under WL conditions. This condition probably made carbohydrates available for amino acid synthesis and, thus, protein synthesis. The continual protein synthesis could have enhanced protein turnover and thus maintained the photosynthetic apparatus, replenished the carbon and N assimilating enzymes, which enabled photosynthesis to continue in RSG 03123 and B35 under water limitation.

Specific proteins by Western Analysis

Chloroplast proteins: LHCPII and OEC33

Chloroplasts are dismantled during the early phase of senescence (Hortensteiner and Feller, 2002), indicating that chloroplast proteins are degraded. In genetic variants with stay-green trait, deconstruction of the photosynthetic apparatus during leaf senescence is partially or completely prevented (Thomas and Donnison, 2000). LHCPII is a major contributor to the overall loss of protein during leaf senescence (Matile, 1992). However, in stay-greens, where chlorophyll catabolism is blocked, LHCPII remains stabilised and proteolytic cleavage is restricted due to a small N-terminal that protrudes into the stroma (Thomas and Donnison, 2000). In R16, the WL plants were more affected, particularly in leaf 4. The changes in RSG 03123 were similar to those in B35, with no difference between the WW and WL plants.

OEC33 (the 33 kDa oxygen-evolving complex protein) is involved in photosynthetic electron transport (Zhang et al., 1998). The OEC33 subunit is known to stabilise the catalytic manganese (Mn) cluster, which is essential for water oxidation (Zhang et al., 1998). The release of OEC33 results in paramagnetic uncoupling and dissociation of two of the four Mn cations from PSII unless more than 100 mM Cl^- is present (Zhang et al., 1998), thus confirming that it is involved in the stabilisation of the Mn cluster. Hence, a reduction in or degradation of OEC33, as a result of drought, would lead to a decline in CO_2 assimilation. The changes in band intensities of OEC33 in B35 and RSG 03123 were similar with little differences between the bands at flowering and physiological maturity. On the other hand, OEC33 was highly reduced in the WL R16 plants. The reduction in OEC33 might have resulted, in part, in the low photosynthesis in WL R16 plants.

POR catalyses the light-dependent reduction of protochlorophyllide *a* to chlorophyllide *a* (Von Wettstein et al., 1995). Most angiosperms have isozymes referred to as POR A and POR B. POR A is dominant in etiolated plants and disappears rapidly when plants are exposed to light, whereas POR B is constitutively expressed and the only remaining POR in light-grown plants (Vavilin and Vermaas, 2002). The amounts of POR B in RSG 03123 were intermediate between those for B35 and R16. PORB might have been involved in the continuous replacement of chlorophyll in mature leaves of B35 and RSG 03123, which enabled these plants to remain photosynthetically active for longer periods compared to the senescent R16 under WL conditions.

Photosynthetic enzymes: PEPC and Rubisco

When the stress is severe, photosynthesis may be more controlled by the capacity of the chloroplast to fix CO_2 than by reduced gL (Faver et al., 1996). Photosynthesis

in C4 plants, including sorghum, involves the enzymes PEPCK, PEPC and Rubisco among others. PEPC occupies a key position as the initial CO_2-fixing enzyme of the C4 pathway and is considered to be a major control point in this pathway (Dever et al., 1997). The band intensities of PEPC for B35 changed very little at physiological maturity, whereas in R16, it was drastically reduced at physiological maturity compared to the band intensities at flowering. Indeed, in the WL R16 it was barely detectable in leaves 1 and 2 and completely degraded in leaves 3 and 4. The retention of PEPC in RSG 03123 was also better than in R16 with sharp bands at physiological maturity, particularly under WL conditions, indicating an improvement in the retention of PEPC in RSG 03123. This could contribute to maintaining higher gas exchange rates in RSG 03123 compared to R16 under WL conditions.

Rubisco catalyses the fixation of CO_2 in the bundle sheath cells (Edwards and Walker, 1983; Hatch, 1987). The abundance of Rubisco in leaves is controlled by the rate of its synthesis and degradation (Parry et al., 2002). In R16, Rubisco was reduced in the WL plants at physiological maturity. Indeed, in these plants what remained in leaves 1 and 2 was being degraded (just as for PEPC). In B35 and RSG 03123, the high amounts of Rubisco retained in the WL plants enabled CO_2 assimilation to continue for longer periods than in the senescent cultivars. Reductions in the large subunit of Rubisco by drought stress have been reported for maize (Prakash and Rao, 1996) and wheat (Martinez et al., 2003) and in the transcripts of the small subunit in tomato (Bartholomew et al., 1991), and rice (Vu et al., 1999), indicating that its degradation was increased and synthesis reduced by water limitation. This is contrary to the arguments that Rubisco is not a primary target of drought stress (Holaday et al., 1992) and that limitation to CO_2 assimilation in drought-stressed leaves is rather caused by a reduction in the supply of CO_2 to Rubisco (Lal et al., 1996). In R16, on other hand, Rubisco was reduced just as in other plants like maize stated earlier. In RSG 03123, Rubisco was unaffected by drought stress confirming the findings by Holaday et al. (1992). Thus, the effect of drought stress on Rubisco in sorghum could be genotype dependent, having less effect in plants possessing the stay-green QTL as demonstrated in RSG 03123 and B35.

Conclusions

In the current study, we have proved that the introgression of the 3 putative SG QTL (*Stg1, Stg3* and *Stg4*) from B35 into the senescent R16 background (RSG 03123) resulted in enhanced retention of GLA, chlorophyll and chloroplast enzymes. This enabled these plants to maintain photosynthesis for longer periods compared to R16. It would, therefore, be interesting to find out if the improvement observed in this study would translate into higher grain yield in RSG 03123 over R16 under field conditions. Furthermore, such improvement could enhance the nutritional contents of these plants, thus providing quality fodder for feeding livestock, particularly, for subsistence farmers. However, RSG 03123 may still require some improvement since its functioning was intermediate between B35 and R16. This study also proved that GLA retention and photosynthesis were enhanced by the SG QTL introgressed into RSG 03123. The maintenance of photosynthesis was due to the retention of chloroplast proteins and enzymes involved in photosynthesis for longer periods compared to the senescent R16 parent. The improvement in these parameters, as observed in RSG 03123 over R16, could be attributed to the SG QTL from B35 introgressed into R16, indicating that SG QTL can function in a senescent background to improve tolerance to water limitation or drought.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

Funding from the Association of Commonwealth Universities, arranged through the University of Cape Coast, Ghana, and Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth University, Wales, UK for carrying out this work is gratefully acknowledged.

REFERENCES

Bachmann MF, Kündig TM, Hengartner H, Zinkernagel RM (1994). Regulation of IgG antibody titers by the amount persisting of immune-complexed antigen. European Journal of Immunology 24(10):2567-2570.

Badigannavar A, Teme N, Costa de Oliveira A, Li G, Vaksmann M, Viana VE, Ganapathi TR (2018). Physiological, genetic and molecular basis of drought resilience in sorghum (*Sorghum bicolor* (L.) Moench). Indian Journal of Plant Physiology 23(4):670-68.

Barth S, Jankowska JM, Hodkinson TR, Vellani T, Klaas M (2016). Variation in sequences containing microsatellite motifs in the perennial biomass and forage grass, *Phalaris arundinacea* (Poaceae). BMC Research Notes 9:184.

Bartholomew DM, Bartley GE, Scolnik PA (1991). Abscisic-acid control of *rbcS* and *cab* transcription in tomato leaves. Plant Physiology 96:291-296.

Blackmer TM, Schepers JS (1995). Use of a chlorophyll meter to monitor nitrogen status and schedule fertilisation for corn. Journal of Production Agriculture 8:56-60.

Blümmel M, Deshpande S, Khlova J, Vadez V (2015). Introgression of staygreen QTL's for concomitant improvement of food and fodder traits in *Sorghum bicolor*. Field Crops Research 180:228-237.

Borland AM, Tecsi L, Leegood RC, Walker RP (1998). Inducibility of crassulacean acid metabolism (CAM) in *Clusia* species; physiological/ biochemical characterisation and intercellular localisation of carboxylation processes in three species which show different degrees of CAM. Planta 205:342-351.

Borrell AK, Bidinger FR, Tao YZ, McIntyre CL, Douglas ACL (2001).

Physiological and molecular aspects of stay-green in sorghum. In: A. K. Borrell and R.G. Henzell (eds). Proceedings from the Fourth Australian Sorghum Conference, Kooralbyn, 5-8 February 2001. CD-ROM format. Range Media Pty Ltd. ISBN: 0-7242-2163-8.

Borrell AK, Bidinger FR, Sunitha K (1999). Stay-green trait associated with yield in recombinant inbred sorghum lines varying in rate of leaf senescence. International Sorghum and Millets. Newsletter 40:31-34.

Borrell AK, Hammer GL (2000). Nitrogen dynamics and the physiological basis of stay-green in sorghum. Crop Science 40:1295-1307.

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

Borrell AK, Van Oosterom EJ, Mullet JE, George-Jaeggli B, Jordan DR, Klein PE, Hammer GL (2014a). Stay-green alleles individually enhance grain yield in sorghum under drought by modifying canopy development and water uptake patterns. New Phytololgy 203:817-830.

Borrell AK, Mullet JE, George-Jaeggli B, van Oosterom EJ, Hammer GL, Klein PE, Jordan DR (2014b). Drought adaptation of stay-green sorghum is associated with canopy development, leaf anatomy, root growth, and water uptake. Journal of Experimental Botany 65:6251-6263.

Boyles RE, Brenton ZW, Kresovich, S (2019). Genetic and genomic resources of sorghumto connect genotype with phenotype in contrasting environments. The Plant Journal 97:19-39.

Brück H, Payne WA, Sattelmacher B (2000). Effects of phosphorus and water supply on yield, transpirational water-use efficiency and carbon isotope discrimination of pearl millet. Crop Science 40:120-125.

Cairns A.J, Cookson A, Thomas BJ, Turner LB (2002). Starch metabolism in the fructan-grasses: patterns of starch accumulation in excised leaves of *Lolium temulentum* L. Journal of Plant Physiology 159 293–305. 10.1078/0176-1617-00644.

Cornic G, Briantais J-M (1991). Partitioning of photosynthetic electron flow between CO_2 and O_2 reduction in a C_3 leaf (*Phaseolus vulgaris* L.) at different CO_2 concentration and during drought stress. Planta 183:178-184.

Crasta OR, Xu WW, Rosenow DT, Mullet JE, Nguyen HT (1999). Mapping of post-flowering drought resistance traits in sorghum: association between QTLs influencing premature senescence and maturity. Molecular and General Genetics 262:579-588.

de Villiers L, Turk K, Thomas H, Howarth C (1993). Analysis and exploitation of the stay-green character in sorghum. Overseas Development Administration Project R4885 Annual Report.

Dever, LV, Bailey KJ, Leegood RC, Lea PJ (1997). Control of photosynthesis in *Amaranthus edulis* mutants with reduced amounts of PEP carboxylase. Australian Journal of Plant Physiology 24:469-476.

Doggett H (1988). Sorghum, 2nd Edition. John Wiley & Sons: New York. pp. 165-199. Duncan RR, Bockholt AJ Miller FR (1981). Descriptive comparison of senescent and nonsenescent sorghum genotypes. Agronomy Journal 73:849-853.

Duvick DN, Smith JSC, Cooper M (2004). Long-term selection in a commercial hybrid maize breeding program. Plant Breeding Reviews 24:109-152.

Edwards GE, Walker DA (1983). C_3, C_4: Mechanisms and cellular and environmental regulation of photosynthesis. Los Angeles, CA: University of California Press.

Faver KL, Gerik TJ Thaxton PM, El-Zik KM (1996). Late season water stress in cotton: II. Leaf gas exchange and assimilation capacity. Crop Science 36:922-928.

Foley JA, Ramankutty N, Braumann KA, Cassidy E, Gerber JS, Johnston M, Mueller ND, O'Connell C, Deepak KR, Zaks DPM (2011). Solutions for a cultivated planet. Nature 478:337-342.

Galyuon IKA, Madhusudhana R, Borrell AK, Hash TC, Howarth CJ (2016). Genetic diversity of stay-green sorghums and their derivatives revealed by microsattelites. African Journal of Biotechnology 15(25):1363-1374.

Gollan T, Passioura JB, Munns R (1986). Soil water status affects the stomatal conductance of fully turgid wheat and sunflower leaves. Australian Journal of Plant Physiology 13:459-464.

Harris K, Subudhi PK, Borrell AK, Jordan D, Rosenow D, Nguyen H, Klein P, Klein R, Mullet J (2007). Sorghum stay-green QTL individually reduce post-flowering drought-induced leaf senescence. Journal of Experimental Botany 58:327-338.

Hash CT, Bhasker Raj AG, Lindup S, Sharma A, Beniwal CR, Folkertsma RT, Mahalakshmi V, Zerbini E, Blümmel M (2003). Opportunities for marker-assisted selection (MAS) to improve the feed quality of crop residues in pearl millet and sorghum. Filed Crops Research 84:79-88.

Hatch MD (1987). C_4 photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure. Biochimica et Biophysica Acta 985:81-106.

Henzell RG, Brengman DS, Fletcher McCosker AN (1992). Relationships between yield and non-senescence (stay-green) in some grain sorghum hybrids grown under terminal drought stress. In: Foale, M. A., Henzell RG, Vance PN (eds) Proceedings of the second Australia Sorghum Conference Gatton, 4th-6th February, 1992. Australian Institute of Agricultural Science, Melbourne pp 355-358.

Hilditch PI, Thomas H, Thomas BJ, Rogers LJ (1989). Leaf senescence in non-yellowing mutant of *Festuca pratensis*: proteins of photosystem II. Planta 177:265-272.

Holaday AS, Ritchie SW, Nguyen HT (1992). Effect of water deficit on gas exchange parameters and ribulose-1,5-bisphospahte carboxylase activation in wheat. Environmental and Experimental Botany 32:403-409.

Hortensteiner S, Feller U (2002). Nitrogen metabolism and remobilization during senescence. Journal of Experimental Botany 53:927-937.

Jagadish KSV, Kavi Kishor PB, Bahuguna RN, von Wiren N, Sreenivasulu N (2015). Staying alive or going to die during terminal senescence - An enigma surrounding yield stability. Frontiers in Plant Science 6:1070.

Johnson SM, Cummins I, Lim FL, Slabas AR, Knight MR (2015). Transcriptomic analysis comparing stay-green and senescent *Sorghum bicolor* lines identifies a role for proline biosynthesis in the stay-green trait. Journal of Experimental Botany 66(22):7061-7073.

Kassahun B, Bidinger FR, Hash CT, Kuruvinshetti MS (2010). Stay-green expression in early generation sorghum (*Sorghum bicolor* (L.) Moench) QTL introgression lines. Euphytica 172:351-362.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680-685.

Lal A, Ku MSB, Edwards GE (1996). Analysis of inhibition of photosynthesis due to water stress in C3 species *Hordeum vulgare* and *Vicia faba*: electron transport, CO_2 fixation and carboxylation capacity. Photosynthesis Research 49:57-69.

Lasky JR, Upadhyaya HD, Ramu P, Deshpande S, Hash CT, Bonnette J, Juenger TE, Hyma K, Acharya C, Mitchell SE, Buckler ES, Brenton Z, Kresovich S, Morris GP (2015). Genome-environment associations in sorghum landraces predict additive traits. Science Advances 1:1-13.

Lu C, Zhang J (1999). Effects of water stress on photosystem II photochemistry and its thermostability in wheat plants. Journal of Experimental Botany 50:1199-1206.

Mace E, Singh V, Van Oosterom E, Hammer G, Hunt C, Jordan D (2012). QTL for nodal angle in sorghum (*Sorghum bicolor* (L.) Moench) co-locate with QTL for traits associated with drought adaptation. Theoretical and Applied Genetics 124:97-109.

Mae T, Thomas H, Gay AP, Makino A, Hidoma J (1993). Leaf development in *Lolium temulentum*: photosynthesis and photosynthetic proteins in leaves senescing under different irradiances. Cell Physiology 34:391-399.

Martinez DE, Luquez VM, Bartoli CG, Guiamet JJ (2003). Persistence of photosynthetic components and photochemical efficiency in ears of water-stressed wheat (*Triticum aestivum*). Physiologia Plantarum 119:519-525.

Mahalakshmi V, Bidinger FR (2002). Evaluation of the stay-green germplasm lines at ICRISAT. Crop Science 42:965-974.

Manschadi AM, Christopher J, deVoil P, Hammer GL (2006). The role of root architectural traits in adaptation of wheat to water-limited environments. Functional Plant Biology 33(9):823-837.

Massacci A, Battisetelli A, Loreto F (1996). Effect of drought stress on photosynthetic characteristics, growth and sugar accumulation of field-gron sweet sorghum. Australian Journal of Plant Physiology 23:331-340.

Matile P (1992). Chloroplast senescence. In: Baker NR Thomas H (eds) Crop Photosynthesis: spatial and temporal determinants. Elsevier, Amsterdam pp. 413-440.
Maxwell K, Johnson GN (2000). Chlorophyll fluorescence – a practical guide. Journal of Experimental Botany 51:659-668.
McBee GG, Miller FR (1982). Carbohydrates in sorghum culms as influenced by cultivars, spacing and maturity over a diurnal period. Crop Science 22:381-385.
McBee GG Miller FR (1993). Stem carbohydrates and lignin concentrations in sorghum hybrids at seven growth stages. Crop Science 33:530-534.
McBee GG, Waskom III RM, Miller FR, Creelman RA (1983). Effect of senescence and nonsenescence on carbohydrates in sorghum during late kernel maturity states. Crop Science 23:372-376.
Oh M-H, Kim Y-J, Lee C-H (2003). Increased stability of LCHIIby aggregate formation during dark-induced leaf senescence in *Arabidopsis thaliana* mutant *ore*10. Plant Cell Physiology 44:1368-1377.
Parry MAJ, Andralojc PJ, Khan S, Lea PJ, Keys AJ (2002). Rubisco activity: Effects of drought stress. Annals of Botany 89:833-839.
Paterson AH, Bowers JE, Bruggmann R, Dubchak I, Grimmwood J, Gundlach H, et al. (2009a). The *Sorghum bicolor* genome and the diversification of grasses. Nature 457:551-556.
Paterson AH, Bowers JE, Feltus FA, Tang HB, Lin LF, Wang XY (2009b). Comparative genomics of grasses promises a bountiful harvest. Plant Physiology 149:125-131.
Payne WA, Drew MC, Hossner LR, Lascano RJ, Onken AB, Wendt CW (1992). Soil phosphorus availability and pearl millet water use efficiency. Crop Science 32:1010-1015.
Pinheiro C, Chaves MM, Ricardo CP (2001). Alterations in carbon and nitrogen metabolism induced by water deficit in the stems and leaves of *Lupinus albus* L. Journal of Experimental Botany 52:1063-1070.
Pinto RS, Lopes MS, Collins NC, Reynolds MP (2016). Modelling and genetic dissection of staygreen under heat stress Theoretical and Applied Genetics 129(11):2055-2074.
Prakash KR, Rao VS (1996). The altered activities of carbonic anhydrase, phosphoenolpyruvate carboxylase and ribulose bisphosphate carboxylase due to water stress and after its relief. Journal of Environmental Biology 17:39-42.
Rosenow DT (1984). Breeding for resistance to root and stalk rots in Texas. In: Mughogho LK (ed) Sorghum root and stalk diseases, a critical review. Proceedings of the Consultative Group discussion of research needs and strategies for control of sorghum root and stalk diseases. Bellagio, Italy, 27th November to 2nd December, 1983, ICRISAT, Pantacheru Andrah Pradesh, India pp 209-217.
Rosenow, DT (1994). Evaluation for drought and disease resistance in sorghum for use molecular marker-assisted selection. In: Witcombe JR, Duncan RR (eds). Proceedings of the use of molecular markers in sorghum and pearl millet breeding for developing countries. Norwich. pp 27-31.
Rosenow DT, Clark LE (1981). Drought tolerance in sorghum. In: Loden HD and Wilkinson D (eds). Proceedings of the 36th Annual Corn and Sorghum Industry Research Conference. Chicago, Illinois, USA. 9th to 11th December, 1981. American Seed trade Association, Washington DC. pp. 18-31.
Rosenow DT, Clark LE, Dahlberg JA, Frederiksen RA, Odvody GN, Peterson GC, Schaefer K, Collins SD, Jones JW, Hamburger AJ (2002). Release of four A/B sorghum parental lines ATx642 through ATx645. International Sorghum and Millets. Newsletter 43:24-3
Rosenow DT, Quisenberry JE, Wendt CW, Clark LE (1983). Drought tolerant sorghum and cotton germplasm. Agriculture Water Managent 7:207-222.
Sanchez AC, Subudhi PK, Rosenow DT, Nguyen HT (2002). Mapping QTLs associated with drought resistance in sorghum (*Sorghum bicolour* (L.) Moench). Plant Molecular Biology 48:713-726.
Sinclair TR Muchow RC (2001). System analysis of plant traits to increase grain yield on limited water supplies. Agronomy Journal 93:263-270.
Spano G, Di Fonzo N, Perrotta C, Platani C, Ronga G, Lawlor DW, Napier JA, Shewry PR (2003). Physiological characterization of 'stay green' mutants in durum wheat. Journal of Experimental Botany 54:1415-1420.
Subudhi PK, Rosenow DT, Nguyen HT (2000). Quantitative trait loci for the stay-green trait in sorghum (*Sorghum bicolor* (L.) Moench): consistency across genetic backgrounds and environments. Theoretical and Applied Genetics 101:733-741.
Ta CT, Weiland RT (1992). Nitrogen partitioning in maize during ear development. Crop Science 32:443-451.
Tao YZ, Henzell RG, Jordan DR, Butler DG, Kelly AM, McIntyre CL (2000). Identification of genomic regions associated with stay-green in sorghum by testing RILs in multiple environments. Theoretical and Applied Genetics 100:1225-1232.
Thomas H (1987). Sid: A Mendelian locus controlling thylakoid membrane disassembly in senescing leaves of *Festuca pratensis*. Theoretical and Applied Genetics 73:551-555.
Thomas H, Donnison I (2000). Back from the brink: plant senescence and its reversibility. In: Bryant JA, Hughes SG Garland JM (eds) Programme cell death in animals and plants. Oxford: Bios pp 149-162.
Thomas H and Howarth, CJ (2000). Five ways to stay green. Journal of Experimental Botany 51:329-337.
Thomas H, Rogers LJ (1990). Turning over an old leaf. University of Wales Reviews of Science and Technology 6:29-38.
Tuinstra MR, Ejeta G, Goldsborough PB (1998). Evaluation of near-isogenic sorghum lines for QTL markers associated with drought tolerance. Crop Science 38:835-842.
Tuinsra MR, Grote EM, Goldsborough PB, Ejeta G (1996). Indentification of quantitative trait loci associated with pre-flowering drought tolerance in sorghum. Crop Science 36:1337-1344.
Tuinstra MR, Grote EM, Goldsborough PB Ejeta G (1997). Genetic analysis of of post-flowering drought tolerance and components of grain development in *Sorghum bicolor* (L.) Moench. Molecular Breeding 3:439-448.
Vadez V, Deshpande S, Kholova J, Ramu P, Hash CT (2013). Molecular breeding for stay-green: progress and challenges in sorghum. In: Varshney R, Tuberosa R (eds) Genomic applications to crop breeding: Volume 2. Improvement for abiotic stress, quality and yield improvement. New York: Wiley pp. 125-141.
Van Oosterom EJ, Jayachandran R, Bindinger F (1996). Diallel nalysis of stay-green trait and its components in sorghum. Crop Science 36:540-555.
Vavilin DV, Vermaas FJ (2002). Regulation of the tetrapyrrole biosynthetic pathway leading to heme and chlorophyll in plants and cyanobacteria. Plant Physiology 115:9-24.
Verma V, Foulkes MJ, Worland AJ, Sylvester-Bradley R, Caligari PDS, Snape JW (2003). Mapping quantitative trait loci for flag leaf senescence as yield determinant in winter wheat under optimal and drought-stressed environments. Euphytica 135:255-263.
Vietor DM, Miller FR, Cralle HT (1990). Nonstructural carbohydrates in axillary branches and main stem of senescent and nonsenescent sorghum types. Crop Science 30:97-100.
Von Caemmerer S, Ludwig M, Millgate A, Farquhar GD, Price D, Badger M, Furbank RT (1997). Carbon isotope discrimination during C4 photosynthesis: Insights from transgenic plants. Australian Journal of Plant Physiology 24:487-494.
Von Wettstein D, Gough S, Kannangara CG (1995). Chlorophyll biosynthesis. Plant Cell 7:1039-1057.
Vu JCV, Gesch RW, Allen LH, Boote KJ, Bowes G (1999). CO_2 enrichment delays a rapid, drought-induced decrease in Rubisco small subunit transcript abundance. Journal Plant Physiology 155:139-142.
Walulu RS, Rosenow DT, Wester DB, Nguyen HT (1994). Inheritance of stay-green trait in sorghum. Crop Science 34:970-972.
Wolfe DW, Henderson DW, Hsiao TC Alvino A (1988). Interactive water and nitrogen effects on senescence of maize. I. Leaf area duration, nitrogen distribution and yield. Agronomy Journal 80:859-864.
Xu L-K, Hsiao TC (2004). Predicted versus measured photosynthetic water-use efficiency of crop stands under dynamically changing field environments. Journal of Experimental Botany 55:2395-2411.
Xu W, Rosenow DT, Nguyen HT (2000a). Stay-green trait in grain sorghum: relationship between visual rating and leaf chlorophyll concentration. Plant Breeding 119:365-367.
Xu W, Subhudi PK, Crasta OR, Rosenow DT, Mullet JE, Nguyen HT (2000b). Molecular mapping of QTLs conferring stay-green in grain

sorghum (*Sorghum bicolor* (L) Moench). Genome 43:461-469.
Yordanov I, Tsonev T, Velikova V, Georgieva K, Ivanov P, Tsenov N, Petrova T (2001). Changes in CO_2 assimilation, transpiration and stomatal resistance of different wheat cultivars experiencing drought under filed conditions. Bulgarian Journal of Plant Physiology 27:20-33.
Yordanov I, Velikova V, Tsonev T (2003). Plant responses to drought and stress tolerance. Bulgarian Journal of Plant Physiology Special pp. 187-206.
Zhang H, Ishikawa Y, Yamamoto Y, Carpentier R (1998). Secondary structure and thermal stability of the extrinsic 23 kDa protein of photosystem II studied by Fourier transform infrared spectroscopy. FEBS Letters 426:347-351.

Vol. 18(26), pp. 564-584, 26 June, 2019
DOI: 10.5897/AJB2018.16621
Article Number: 826E5CC61345
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Microencapsulation of *Aspergillus niger* phytases produced in triticale by solid fermentation and microencapsulates characterization

José Daniel García García[1], Ana Iliná[1], Janeth Ventura[1], Georgina Michelena[2], Erika Nava[3], Carlos Espinoza González[4] and José Luis Martínez[1]*

[1]Nano Bioscience Academic Group, Health Science School, Autonomous University of Coahuila, México.
[2]Cuban Institute for Research on Sugarcane Derivatives, Cuba.
[3]The National Institute for Forestry, Agriculture and Livestock Research, Mexico.
[4]Research Center for Applied Chemistry, México.

Received 15 August, 2018; Accepted 18 April, 2019

This paper aimed to carry out the stabilization of phytase produced in solid-state fermentation by means of Aspergillus níger 7A-1 in triticale as substrate. The crude extract obtained in liquid state was stabilized with glycerol and maltose. These materials presented a synergic effect that improve phytase stability around 80% with ki of 0.031 and a shelf-time of 156 days (22.35 weeks) with glycerol 30% and maltose 20%. Besides, microencapsulation by spray drying was used as well to analyze the potential of whey and guar gum to obtain microencapsulates of phytase for monogastric animals. Statistical analysis allowed establishing the suitable temperature at 110°C with a flow of 10% (3.3 ml/min), guar gum concentration of 1% and yield process at 93%. Characterization allows us to observe that microencapsulates with whey and guar gum is a good alternative to be used in spray drying. SEM showed the formation of aggregates with pores. Thermogravimetric analysis demonstrated that thermal stability of guar gum is improved when it is mixed with whey. Infrared showed the signal of protein groups in samples with whey and guar gum. Stability assessment was performed with two different containers, Eppendorf and Ziploc bags, in which it was demonstrated that microencapsulation with whey and guar gum allow the retention of 85% for both containers. At 35°C, phytase activity decreases until 55%. In this study, color was evaluated as well in Ziploc bags with the color observing change during the 8 weeks; this change was not observed in Eppendorf container and it was possible to observe that color does not affect enzymatic stability. Finally, digestibility evaluation demonstrated that whey and guar gum provide protection in gastrointestinal conditions. After digestive system simulation, 90% of activity is retained which allows observing the potential of whey and guar gum to protect enzymes under gastrointestinal condition.

Key words: Phytases, phosphorus, phytate, guar gum, microencapsulation.

INTRODUCTION

After energy and protein, phosphorus is the third element more important in animal diets (Rodehutscord, 2013). It could be found in cereals as a compound known as phytate, which is formed of phytic acid with minerals or

*Corresponding author. E-mail: jose-martinez@uadec.edu.mx.

proteins. Phytate has a negative effect upon phosphorus' bioavailability. This leads to lower absorption of phosphorus and the needing of alternative phosphorous sources (Tang et al., 2018). Phytase (myo-inositol hexaphosphate phosphohydrolase) catalyzes the hydrolysis of phytate to inositol phosphates, myo-inositol, and inorganic phosphate. Phytase can be found in cereals but in low concentration, researches have been carried out to improve the expression of phytase genes and in this way promote the synthesis of this enzyme (Tan et al., 2017). Solid fermentation is the most used procedure to obtain this enzyme and then, the crude extract is recovered in aqueous solution. However, when proteins are in aqueous solution the hydration of the molecule and denaturalization of quaternary structure proteins is the main risk. One of the main challenges in livestock industry is to maintain the enzymatic activity and stability in aqueous solution. Stabilization with some additives as sugars, salts, amino acids, amines, polyols and buffers systems are as co-solvents which has been employed for enzyme stabilization and these additives have a positive effect upon the enzymes degradation.

High temperatures of ~70–90°C are used in the feed-pelleting procedure; phytase is easily inactivated to 30% or less of its original activity. Microencapsulation is a process widely used due to its low cost and it is the most common method to dry liquid compositions. It provides protection by carriers and additives by means of spray drying and, in this way, avoiding enzyme inactivation. Nowadays, many different compounds have been dried by this method like some oils (Aghbashlo et al., 2012; Gangurde et al., 2015; Koç et al., 2015), lactobacillus (Eckert et al., 2017), DNA (Alexakis et al., 1995), food (Pérez-Alonso et al., 2009), volatile aromas (Brückner et al., 2007), enzymes (Anjani et al., 2007; Dutta and Bhattacharjee, 2017; Estevinho et al., 2014; Gupta et al., 2014; Jesus and Filho, 2014), among others. The greatest challenge in the spray drying of proteins is denaturalization due to heating. High temperatures are involved in this process; spray drying is based on generation of fine droplets by a nozzle or a rotary atomizer into a hot dry air stream normally at 180-220°C; and enzymes cannot support these temperatures. Nevertheless, there are carriers that can be used to protect enzymes. Spray drying technique consists of three steps: atomization, dehydration, and powder collection. The feed liquid, which include carriers, additives and compounds to encapsulate (like enzymes), is sprayed through an atomizing nozzle into a drying chamber (Ganju and Gogate, 2017; Hamin et al., 2017), in which a hot air flow dry water and finally the powder is recollected. There are few reports of phytase microencapsulation through spray drying with polysaccharides (Spier and Greiner, 2015).

Whey is considered as a waste for dairy industry, which is a by-product and it is produced in significant quantum due to the volume of required milk products (Ganju and Gogate, 2017). It is mainly composed of proteins, lactose, minerals and dry matter, and could be used in fermentations, beverages or additives in food in order to obtain a functional one (Teniza, 2008). Whey proteins concentrated and isolated have been recovered by the utilization of membranes, bio-catalysis or high performance tangential flow ultrafiltration. However, the residues of the separation of proteins remain considered as waste. For this reason, new alternatives for the utilization of whey are being searched for. When whey is dried, water can be promptly removed from the dry matter, minimizing heat damage and heating costs. Some components have been used as a carrier or additive to encapsulate different compounds. Guar meal is the main by-product of guar gum (GG) production with a high protein content (Ahmed and Abou-Elkhair, 2016). GG has been used as a carrier to encapsulate, and it has shown positive effects during drying, protecting the product even at high temperatures (Kuck and Noreña, 2016; Mehyar et al., 2014).

The poultry industry has become a specific area of producing enzymes for animals. The demand for complementary enzymes for animals diets have been increasing (Ahmed and Abou-Elkhair, 2016). Phytases are one of this example, which is estimated by Global Phytases Market Report (2017) with an annual growth rate of 2.78% from 2012 to 2013, implying 315 to 342 million dollars respectively (Global Phytases Market Report, 2017). There are predictions for 2021-2022 of 520 million dollars for phytases market. This is the reason why the huge intake of cereals and legumes in animal diets that are complemented with phytases allowing the development of a process to produce the enzyme, recover and purify this kind of enzymes with higher profitability. Phytases hydrolyze phytate producing inorganic phosphorous and myo-inositol (Neira et al., 2013). There are different products currently commercialized, most of which are presented in liquid, with transportation and availability in process of food pelleting as disadvantage (Menezes-Blackburn et al., 2015). Microencapsulation is the drying of liquid solutions by wrapping compounds with a thin polymeric coating to obtain micro particles powders with a normal size from 5 – 5000 µm. At present, microencapsulation has been widely used in textile, cosmetic, pharmaceutical and food industry. Depending on the method used, characteristics of microencapsulates will differ (Das et al., 2011). Chemical, physic and physico-chemical are methods of microencapsulation. Spray drying is a physico-chemical process suitable for use in industry due to its low cost commercial process. It is useful for compounds that are heat sensitive, such as proteins or peptides like enzymes; however, with the appropriate coating material, encapsulation could be accomplished (Nava et al., 2015). In addition, spray drying method is a rapid and reproducible method for microencapsulate and is easy to scale up. There are a lot of materials that can be used as

coating materials like gums, waxes, proteins, polysaccharides, carbohydrates, etc. (Eckert et al., 2017; Lupo et al., 2012; Mehyar et al., 2014). Nevertheless, aspects such as properties materials, feed rate, viscosity, glassy transition temperature, inlet and outlet temperature, vacuum, equipment conditions, uniformity as well as the compound to be encapsulated, must be considered in drying process. These aspects will have an influence not only on the objective desired, but also in the physical characteristics. Techniques of characterization which are great tools to elucidate physical characteristics, thermogravimetric analysis, X-Rays, infrared, scanning electronic microscopy, among others has been used. In this way, it is possible to determine physical structures of microcapsules. All products tend to deteriorate during storage, but in different rates (Sousa-Gallagher et al., 2016). For this, storage assessment is important, as it is possible to determine the time in which products will preserve their qualities that make them a stable product. Products can suffer different changes during storage like enzymatic browning, non-enzymatic browning, oxidation-reduction reaction, microbial damage, change in color, etc. Another important element to take into consideration is the choice of packing material as well, so that the integrity of the product could be preserved for a long duration. This is the reason why a suitable assessment of storage should be carried out. Furthermore, digestibility evaluation will provide relevant information about the bioavailability of microencapsulates in gastrointestinal conditions. Digestibility is defined as the percentage of foodstuff available to be absorbed by the organism. Poultry have a gastrointestinal condition like those of humans. α-amylase, pepsin and pancreatic enzymes (α-amylase, lipase and proteases) are enzymes presented in gastrointestinal tract. Nowadays, there are different models for simulation of gastrointestinal condition, and they could be applied according to the purpose of interest.

The aim of this work was the stabilization of phytase liquid extract using maltose and glycerol to improve its shelf-time. Besides, evaluation of whey and guar gum intended to be used as materials for phytase encapsulation in spray drying was carried out as well, to determine physical characteristics of phytase microencapsulates obtained under the conditions selected and to evaluate the digestibility of the microencapsulates in an *in vitro* system simulating gastrointestinal conditions.

METHODOLOGY

Microorganism

The microorganism used to produce phytase was a fungus of the genus *Aspergillus*, the *niger* species, provided by Nanobioscience Group of the Autonomous University of Coahuila, Mexico with code 7A-1. *A. niger* 7A-1 strain was selected by Neira et al., (2013).

Phytase production

Phytase was produced in solid state fermentation (SSF) following the methodology used by Costa et al., (2010) and modified by Neira et al., (2013). The strain was grown in potato dextrose agar (PDA) at 37°C for 5 days. Spores were recovered with 0.1% Tween 80 and stirred for 15 min. The liquid was filtrated, and spores concentration quantified. The inoculum was prepared with dextrose (20 g/L) and yeast extract (4 g/L), spores were added into solution in concentration of 1×10^6 spores per milliliter and stirring at 180 rpm for 36 h. The triticale substrate used was selected by Neira et al., (2013). Triticale was used for 159 days in a proportion of 3:1 spike-stem and was submerged in sodium hypochlorite (5 ml/L) for 5 min. Substrate was rinsed with distillated water and ground in a grain mill electric Thomas Willey with wire mesh size of 0.5 mm. Triticale was used as substrate and it was provided by the Antonio Narro Autonomous Agrarian University (UAAAN), Mexico. 5 g of substrate were placed in Petri dish and sterilized at 121°C for 30 min. After cooling, 3 ml inoculum and 2.5 ml $(NH_4)_2SO_4$ were added to each Petri dish and incubated at 37°C for 5 days. In order to analyze the growing, a kinetic was performed for seven days and every day one Petri dish was sacrificed, with sugars, proteins and phytase activity evaluated. Crude extract was obtained by adding 5 ml H_2O per gram of dry substrate and stirred at 200 rpm for 30 min. The liquid was centrifugated at 10,000 rpm for 10 min. The supernatant was recollected, filtrated to eliminate spore and stored at 4°C.

Phytase activity

Phytase activity was measured spectrophotometrically according to the technique used by Harland and Harland (1980). The reaction mixture consisted of addition of 1 ml of 100 mM $MgSO_4$ in 200 mM acetate buffer (pH 5.15); 2.4 ml of 6.82 mM phytic acid prepared in 200 mM acetate buffer (pH 5.15) and 0.6 ml of crude extract containing phytase. The reaction mixture was stirred and incubated for 60 min at 55°C. After this time, reaction was stopped with the addition of 0.5 ml of trichloroacetic acid 10%. 1 ml of distilled water was added to adjust volume, and 2.4 ml of freshly prepared reagent of Taussky-Schorr (10 mL of 10 N H_2SO_4, 1 g of $(NH_4)Mo_7O_{24}*4H_2O$ and 5 g of $FeSO_4*7H_2O$ diluted to the mark in 100-ml flask with distilled water) to generate a blue chromophore were added. The contents were mixed and incubated for 30 min and the inorganic phosphate released was measured at 660 nm. One unit of phytase activity (U) was expressed as the amount of enzyme releasing 1 µmol of phosphorus per minute under standard assay conditions. Calibration curve was performed by triplicate in a range of 0.04 µmol/ml of inorganic phosphorus with KH_2PO_4. 28 mg of KH_2PO_4 was used, dissolved in acetates buffer (0.2 M pH 5.15) and diluted to 100 ml.

Stabilization of phytase

Glycerol (Jalmek, Mexico) and maltose (Sigma-Aldrich, Japan) were employed to study the stability of the crude extract containing phytase. The study was performed under two conditions for glycerol (10 and 30%) and maltose (0 and 20%) in a statistic design of 2^2.

Twenty-seven samples were prepared and stored at 4°C for eight weeks. Each week, three samples were sacrificed to determine phytase activity according to Harland and Harland method described previously. To predict the stability of the enzymes, the inactivation constants were determined using the Arrhenius equation. Shelf-time was calculated with equation number 1.

$$t_{1/2} = \frac{\ln(2)}{k} \quad (1)$$

Shelf-time equation for first order reactions (Brown et al., 2004).

Phytases microencapsulation

Characterization of whey

Whey was acquired from the company “Quesos de Saltillo”, Coahuila, México. Whey were analyzed for physicochemical parameters according to Association of Analytical Communities (1990). Total solids were evaluated using Thermobalance OHAUS MB23 at 105°C (method No. 990.20), ash using a muffle furnace Felisa FE-340 at 550°C (method No. 968.08), fat by using the Soxhlet method (method No. 922.06), protein by using the Kjeldahl method (method No. 991.20) and carbohydrates by using the difference method (method No. 986.25). pH was evaluated using a HANNA HI2550 potentiometer whereas samples were by triplicate.

Liquid feed, dryer equipment and operating conditions

Guar gum has been shown to be a good carrier for protecting different compounds in spray drying. According to literature, more than 1% of guar gum increases the viscosity of the liquid and makes it more difficult to dry samples. In addition, there are some reports where it is mentioned that more than 7.5 - 10% of guar meal in animal diets can cause severe growth depression and sticky feces (AOAC, 1990; Jellouli et al., 2011). Even though guar meal is a by-product of guar gum production, guar gum can present a few effect when more than 5% of it is used (Rao et al., 2016). Therefore, 0.1 and 1% were GG concentration in the liquid feed which contains 80% of whey/GG and 20% of extract crude containing phytase.

Phytase microencapsulation process

Spray drying was carried out in a Mini Spray Dryer B-290 (Büchi Labortechnik AG, Flawil, Switzerland). The liquid was fed into the atomizer under two-flow condition: 10 and 30%, respectively. To determine the suitable flow for this specific liquid, every sample was 100 ml and analyzed in triplicate. 100-ml liquid was dried, time was measured with a chronometer and flow rate was calculated. Nozzle specification was 0.7 mm. Previous experiments were conducted at 150°C according to the results obtained, and the inlet temperatures evaluated were 110 and 130°C. Central points were 0.55% for guar gum concentration, 120°C for temperature and 20% for flow. There are some important factors involved in drying, which can lead to a stress state for protein, dehydration stress, temperature and moisture. This study was performed by a factorial design with one central point.

Microencapsulates assessment

Enzymatic activity

100 mg of the powder were dissolved in 10 ml of saline solution to liberate enzyme and stirred. Phytase activity was evaluated by Harland and Harland (1980) method previously described.

Yield of encapsulation

Yield was calculated using total solids amount as reference and determined in the same way described before. The yield was calculated with the Equation 2.

$$Yield = \frac{Powder\ recovered\ (grams)}{Total\ solids\ (grams)} * 100 \quad (2)$$

This is recovery yield equation.

Product moisture content and water activity

Moisture content of the samples was calculated from weight loss after heating the sample at 60°C for 24 h. Water activity (Aw) was measured by direct reading in electronic meter (Aqualab 3TE-Decagon, Pullman). Both parameters were evaluated according to AOAC (1990). The analyses were performed in triplicate.

Color assessment of microencapsulates

Color of the microencapsulated powders was measured using a NH310 Portable Colorimeter. CIELAB system define L*, a*, b*, where L* indicates light-ness (0 = black and 100 = white), a* and b* are coordinates for green (-a*)/red (+a*), and blue (-b*)/yellow (+b*) (Kuck and Noreña, 2016).

Statistical analysis

The data produced were analyzed for mean, standard deviation and standard error using Microsoft Office Excel (Tukey test) and Stat Graphics (ANOVA). All the results are expressed as mean of three replicates, unless otherwise stated.

Microencapsulates characterization

Morphology

Structural analysis of the surface of the particles was performed by scanning electron microscopy (SEM) in Polymer Department, Autonomous University of Coahuila. Samples were covered with gold/palladium and placed on metallic stubs with carbon tape in an argon atmosphere with Quorum Q 50 RES. SEM microcapsules analyses were performed in HI-0873-0004 Scanning Electronic Microscope. Images were obtained using a potential of 3 kV and analyses were performed at room temperature (20°C).

TGA and IR analysis

Thermogravimetric analyses (TGA) were carried out from 30 - 800°C at 10°C/min in an oxygen atmosphere in order to observe degradation completely. IR was carried out from 600 to 3750 nm. These analyses were performed in TGA 4000 – Spectrum™ 100 FT-IR with TL-8000 Transfer Line, Perkin Elmer, USA. Data was plot in Origin Pro 2016, Graphic and Analyses.

Storage availability assessment

Temperature and container were the parameters evaluated to determine the storage-availability. Nowadays phytase is commercialized in bottles and bags; for this reason, microcapsules were placed in Ziploc bags and Eppendorf tubes. Samples were stored at 4°C, which represents refrigeration temperature (25°C), as well as room temperature (35°C), according to Tonon et al. (2010); this temperature is recommended for storage assessment. Every,

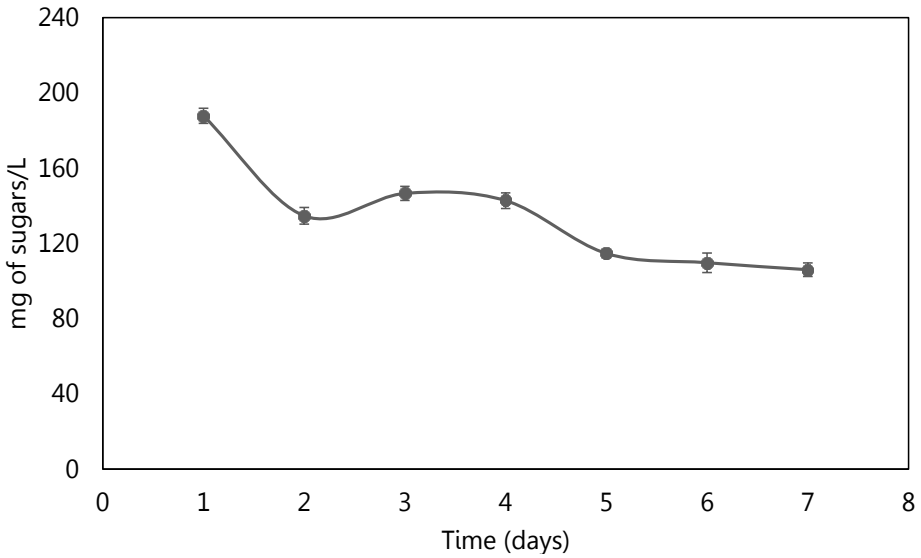

Figure 1. Fermentation process graphic of sugars in which is possible to observe a fluctuating behavior.

week one sample was taken until the 8th week. Phytase activity as well as color was evaluated. To predict the stability of the enzymes the inactivation constants were determined using Arrhenius equation and shelf life was also calculated.

Digestibility and bioavailability assessment

To study the effect of the simulated digestive tract conditions upon the stability of the microencapsulates, the conditions described by Madureira et al. (2011) were followed. Samples were prepared by dissolving microencapsulates with food and without food; as control, microencapsulates without enzyme was dissolved in a saline solution. Mouth digestion simulation was carried out adjusting pH in all tested solutions to values between 5.6 and 6.9, using HCl 1 M. Artificial saliva was simulated by using amylase (Novozymes, Denmark) at 100 U/mL and added to solutions digestion. Incubation was made within 1 min at 37°C and 200 rpm. pH was adjusted to 2.0 using HCl 1 M to simulate stomach digestion. Gastric juice was simulated by dissolving pepsin (Sigma-Aldrich Chemistry, USA) (25 mg/mL). Incubation lasted 60 min (that is, short digestion) at 37°C and 130 rpm. Simulation of gut conditions was performed as follows: intestinal juice was simulated by dissolving 2 g/L of pancreatic enzymes (Sigma-Aldrich Chemistry, USA); and pH was adjusted to 6.0 using $NaHCO_3$ 1 M. All assays were performed in duplicate. Samples were submitted to an extraction process to evaluate phosphorus release during the GIT, were thereafter stored at 4°C and subsequently analyzed.

RESULTS AND DISCUSSION

Fermentation process

Figure 1 shows a fluctuating graphic for sugars. The higher sugar amount was observed in the first day, then, as long as time is going on, the amount of sugar presents a fluctuating behavior. In day three and four, sugar amount increase and from day four, sugar amount decreases. This behavior could be because the microorganism produces sugars or hydrolyzes them from the substrate used. The level decreased in the first day, in association with microbial growth wherein micro-organism consumes sugar presented from medium; however, in the following days it was fluctuating. This may be related to the exponential phase in which the microorganism synthetizes a great quantity of metabolites such as sugars. Besides, protein production is increased in the first day. Neira et al., (2013) observed the same behavior and asserted that in the third day there could be a process of adaptation, which can promote the production of enzymes. In addition, Neira et al., (2013) commented that this sugar production come from the hydrolysis of vegetal material, with each other, sugars from cell wall and other structures. There are reports that phytases are involved in dephytinization process in cereals, in which inorganic phosphorus, proteins and reducing sugars are being released (Turki et al., 2011). Therefore, this fluctuating behavior could be related to sugar produced by the metabolism of microorganism and the releasing of sugars from the substrate.

Figure 2 shows the results related with the fermentation process. It was possible to observe the protein production in the first day which was increasing until the maximum production in the fifth day. In this stage, the microorganism is found in the exponential phase, where different kinds of protein are produced, like enzymes, to get used to the medium. According to Costa et al., (2010), the microorganism uses the proteins produced as a nutrient, which could be used as nitrogen source. This value is similar to the phytase activity (Figure 2), which is related to the content of protein. It was possible to

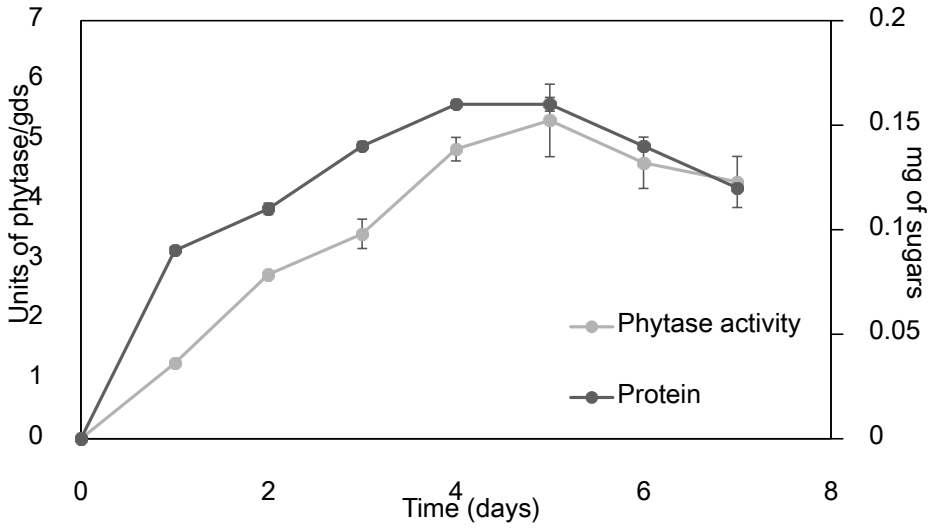

Figure 2. Phytase activity and protein plot in relation with time for eight days.

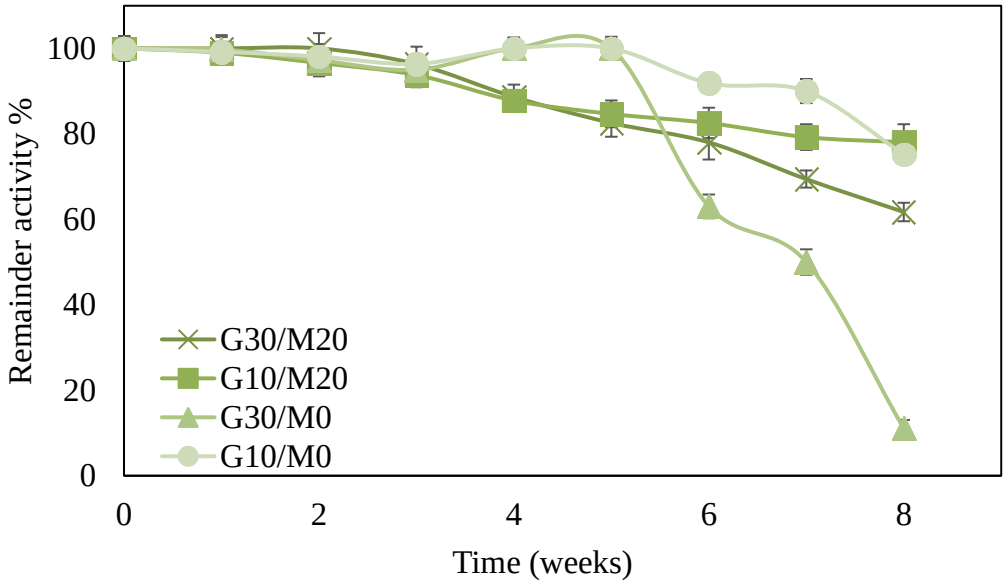

Figure 3. Phytase stability for 8 weeks. G30/M20: Glycerol 30% (v/v)/Maltose 20% (w/v), G10/M20: Glycerol 10% (v/v)/Maltose 20% (w/v), G30/M0: Glycerol 30% (v/v)/Maltose 0% (w/v), G10/M0: Glycerol 10% (v/v)/Maltose 0% (w/v).

corroborate what Neira et al., (2013) found and selected as the maximum production on the fifth day.

Phytase stabilization

Stabilization was carried out with glycerol (G) and maltose (M) as Figure 3 shows the behavior during the 8 weeks. It was possible to observe that for G30/M20 the activity was lost gradually until 62% from his initial activity. G10/M20 retained an activity of 78% after 8 weeks. Both samples contain maltose 20% (w/v) which can be related to this behavior. It has been reported that some sugars allow the stability of proteins by the creation of bonds among the enzyme and glycerol (Crowe, 2002; Singh et al., 2015). Though there is a difference when maltose is not in the sample.

In week eight, G30/M0 lost a little bit more than 90% from his initial activity, and G10/M0 lost 25%. This could be due to lack of maltose. The great difference among

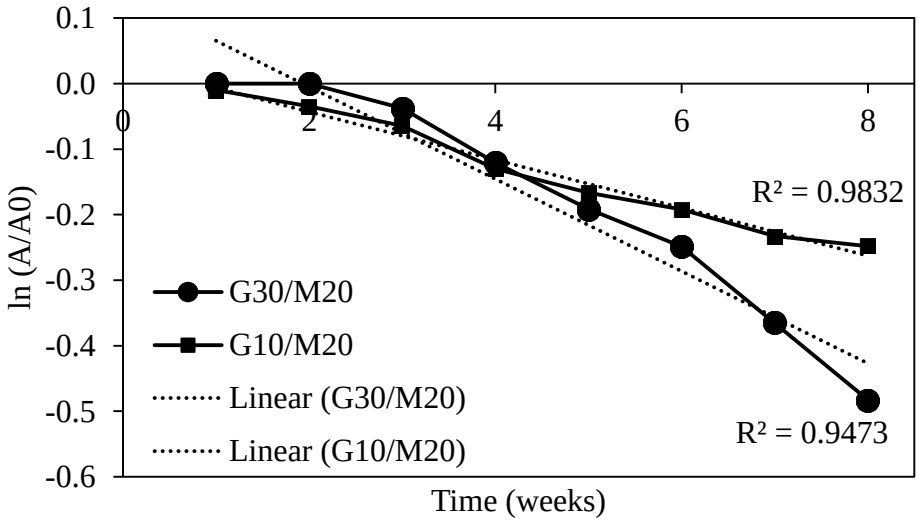

Figure 4. Linearization chart using Arrhenius equation. G30/M20: Glycerol 30% (v/v)/Maltose 20% (w/v), G10/M20: Glycerol 10% (v/v)/Maltose 20% (w/v).

Table 1. ki and half-time (t1/2) for the stability treatments that present a first order kinect.

Treatment	k_i	$t_{1/2}$
G30/M20	0.031	22.35
G10/M20	0.036	19.46

this compared to samples is bigger and this allow determination of the influence of maltose and its interaction with glycerol upon the stability of phytase.

The samples with maltose and glycerol were selected according to regression value where both samples showed straight lines with high regression coefficient values (R^2>0.95) (Figure 4). This demonstrates that thermal inactivation kinetics for both preparations follow a first-order kinetic behavior in which concentration decrease proportional to time. k_i and half time ($t_{1/2}$) were calculated and shown in Table 1. The values for ki are similar among them and it was possible to deduce that maltose together with glycerol could be the elements that provides this stability to phytase.

Rodríguez-Fernández et al. (2013) described that the inactivation of the phytase could be owing to irreversible chemical degradation process that is related to the amount of water in the samples. When the enzyme is in liquid or solution, proteins can get hydrated through the formation of hydrogen bounds (Costa et al., 2002). Neira et al. (2018) also reported similar ki; they used glycerol, maltose and phenyl methyl sulphonyl fluoride (PSMF), with the last one acting as proteases inhibitor. The present study shows values similar to Neira et al., (2018) without the addition of PSMF. They attribute it to peptides folding and stabilization of third and quaternary structure, due to the formation of stronger bounds among enzyme, glycerol and maltose, while PSMF inhibits the effect of proteases in the medium (Beg and Gupta, 2003).

Half-life results indicate that both samples present small differences, which can be due to the presence of maltose. Mensink et al. (2017) proposed two ways of how sugar protects proteins in solution. Vitrification and water replacement are the two ways, but the complete mechanism has not yet been understood. It is possible to determine that sugar could create a rigid matrix around the protein which avoids the movement of enzyme and preserves the structure. It has been shown that smaller and molecular oligosaccharides are better able to stabilize due to its molecular flexibility. Sugars produce stronger interactions and a tighter packing reducing free volume (Kamerzell et al., 2011).

One possible reason of this may be related to the fact that smaller sugars fill smaller cavities in the protein structures (Mensink et al., 2017). Besides, sugar can also interact with aromatic protein groups presented in enzymes. However, although there are two possible explanations on sugar stabilization of proteins, Grasmeijer et al. (2013) proposes that these two explanations are related with glass transition temperature (Tg); vitrification mechanism is presented if the temperature used is below Tg. Above Tg, protein mobility is reduced and water

Table 2. Elemental composition for whey compared with literature.

Parameter	Result (%)	Range (%)	Reported (%)	Reference
Color	L = 60.66 ± 0.2	-	55-70	Hernández-Rojas and Vélez-Ruiz (2014)
Fat	6.7 ± 0.02	6-8	-	Teniza (2008),
Total solids	8.6 ± 0.01	4-5	-	Poveda (2013)
Protein	1.73 ± 0.25	0.7 - 1.2	0.1 - 10	De Jesus and Maciel Filho (2014)
Ashes	0.63 ± 0.01	0.6 - 0.8	-	Gupta et al. (2014)
pH	4.9 ± 0	<5.0	4.5 - 5.5	Neira (2017)
Lactose	4.89 ± 0.005	4 - 5	5	Gupta et al. (2014)

Table 3. Preliminary test in which it was evaluated whey and guar gum at 150°C to observe the response at this temperature.

Treatment (%)	Temperature/pump	Yield
Whey/GG 0.0	150°C/30%	85% ±0.91
Whey/GG 0.25	150°C/20%	NY
Whey/GG 0.50	150°C/20%	NY
Whey/GG 1.0	150°C/20%	NY

NY: No yield.

replacement mechanism becomes the most suitable explanation. Protein which are in aqueous solution might be maintained with polyols that replace the bonds of water and the formation of a water shell around prevents protein from unfolding.

Phytase microencapsulation

Whey characterization

In order to determine the whey elemental composition and evaluate the properties that make it affordable for microencapsulation, physicochemical analysis was carried out. Table 2 allows observing variations with previous reports. These variations could be related with the origin of whey. There are reports which indicate that variations in whey compositions are as a result of animal feed, animal growth stage, milk composition and kind of cheese produced (Poveda, 2013).

For this study, lactose, protein, pH and ashes (mainly NaCl and CaCl) (Tsakali et al., 2010) were the elements more important in whey composition. This is because whey is characterized by high protein content, which is beneficial to be used for different purposes (Ganju and Gogate, 2017). Both isolated and concentrated whey protein have been used as binders (Aghbashlo et al., 2012; Koç et al., 2015; Mehyar et al., 2014). Lactose has been used as a stabilizer and has shown a great effect over the encapsulated material (Gonçalves et al., 2013). Whey contains minerals as NaCl and CaCl, which have been used as stabilizer as well (Pescuma et al., 2015). According to Neira et al., (2018), phytase from *A. niger* has an optimum pH of 4.5-5.5. Whey pH is suitable for maintaining the phytase bioavailability. This study shows the potential of the use of whole whey.

Liquid feed, dryer equipment and operating conditions

Aimed at observing the condition in which whey with guar gum should be dried, a preliminary test was carried out and results obtained (Table 3). Whey without guar gum was well dried with a considerable yield, but when guar gum was added and dried, it was not possible to obtain microencapsulates. Glass transition temperature (Tg) could be defined as the temperature at which a material turns from a hard material to a glassy material and it must be considered when microencapsulation is used. When drying temperature is higher than Tg, a sticky solution will appear in spray cylinder.

Nevertheless, it is possible to avoid this phenomenon, as mixing materials with high Tg could increase the protection and prevent the sticky solution formation. Also, since it was not possible to observe that in this material GG has a Tg of 120°C, (Mudgil et al., 2012), it was then necessary to decrease inlet temperature.

Taking these results into consideration, this experiment was performed by factorial design with one central point. Statistical analysis allows determining the better conditions to dry samples. From numbers 1 – 8, values

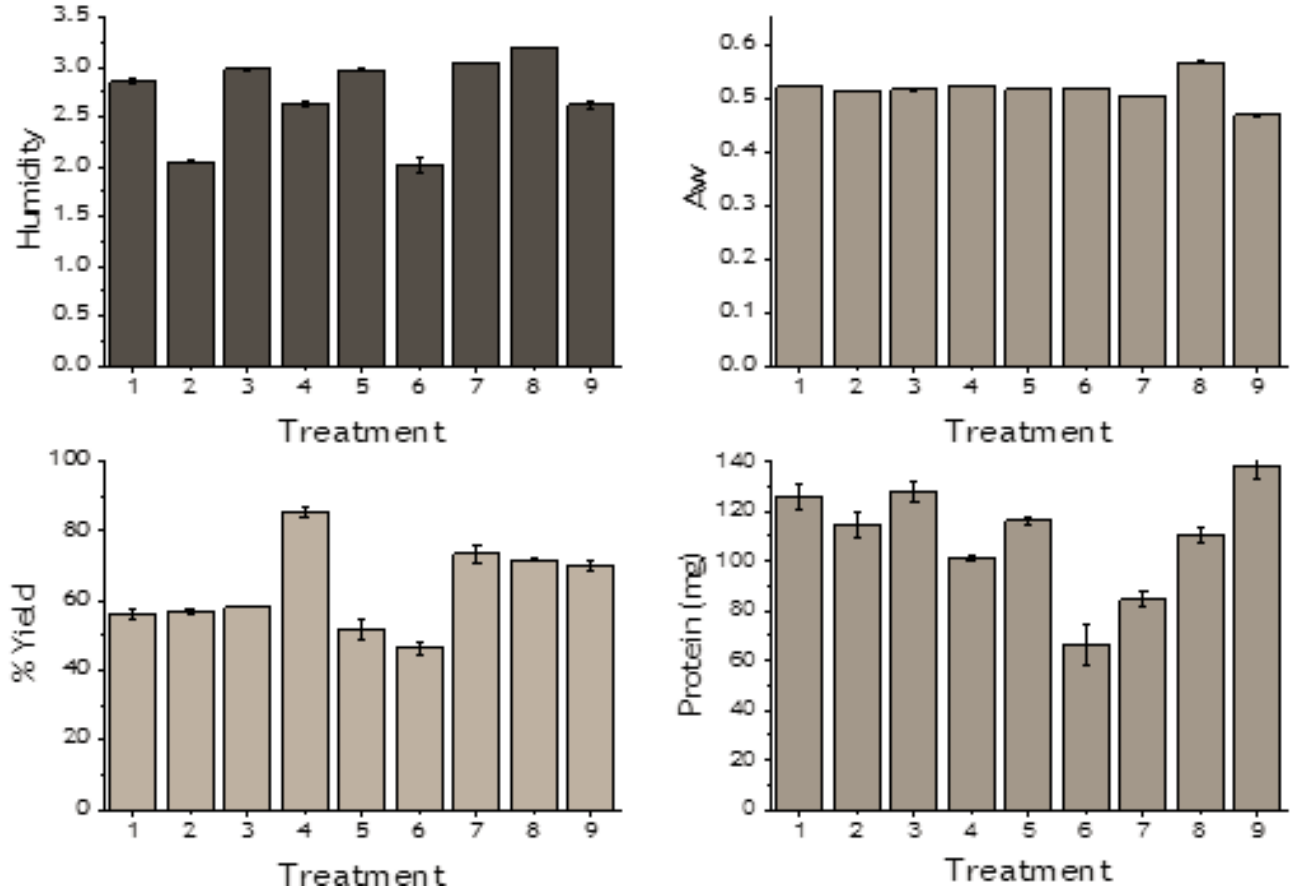

Figure 5. Treatments graphics for the factorial design. a) humidity, b) water activity, c) yield, d) protein, e) phytase activity, f) color.

from odd numbers treatments (ONT) are samples dried at 130°C, even numbers treatments (ENT) are samples dried at 110°C and central point treatments (CPT) is number nine (120°C). Results are shown in Figure 5.

Humidity and water activity

Humidity results are shown in Figure 5a. Micro-encapsulates presents low humidity no more than 3.5% and is related to the drying, which is one of the advantages of spray drying process. This value is important because higher humidity values could promote the growing of microorganism and deteriorate encapsulate (De Castro-Cislaghi et al., 2012). In the Figure 5b, it can be observed that the result of water activity (Aw) is an important data as it allows predicting possible changes in the product as Maillard reaction. Some authors described that lower values to 0.6 could promote a long-time storage availability (Beg and Gupta, 2003; Poveda, 2013). In this study, the values of all samples were below the reference. Outcomes obtained can be a favorable factor for preserving phytase due to growth of microorganisms when water activity exceeds 0.8, wherein fungus and bacteria tend to grow.

Yield

In the case of yield, treatment numbers 4, 7, 8 and 9 had a yield above 60% (Figure 5c). A yield above 60% represents an efficient drying. This could be due to the flow applied. Microencapsulation by means of spray drying of enzymes comes with great challenges due to some materials that must be dried at high temperatures to obtain higher yields. However, proteins like enzymes, cannot resist high temperature; hence it is necessary that low temperatures be used, and is the reason why higher yields are difficult to obtain. Some examples of yield are presented in Table 4.

There is a report on phytase microencapsulation in which the maximum yield obtained was 68%, different materials were tested and yield obtained was from 35 - 68%, with maltodextrin the most unsuitable material due to low yield at low temperatures (Spier and Greiner, 2015).

Table 4. Spray drying process of enzymes.

Enzyme	Carrier or binder	Temperature Inlet/outlet (°C)	Yield (%)	Enzymatic activity (%)	Reference
Xylanase	Magnesium sulfate and malt extract	140/65.2	62.14	99.4	Gupta et al. (2014)
α-Amylase	None	145/90	Data not mentioned	83	De Jesus and Maciel Filho et al. (2014)
Lipase	Skim milk powder, Maltodextrin, Arabic gum	160/85	100	64	Souza et al. (2014)
β-galactosidase	Arabic gum	115/58	56	20%	Estevinho et al. (2014)
Phytase	Starch	100/68	63.1	41.2	Spier and Greiner (2015)

Table 5. Color assessment from factorial design treatments.

Treatment	*L**	*a**	*b**	*c**	*h**
1	71.95 ± 0.44	5.61 ± 0.12	18.16 ± 0.29	19.00 ± 0.30	72.83 ± 0.92
2	60.06 ± 0.68	7.57 ± 0.13	22.15 ± 1.03	23.40 ± 1.10	71.12 ± 0.34
3	71.64 ± 0.90	5.64 ± 0.60	17.97 ± 1.08	18.84 ± 1.49	72.58 ± 1.75
4	54.61 ± 0.67	8.47 ± 1.37	23.60 ± 0.81	25.08 ± 1.5	70.27 ± 1.55
5	74.27 ± 1.17	5.11 ± 1.29	16.66 ± 0.95	17.43 ± 1.75	72.97 ± 1.15
6	60.24 ± 0.91	7.21 ± 1.39	21.94 ± 0.65	23.10 ± 1.42	71.83 ± 1.68
7	74.52 ± 1.27	4.65 ± 1.25	15.15 ± 1.46	15.85 ± 0.85	72.91 ± 0.94
8	50.84 ± 1.12	8.00 ± 0.32	21.9 ± 1.54	23.32 ± 1.64	69.92 ± 1.30
9	72.65 ± 1.69	5.47 ± 0.57	16.28 ± 1.22	17.23 ± 0.69	71.54 ± 0.59

L* indicates light-ness (0 = black and 100 = white), a* and b* are coordinates for green (-a*)/red (+a*), and blue (-b*)/yellow (+b*). Hue angle (H* = tan-1 b*/a*).

In this study, the Treatment 7 shows higher yield. Protein was measured, and results are shown in Figure 5d. High protein content was observed, which is normal considering that the majority of whey composition is protein. Besides, the protein was related with whey. The crude extract used is not purified; also, the presence of other enzymes could be the reason why this protein content is considerable. *A. niger* produces phytases, lipases, cellulases, xylanases, β-xylosidase, tannase, among others, however, the process used promote the production mostly of phytase, without the exclusion of other enzymes.

Color assessment

The color was also evaluated using the color values result shown in Table 5. L* value is scaled from 0-100, in which 0 is black color and 100 is white color. It was possible to obtain results from 50-70. Compared with what was observed in elemental composition, this value is similar to the one after spray drying. a* values tend from green (-) to red (+) while b* values tend from blue (-) to yellow (+), with results in a yellow range, according to (De Castro-Cislaghi et al. (2012), this color presented in the samples may be indicative of Mallard reaction due to the presence of lactose. Due to the purpose of this work which was to produce a highly drying process with high enzymatic activity, phytase activity was taken into consideration during statistical analysis (Figure 6).

Pareto chart shows that all factors and their interactions influenced phytase activity (Figure 7). Statistical analysis indicates the most suitable conditions for temperature as well as flow in low values, whereas for guar gum concentration, the higher values are better.

During drying, proteins are submitted to high shear forces while atomization is being carried out along with high-temperature air flow. These conditions result in an irreversible thermal denaturation. In this way, it was possible to set the better conditions, in which temperature is 110°C, 10% flow and guar gum 1%.

Microencapsulates characterization

Morphology

Figure 8 shows the microencapsulates micrographs, in which it is possible to observe the formation of aggregates and irregular shapes that are characteristic of

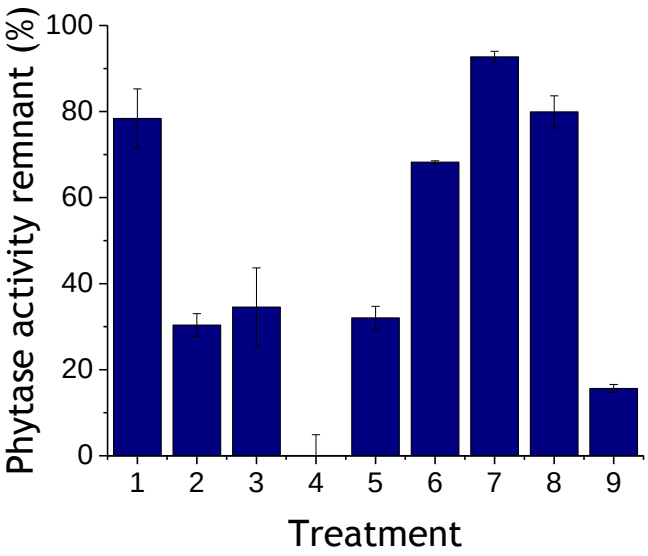

Figure 6. Phytase activity remnant for microencapsulates of experimental design.

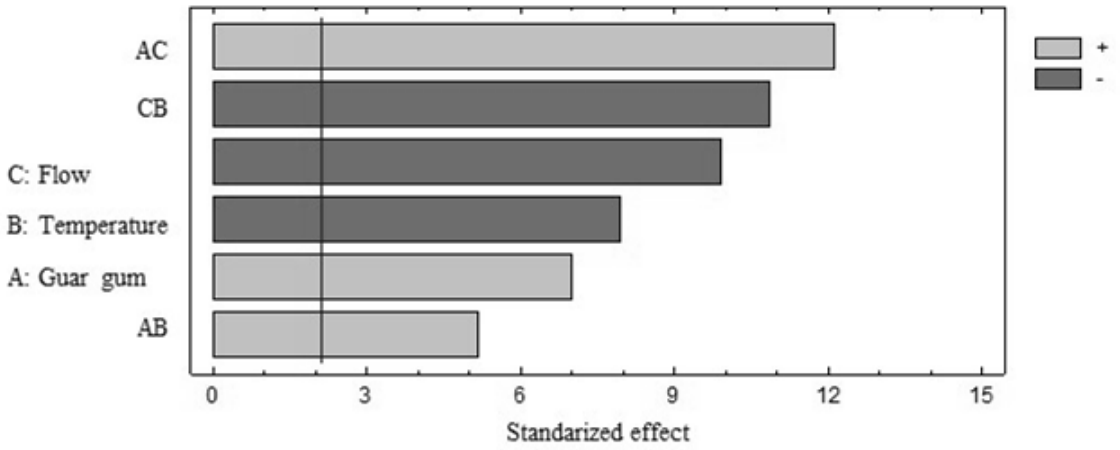

Figure 7. Pareto chart for phytase activity drying condition.

spray drying process. In Figure 8a and 8b, microencapsulates present soft surfaces and with the presence of pores; whereas in Figure 8c and 8d, the surface is smooth, but aggregates are in more compact form than microencapsulates at 130°C. This kind of shapes are typical of spray drying process (Favaro-Trindade et al., 2010). Rodriguez et al. (2005) commented that temperature and material are the reasons why these shapes are presented at low temperatures and carbohydrates, and agglomerates are promoted to be formed (Rodríguez-Huezo et al., 2007). This differences in surfaces, between microencapsulates dried at 110 and 130°C could be due to condition process or that the material make the formation of a tight matrix difficult owing to quick evaporation of water (Favaro-Trindade et al., 2010). However, Cunha et al., (2005) observed that GG tend to form microencapsulates with pores, which is because there is a cross-linking formed between hydrogen bonds and enzyme, which could protect the enzyme from being encapsulated (Cunha et al., 2005).

Currently, the reason why formation of aggregates is not well known may be that the phenomenon is influenced by different factors as earlier mentioned. The knowledge of this process could be useful for physicochemical explanation. However, Benavent et al. (2018) worked on a study in which he observed the formation of aggregates around some capsules. They

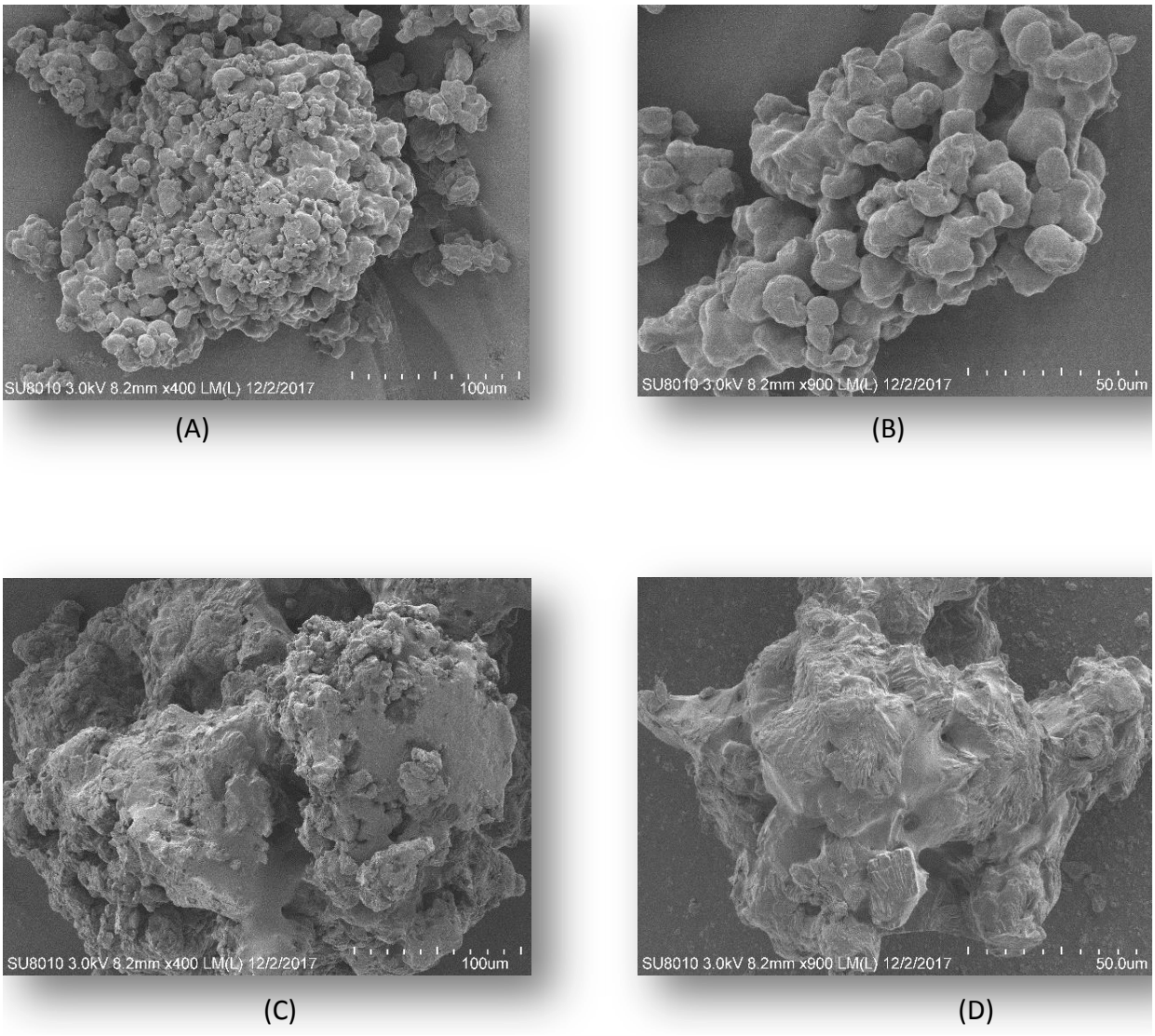

Figure 8. Micrograph of microencapsulates: a) 130 °C/GG 1% a 400x, b) 130 °C/GG 1% a 900x, c) 110 °C/GG 1% a 400x, d) 110 °C/GG 1% a 900x. The presence of aggregates is observed.

commented that the formation of aggregates is due to the interaction among materials used (Benavent-Gil et al., 2018; Cunha et al., 2005). These results could be compared with the explanation by Costa et al., (2002) who worked with starch and gelatin and observed the formation of aggregates as well. They attributed this to the presence of gelatin protein, which leads to this observatio and pointed out that when starch without gelatin is dried, microencapsulates are more scattered but, when gelatin protein is added, the formation of aggregates are induced (Beirão-Da-Costa et al., 2011). This was mentioned previously by Zhao and Whistler (1994), who demonstrated that these aggregates are produced when suspensions are submitted to spray-drying if bonding agents are presented like polysaccharides or proteins at any temperature.

Determination of weight loss by thermogravimetrical analysis

Thermal analysis demonstrated that for whey and whey with guar gum samples, the thermal behavior was similar; however, for the enzyme, extract and guar gum was completely different. Both samples suffered the first weight loss around 120-200°C, mostly water; whereas the second weight loss from 200 to 450°C was slow and could be related to thermal decomposition with the formation of volatile reaction products. The next mass loss takes place between 450 to 550°C, with a residue of 15%. Guar gum thermogram showed the first loss around 75 to 150°C, probably due to the amount of humidity. The second loss for guar gum is very fast, beginning from 290°C and ending at 320°C, and was attributed to

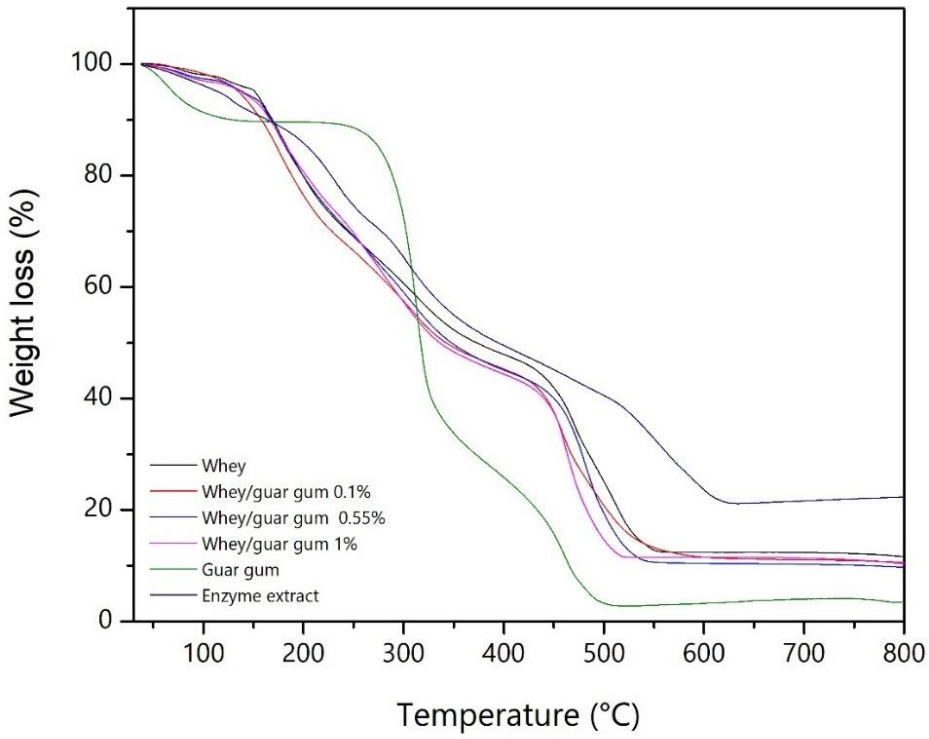

Figure 9. Thermogram for samples of Whey, Whey/GG 0.1%, Whey/GG 0.55%, Whey/GG 1%, GG and enzyme extract.

polymer decomposition. Gliko-Kabir (1999) reported values for this mass loss in a range of 280 to 310°C (with some differences of 10 to 30°C), in which lower temperature is required to cause 65% of weight loss (Gliko-Kabir et al., 1999). The last mass loss is observed from 330 to 500°C with a low residual of 5% which is due to guar gum composition that is mainly of organic compounds that are quickly lost and burns almost completely. For enzyme extract crude, it was possible to observe three mass losses: the first one related with water from 70 to 200°C; the second was less than the other samples and was from 200 to 500°C; and the third was from 500 to 600°C with a remnant of 20% which could be related to minerals and inorganic carbon presented in sample.

According to outcomes, it was possible to observe that the interaction of whey with guar gum improve the stability of guar gum without changing thermal behavior of whey. However, enzyme extract behavior is not improved or maintained, which may be due to the presence of minerals and residues of fermentation process, it is worth highlighting that enzyme extract was obtained by lyophilization after the recovery in solid state fermentation.

The thermal stability is important because in some cases during the process in which microencapsulates are applied, high temperatures are involved (Cunha et al., 2005). For this reason, the knowledge of thermal behavior is an important characteristic for materials used in biological process.

X-Ray diffraction

Diffractogram for this samples indicate that samples analyzed have amorphous structure (Figure 10). Guar gum diffractogram show a signal but this is not enough to affirm that guar gum has a crystalline structure. It is worth highlighting that when whey and enzymatic extract was added, the signal observed in guar gum decreased in microencapsulates; however there are not differences among samples with whey and whey/GG. Costa et al., (2011) found that when starch and material considered as semi-crystalline was examined with XRD it was possible to observe clear peaks; but when gelatin was added, the shape and intensity of some peaks undergo small changes for gelatin (Beirão-Da-Costa et al., 2011). Gelatin is mainly composed of fibrous protein by means of denaturation of collagen (Jellouli et al., 2011).

Protein content in gelatin could be different among materials, approximately from 8.8 - 12.8% (Silva et al., 2014); this protein is responsible for this decrease in shapes and intensity. Whey is formed by proteins, mainly β-lactoglobulin and α-lactalbumin(Ganju and Gogate,

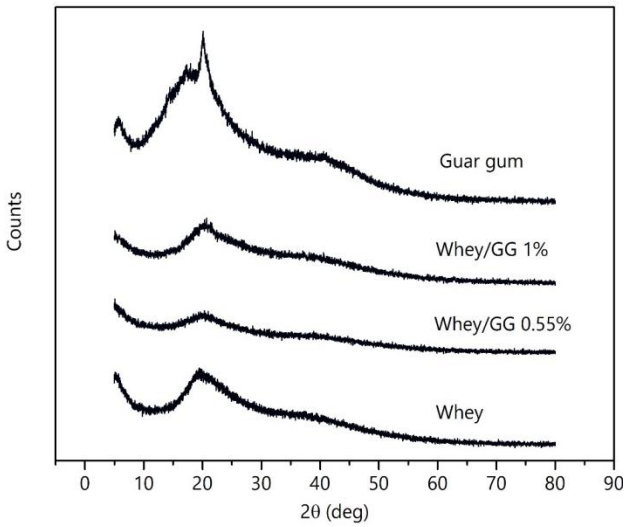

Figure 10. Diffractogram for Whey, Whey/GG and GG in which is possible to observe an amorphous structure for the mix of whey and guar gum in any concentration.

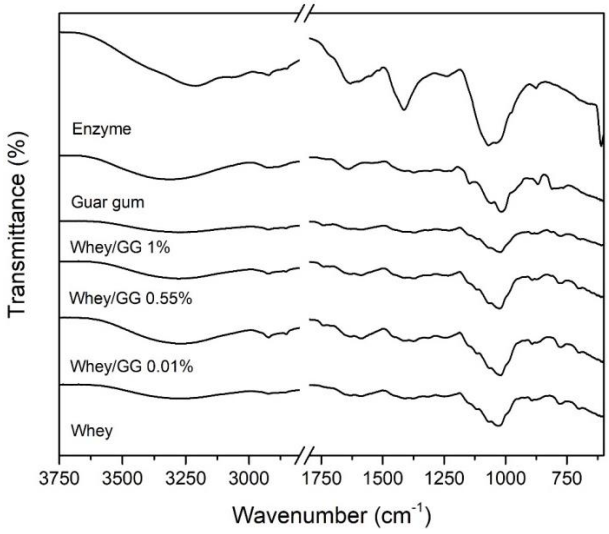

Figure 11. IR spectrums for Whey, Whey/GG 0.1%, Whey/GG 0.55%, Whey/GG 1%, GG and enzyme extract.

2017), and is the reason why the shape and intensity decreased. The interaction of whey protein and guar gum is reflected in the diffractogram. It was possible to observe the effect of protein over the semi-crystallinity structure of guar gum.

Infrared

It is possible to observe in IR spectrums (Figure 11) an absorption band at around 3450 to 3250 cm^{-1} for the O-H stretching vibration. At 2950 to 2940 cm^{-1}, a signal was observed that corresponded to C-H stretching vibration. Then, guar gum structure (Figure 12) present a plane bending, which can be observed in 1423.55 cm^{-1}, with the stretching vibration of alcoholic group of guar gum at 1249 cm^{-1} (Gliko-Kabir et al., 1999; Zhao and Whistler, 1994). These peaks are found in GG and whey/GG, through which it is possible to confirm the presence of GG in microencapsulates.

Signal for proteins are from 1800 to 800 cm^{-1}. Ven et al. (2002) characterized casein and whey and found that

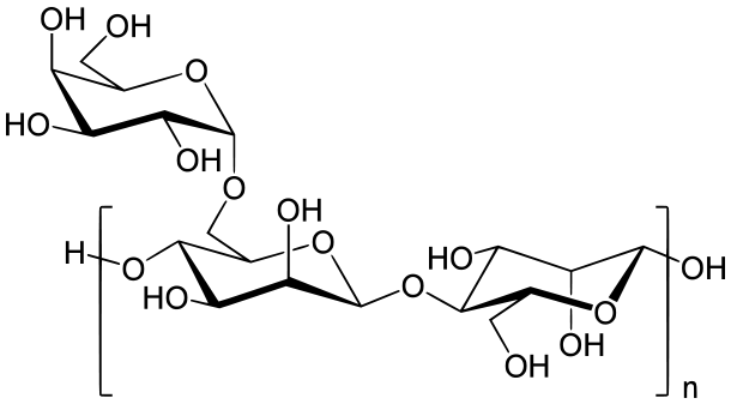

Figure 12. Guar gum molecular structure.

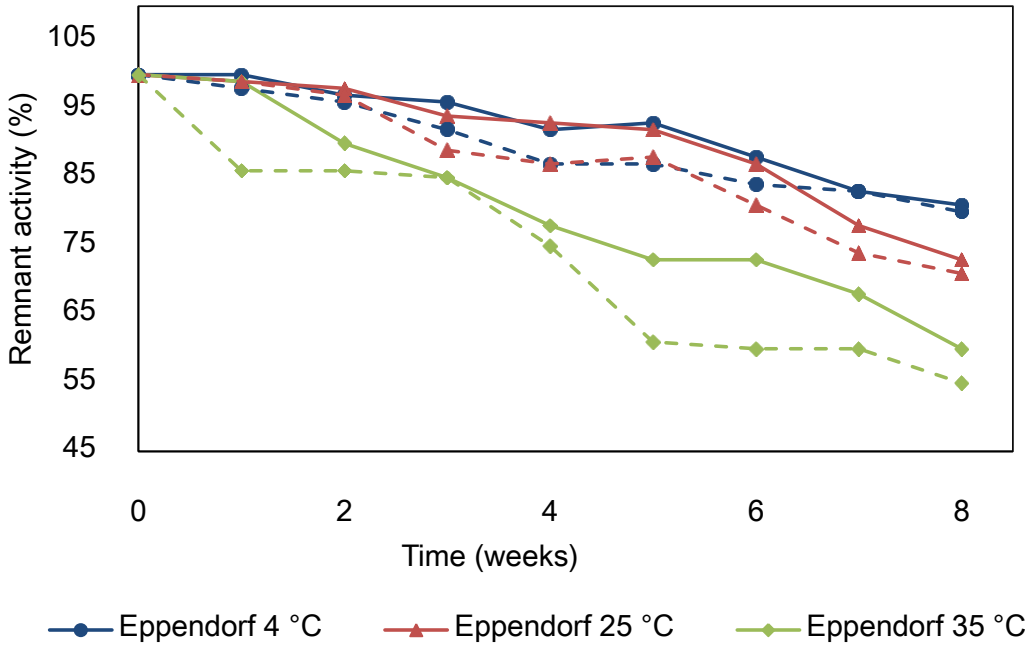

Figure 13. Remnant activity graph for storage availability in different containers over eight weeks.

both samples present same signal around 1700 to 1485 cm^{-1}, values that may be related with proteins (casein and whey proteins) (Van der Ven et al., 2002). Forato et al., (1998) investigated the IR of proteins, in which they found that the secondary structure is preserved by means of the observation of amides (A, I, II and III) (Forato et al., 1998). Chanphai (2017) observed the amide I around 1660–1650 cm^{-1} and the amide II at 1550–1530 cm^{-1} (Chanphai and Tajmir-Riahi, 2017). These signals are presented in IR spectra, specifically in enzyme extract, which could be due to enzymes in the extract. There are a few reports of whey protein, due to there being a great variety of proteins, and every protein is characterized by a specific structure.

Storage availability

In Figure 13, the graphics for storage assessment are presented. Continuous lines belong to Eppendorf containers, while discontinued lines belong to Ziploc bags. It is possible to observe that for both containers a similar behavior was observed at each temperature. For 4°C (blue lines), microencapsulates retain 80% of remnant activity after 8 weeks, while for 25°C temperature (orange lines), only around 75% is retained for both

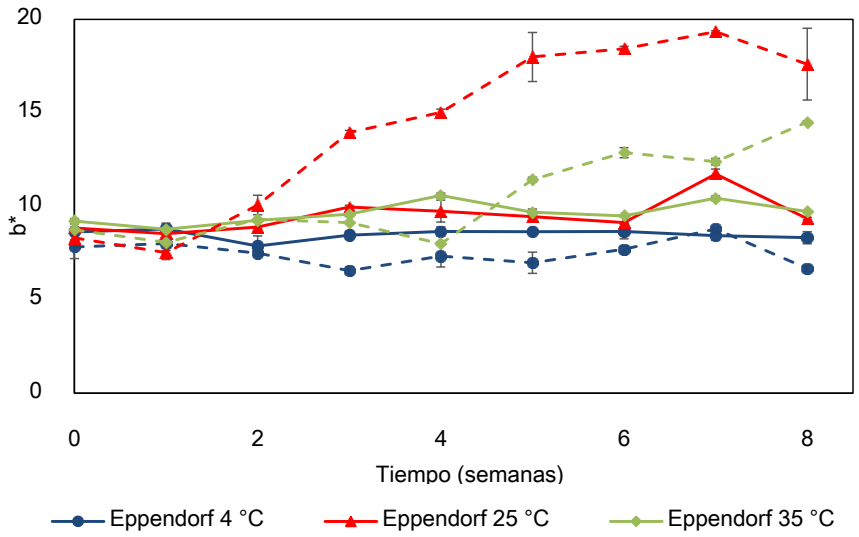

Figure 14. Color graph for storage availability for assessment of the influence over enzymatic activity.

systems. Some authors have reported phytases shelf life of 14 to 20 days at 4°C (Mudgil et al., 2012; Sweeti and Sawarkar, 2015; Van der Ven et al., 2002). Spier et al., (2015) preserves phytase testing of different materials. Microencapsulates with Na-alginate retained 52% at room temperature for 60 days. Outcomes of the present investigation were better as the use of whey and guar gum is a promising alternative as protective material for phytase. It was expected that for 35°C, the loss of enzymatic activity was less than the other temperatures; however, it was possible to observe 55% of remnant activity.

Color assessment during storage analysis

In Figure 14, the color analysis in which continuous lines belong to Eppendorf containers, whereas discontinued lines belong to Ziploc bags is shown. Microencapsulates in Eppendorf container does not present a color variety, as for three temperatures evaluated, the color is kept as in the beginning. Different results are presented in Ziploc bags for 4°C as there is no difference with Eppendorf container; but for 25 and 35°C, color tend to change slowly from white to light yellow (Figure 15). The change in color is attributed mainly to Maillard reactions. At low temperatures, Maillard reactions are not promoted, while at other temperature (like 25°C room temperature and 35°C), Maillard reaction appear. This is owing to the lactose present in whey (Van Boekel, 1998). It is noted that whey was used completely without separating its components. Some of the major consequences of this reaction includes agglomeration of proteins causing inactivation and browning process (Zin El-Din and Aoki, 1993). The material properties, like permeability may be the reason why the color changes. Results shown in this work demonstrated that although color is different between both containers, phytase activity is not affected by this phenomenon. For this reason, there is no difference in which containers phytase is stored. However, there are other factors that could affect the stability of phytase microencapsulates as permeability. Ziploc bags are made of low density polyethylene (LDPE), a material that is very common in packing, but does not prevent permeability against humidity which could affect stability and appearance of micro-encapsulates. Furthermore, Eppendorf containers are made of polypropylene (PP), which is a material more high than LDPE and PP is impermeable to humidity (Huanchi, 2013).

Linearization of stability data and determination of k_i and $t_{1/2}$

When data is linearized, it is possible to observe reaction of first order, in which concentration is decreasing with time. Once this data is obtained, k_i is calculated, showing low values and, when shelf time was calculated ($t_{1/2}$), the number of weeks in which stability will be preserved was determined. These outcomes are presented in Table 6. k_i values are a little higher than those obtained by Spier et al., (2015) in which k_i of 0.0052 day^{-1} was calculated with a half-life of 132.10 days (18.9 weeks). Although values are in days, it is possible to observe that results presented in this work with whey and guar gum could be used on industrial process as carrier for spray drying.

In Table 7, the currently commercialized phytases is presented. It is possible to observe that more superior products with higher shelf-time can be obtained based on

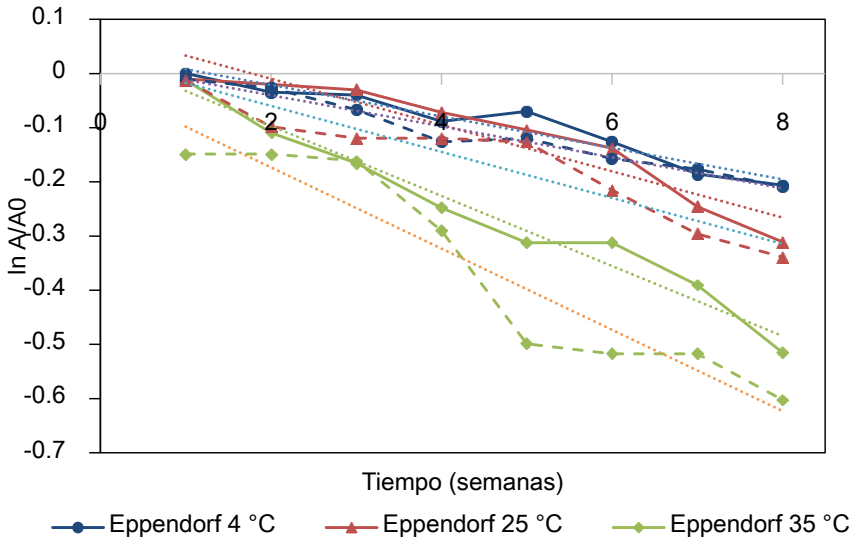

Figure 15. Linearization chart for microencapsulates stability in 8 weeks to calculate kinect reaction.

Table 6. Values for R^2, k_i and $t_{1/2}$ for storage stability assessment.

T °C	Eppendorf			B. Ziploc		
	R^2	k_i	$t_{1/2}$	R^2	k_i	$t_{½}$
4	0.9342	0.0258	26.88	0.9005	0.0262	26.5
25	0.9690	0.0389	17.82	0.9097	0.0424	16.36
35	0.9714	0.0643	10.77	0.9032	0.0753	9.2

Table 7. Commercialized phytases in powder presentation.

Study	Time (weeks)	Temperature	Color
Probioway[a]	52	Room temperature	Light yellow
Yonkong[b]	26	4°C	Light yellow
Habio[c]	26	4°C in absence of light and humidity	Light yellow
This work	27	4°C	Light yellow

[a] https://probioticspbw.en.made-in-china.com; [b] https://yonkong.en.made-in-china.com/ [c] http://en.habio.net/

this study. Nevertheless, there are other enzymes with similar values and at the same temperature, which make it competitive against those currently sold.

Digestibility and bioavailability assessment

Figure 16 shows the results for digestibility evaluation. Digestibility is the amount of food that is available to be absorbed; this factor is very important due to gastrointestinal condition that could affect the food or product, preventing the bioavailability of the compound. In mouth phase, for both samples (microencapsulates and microencapsulates with food), a low decrease in phytase activity of around 97% was observed; nevertheless, this activity is retained in stomach phase at low pH. At the end of stomach phase, phytase activity decreased to 95%. After this phase, a gradual decrease until end of intestinal phase could be observed. Around 90% of enzymatic activity is preserved after digestion process. It is worth highlighting that digestive simulation was for monogastric animals like poultry. It is well known that phytases in presence of proteases will be inactivated (Neira et al., 2018); for this reason, evaluation of phytases

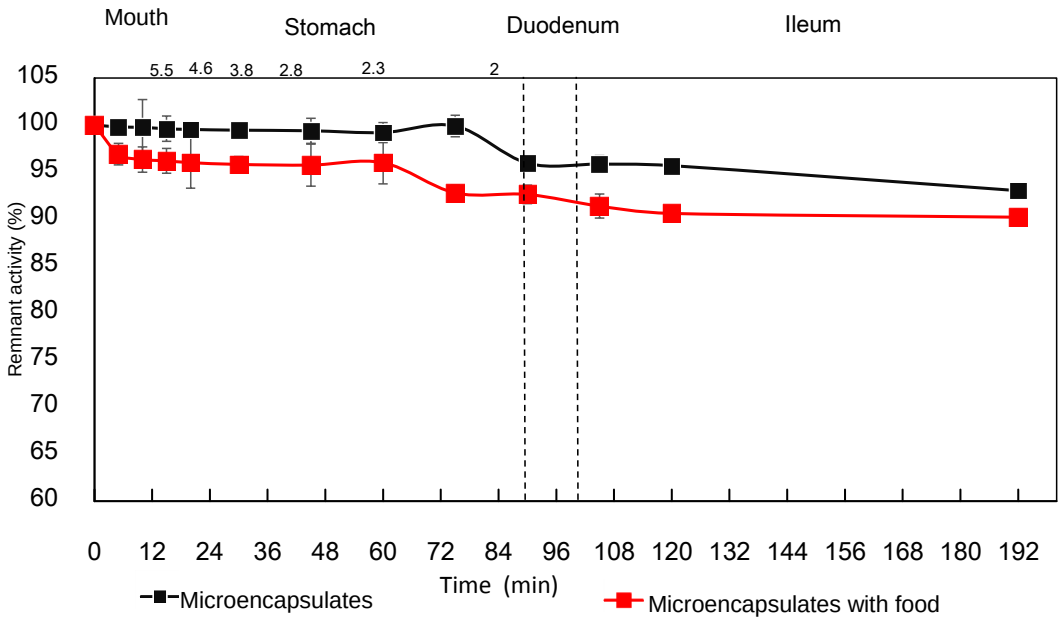

Figure 16. Remnant activities for microencapsulates and microencapsulates with food in a gastrointestinal system *in vitro*.

in gastro-intestinal system was not considered. Menezes et al. (2015) evaluated seven commercial phytases in an *in vitro* simulation of poultry digestive tract. Six phytases evaluated were in liquid presentation, with only one of them in powder presentation. This last one, after the assessment, presented just 47% of its initial activity. They attributed these results to the susceptibility of phytases to proteolytic cleavage by pepsin (Menezes-Blackburn et al., 2015). Currently, GG has been used to delay the releasing of drugs in pharmacy industry with good results (Patel et al., 2014). It has been demonstrated that GG can be used to carry drugs until colon releases the drug with higher availability (Spier et al., 2015). Li et al. (2011) observed that whey and guar gum at different pH retain the structure due to the uneasily dissociated shells formed by hydrogen bonding and Van der Waals force. Pepsin can accelerate the releasing of the compound encapsulated by means of enzymolysis. They observed that the addition of polysaccharides which are absorbed on the surface of microencapsulates forms a barrier that blocks the penetration of pepsin preventing the dissociation of microcapsules (Li et al., 2011).

It has been found that whey protein with gums can generate many lipid–water bonds in other systems (Li et al., 2011) allowing the releasing of compounds microencapsulated in intestinal phase.

Conclusion

It was possible to set conditions to dry proteins like phytases with great enzymatic activity after drying. Besides, materials used provide protection in gastrointestinal conditions. As seen from results, it is possible to use phytase solution with some sugars and polyols, making it a stable product. Outcomes demonstrated that glycerol and maltose is a great and promising cryopreservation method, besides being a reducer of aqueous activity in molecular level and in this way, prevent protein denaturalization. This study demonstrated the use of whey as a material for microencapsulation because of its composition. Currently, whey protein isolated (WPI) has been used as carrier in spray drying; however, the purification process to obtain WPI causes waste which continue to serve as an environmental pollutant. This work proposes the use of whey without purification. Results demonstrated that it is possible to combine whey with other materials as guar gum. Microencapsulation with guar gum and whey is an alternative for improving stabilization of enzymes due to the ability to protect enzyme and obtain higher yields.

Overall, it was possible to observe interactions between whey and guar gum. Functional groups of guar gum and proteins was seen in IR, which affirms the possibility of using guar gum as carrier and retain its identity together with whey protein. Characterization indicated that the microencapsulation of phytases with guar gum and whey is possible and produces higher results than other authors. Besides, storage availability assessment demonstrates the potential to obtain a product that can compete with those currently commercialized; however, the evaluation of materials or even minerals which help to improve stability is necessary. It was possible to affirm that color, promoted by Maillard reaction, do not affect

enzymatic activity. Digestive evaluation *in vitro* showed that whey with guar gum can preserve phytases against condition of gastro-intestinal system. These results have important contributions for the microencapsulation process of enzymes, which continues to present serious challenges to the enzymes. The effect of whey and guar gum was examined and the bioavailability of phytases is improved in order to promote the hydrolysis of phytate on intestinal phase, allowing the absorption of native phosphorus in cereals. These contributions may be reflected in environmental aspects, due to phytate causing eutrophication; nevertheless, the study of how microencapsulates are being released *in vivo* is necessary for future studies.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Aghbashlo M, Mobli H, Rafiee S, Madadlou A (2012). Energy and exergy analyses of the spray drying process of fish oil microencapsulation. Biosystems Engineering 111(2):229-241. https://doi.org/10.1016/j.biosystemseng.2011.12.001.

Ahmed HA, Abou-Elkhair RM (2016). Potential application of guar meal in broiler diet. Asian Journal of Animal and Veterinary Advances 11(5):280-287. https://doi.org/10.3923/ajava.2016.280.287.

Alexakis T, Boadi DK, Quong D, Groboillot A, O'Neill I, Poncelet D, Neufeld RJ (1995). Microencapsulation of DNA Within alginate microspheres and crosslinked chitosan membranes for in vivo application. Applied Biochemistry and Biotechnology 50(1):93-106. https://doi.org/10.1007/BF02788043.

Anjani K, Kailasapathy K, Phillips M (2007). Microencapsulation of enzymes for potential application in acceleration of cheese ripening. International Dairy Journal 17(1):79-86. https://doi.org/10.1016/j.idairyj.2006.01.005.

AOAC (1990). Official Methods of analysis. Association of Official Analytical Chemist. EUA.

Beg QK, Gupta R (2003). Purification and characterization of an oxidation-stable, thiol-dependant serine alkaline proteas from Bacillus mojavensis. Enzyme and Microbial Technology 32(2):294-304..

Beirão-Da-Costa S, Duarte C, Moldão-Martins M, Beirão-Da-Costa ML (2011). Physical characterization of rice starch spherical aggregates produced by spray-drying. Journal of Food Engineering 104(1):36-42.

Benavent-Gil Y, Rodrigo D, Rosell CM (2018). Thermal stabilization of probiotics by adsorption onto porous starches. Carbohydrate Polymers 197:558-564. https://doi.org/10.1016/j.carbpol.2018.06.044.

Brown TL, LeMay HE, Bursten BE (2004). Chemestry, The Central Science. pp. 506-520.

Brückner M, Bade M, Kunz B (2007). Investigations into the stabilization of a volatile aroma compound using a combined emulsification and spray drying process. European Food Research and Technology 226(1-2):137-146. https://doi.org/10.1007/s00217-006-0518-3.

Chanphai P, Tajmir-Riahi HA (2017). Trypsin and trypsin inhibitor bind milk beta-lactoglobulin: Protein-protein interactions and morphology. International Journal of Biological Macromolecules 96:754-758.

Costa M, Torres M, Magariños H, Reyes A (2010). Producción y purificación parcial de enzimas hidrolíticas de Aspergillus ficuum en fermentación sólida sobre residuos agroindustriales Production and partial purification of Aspergillus ficuum hydrolytic enzymes in solid state fermentation of agro industrial residues. Revista Colombiana de Biotecnología 12(2):163-175.

Costa SA, Tzanov T, Filipa A, Paar A, Gübitz GM, Cavaco-Paulo A (2002). Studies of stabilization of native catalase using additives. Enzyme and Microbial Technology 30(3):387-391.

Crowe LM (2002). Lessons from nature: The role of sugars in anhydrobiosis. Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology 131(3):505-513. https://doi.org/10.1016/S1095-6433(01)00503-7.

Cunha PLR, Castro RR, Rocha FAC, De Paula RCM, Feitosa JPA (2005). Low viscosity hydrogel of guar gum: Preparation and physicochemical characterization. International Journal of Biological Macromolecules 37(1-2):99-104.

Das SK, David SRN, Rajabalaya R, Mukhopadhyay HK, Halder T, Palanisamy M, Nanda A (2011). Microencapsulation Techniques and its Practices. International Journal of Pharmaceutical Science and Technology 6(2):1-23.

De Castro-Cislaghi FP, Silva CDR, Fritzen-Freire CB, Lorenz JG, Sant'Anna ES (2012). Bifidobacterium Bb-12 microencapsulated by spray drying with whey: Survival under simulated gastrointestinal conditions, tolerance to NaCl, and viability during storage. Journal of Food Engineering 113(2):186-193.

De Jesus SS, Maciel Filho R (2014). Drying of α-amylase by spray drying and freeze-drying - A comparative study. Brazilian Journal of Chemical Engineering 31(3):625-631.

Dutta S, Bhattacharjee P (2017). Microencapsulation of enzyme-assisted supercritical carbon dioxide extract of small cardamom by spray drying. Journal of Food Measurement and Characterization 11(1):310-319.

Eckert C, Serpa VG, Felipe dos Santos AC, Marinês da Costa S, Dalpubel V, Lehn DN, Volken de Souza CF (2017). Microencapsulation of Lactobacillus plantarum ATCC 8014 through spray drying and using dairy whey as wall materials. LWT-Food Science and Technology 82:176-183.

Estevinho BN, Damas AM, Martins P, Rocha F (2014). Microencapsulation of β -galactosidase with different biopolymers by a spray-drying process. Food Research International 64:134-140.

Favaro-Trindade CS, Santana AS, Monterrey-Quintero ES, Trindade MA, Netto FM (2010). The use of spray drying technology to reduce bitter taste of casein hydrolysate. Food Hydrocolloids 24(4):336-340.

Forato LA, Bernardes-filho R, Colnago LA (1998). Protein Structure in KBr Pellets by Infrared Spectroscopy Protein Structure in KBr Pellets by Infrared Spectroscopy. Analytical Biochemistry 259(1):136-141.

Gangurde AB, Fule RA, Javeer SD, Patole RK, Pawar JN, Amin PD (2015). Microencapsulation using aqueous dispersion of lipid matrix by fluidized bed processing technique for stabilization of choline salt. Journal of Pharmaceutical Investigation 45(2):209-221

Ganju S, Gogate PR (2017). A review on approaches for efficient recovery of whey proteins from dairy industry effluents. Journal of Food Engineering 215:84-96.

Gliko-Kabir I, Penhasi A, Rubinstein A (1999). Characterization of crosslinked guar by thermal analysis. Carbohydrate Research 316(1-4):6-13.

Global Phytases Market Report (2017). Global Phytases Market Report. Retrieved from https://www.marketresearchstore.com/report/global-phytases-market-rport-2017-150562.

Gonçalves HB, Jorge JA, Oliveira WP, Souza CRF, Guimarães LHS (2013). Extracellular β-fructofuranosidase from Fusarium graminearum: Stability of the spray-dried enzyme in the presence of different carbohydrates. Journal of Microencapsulation 30(7):624-631.

Grasmeijer N, Stankovic M, Waard H, Frijlink HW, Hinrichs WLJ (2013). Unraveling protein stabilization mechanisms: Vitrification and water replacement in a glass transition temperature controlled system. BBA - Proteins and Proteomics 1834(4):763-769.

Gupta G, Sahai V, Mishra S, Gupta RK (2014). Spray-drying of xylanase from thermophilic fungus melanocarpus albomyces- Effect of carriers and binders on enzyme stability. Indian Journal of Chemical Technology 21(2):89-95.

Hamin YAN, Barbosa CL, Cabral LA (2017). Box-Behnken Analysis and Storage of Spray-Dried Collagenolytic Proteases From Myceliophthora Thermophila Submerged Bioprocess. Preparative Biochemistry and Biotechnology 47(5):473-480.

Harland BF, Harland J (1980). Fermentative reduction of Phytate in Rye, White , and Whole Wheat Breads. Cereal Chemistry 57(3):226-

229.
Hernández RM, Vélez-Ruiz JF (2014). Whey and its aplication to funtional food elaboration. Selected Topics of Food Engineering 8(2):13-22.
Huanchi R (2013). Determination of the permeability of plastic packaging. Universidad Nacional de la Amazonia Peruana. Retrieved from http://repositorio.unapiquitos.edu.pe/bitstream/handle/UNAP/2449/Determinación de la permeabilidad en empaques plásticos.pdf?sequence=1andisAllowed=y.
Jellouli K, Balti R, Bougatef A, Hmidet N, Barkia A, Nasri M (2011). Chemical composition and characteristics of skin gelatin from grey triggerfish (Balistes capriscus). LWT-Food Science and Technology 44(9):1965-1970.
Jesus SS, Filho RM (2014). Drying of α-amylase by spray drying and freeze-drying a comparative study. Brazilian Journal of Chemical Engineering 31(3):625-631.
Kamerzell TJ, Esfandiary R, Joshi SB, Middaugh CR (2011). Protein-excipient interactions: mechanisms and biophysical characterization applied to protein formulation development. Advanced Drug Delivery Reviews 63(13):1118-1159.
Koç M, Güngör Ö, Zungur A, Yalçın B, Selek İ, Ertekin FK, Ötles S (2015). Microencapsulation of Extra Virgin Olive Oil by Spray Drying: Effect of Wall Materials Composition, Process Conditions, and Emulsification Method. Food and Bioprocess Technology 8(2):301-318.
Kuck LS, Noreña CPZ (2016). Microencapsulation of grape (*Vitis labrusca* var. Bordo) skin phenolic extract using gum Arabic, polydextrose, and partially hydrolyzed guar gum as encapsulating agents. Food Chemistry 194:569-576.
Li B, Jiang Y, Liu F, Chai Z, Li Y, Leng X (2011). Study of the encapsulation efficiency and controlled release property of whey protein isolate-Polysaccharide complexes in W1/O/W2double emulsions. International Journal of Food Engineering 7(3). https://doi.org/10.2202/1556-3758.2321.
Lupo B, González C, Maestro A (2012). Microencapsulation in alginate for food. Technologies and applications. Venezuelan Journal of Food Science and Technology 3(1):130-151.
Madureira AR, Amorim M, Gomes AM, Pintado ME, Malcata FX (2011). Protective effect of whey cheese matrix on probiotic strains exposed to simulated gastrointestinal conditions. Food Research International 44(1):465-470.
Mehyar GF, Al-Isamil KM, Al-Ghizzawi HM, Holley RA (2014). Stability of Cardamom (Elettaria Cardamomum) Essential Oil in Microcapsules Made of Whey Protein Isolate, Guar Gum, and Carrageenan. Journal of Food Science 79(10):C1939-C1949..
Menezes-Blackburn D, Gabler S, Greiner R (2015). Performance of Seven Commercial Phytases in an in Vitro Simulation of Poultry Digestive Tract. Journal of Agricultural and Food Chemistry 63(27):6142-6149.
Mensink MA, Frijlink HW, Voort Maarschalk K, Hinrich WLJ (2017). How sugars protect proteins in the solid state and during drying (review): Mechanisms of stabilization in relation to stress conditions. European Journal of Pharmaceutics and Biopharmaceutics 114:288-295.
Mudgil D, Barak S, Khatkar BS (2012). X-ray diffraction, IR spectroscopy and thermal characterization of partially hydrolyzed guar gum. International Journal of Biological Macromolecules 50(4):1035-1039.
Nava RE, Álvarez M, Hernández MJL (2015). Microencapsulation of bioactive components. Research and Science of the Autonomous University of Aguascalientes.
Neira VA, Nava RE, Iliná A, Álvarez GM, Gerardo J, Lozano G. (2013). Fundamentals aspects of phytases. Research and Science from Autonomus University of Aguascalientes 21(57):58-63.
Neira VA, Aguilar CN, Ilyin A, Contreras-Esquivel JC, Carneiro-da-Cunha MG, Michelena-Álvarez G, Martínez-Hernández JL (2018). Purification and biochemical characterization of an Aspergillus niger phytase produced by solid-state fermentation using triticale residues as substrate. Biotechnology Reports 17:49-54.
Neira VA (2013). Production of phytases with native strains of *Aspergillus spp*. by fermentation in solid medium of agroindustrial waste. Autonomus University of Coahuila.
Neira VA (2017). Production of phytases by *Aspergillus niger* at the pilot level for the formulation of a product as a functional additive for the diets of non-ruminant animals. Autonomus University of Coahuila-Federal University of Pernambuco.
Patel JJ, Karve M, Patel NK (2014). Guar gum: a versatile material for farmaceutical industries. International Journal of Pharmacy and Pharmaceutical Sciences 6(8):13-19.
Pérez-Alonso C, Fabela-Morón MF, Guadarrama-Lezama Y, Barrera-Pichardo JF, Alamilla-Beltrán L, Rodríguez-Huezo ME (2009). Interrelationship between the structural features and rehydration properties of spray dried manzano chilli sauce microcapsules. Mexican Journal of Chemical Engineering 8(2):187-196.
Pescuma M, de Valdez GF, Mozzi F (2015). Whey-derived valuable products obtained by microbial fermentation. Applied Microbiology and Biotechnology 99(15):6183-6196.
Poveda EE (2013). Whey, generalities and potential use as a source of high bioavailability calcium. Chilean Journal of Nutrition 40(4):397-403.
Rao SVR, Raju MVL, Prakash B, Reddy EPK (2016). Effect of dietary inclusion of toasted guar (Cyamopsis tetragonoloba) meal as a source of protein on performance of White Leghorn layers. British Poultry Science 56(6):733-739.
Rodehutscord M (2013). Determination of phosphorus availability in poultry. World's Poultry Science Journal 69(3):687-698.
Rodríguez-Fernández DE, Parada JL, Medeiros ABP, Carvalho JC, Lacerda LG, Rodríguez-León JA, Soccol CR (2013). Concentration by ultrafiltration and stabilization of phytase produced by solid-state fermentation. Process Biochemistry 48(2):374-379
Rodríguez-Huezo ME, Durán-Lugo R, Prado-Barragán LA, Cruz-Sosa F, Lobato-Calleros C, Alvarez-Ramírez J, Vernon-Carter EJ (2007). Pre-selection of protective colloids for enhanced viability of Bifidobacterium bifidum following spray-drying and storage, and evaluation of aguamiel as thermoprotective prebiotic. Food Research International 40(10):1299-1306.
Silva RSG, Bandeira SF, Pinto LAA (2014). Characteristics and chemical composition of skins gelatin from cobia (Rachycentron canadum). LWT-Food Science and Technology 57(2):580-585.
Singh N, Kumari A, Gakhar SK, Singh B (2015). Enhanced cost-effective phytase production by Aspergillus niger and its applicability in dephytinization of food ingredients. Microbiology 84(2):219-226.
Sousa-Gallagher MJ, Tank A, Sousa R (2016). *T*he Stability and Shelf Life of Food. (P. Subramaniam, Ed.), The Stability and Shelf Life of Food (Second Edition). USA: Woodhead Publishing 612 p.
Souza CRF, Oliveira WP, Said S (2014). Characterization and spray drying of lipase produced by the endophytic fungus Cercospora kikuchii. Brazilian Journal of Chemical Engineering 31(4):849-858.
Spier MR, Behsnilian D, Zielinski A, Konietzny U, Greiner R (2015). Studies Towards the Stabilisation of a Mushroom Phytase Produced by Submerged Cultivation. Protein Journal 34(5):367-379.
Sweeti M, Sawarkar B (2015). Recovery and Synthesis of Guar Gum and its Derivatives. International Journal of Advanced Research in Chemical Science 2(5):33-40.
Tan Y, Tong Z, Yang Q, Sun Y, Jin X, Peng C, Wang X (2017). Proteomic analysis of phytase transgenic and non-transgenic maize seeds. Scientific Reports 7(1):9246.
Tang Z, Jin W, Sun R, Liao Y, Zhen T, Chen H, Li C (2018). Improved thermostability and enzyme activity of a recombinant phyA mutant phytase from Aspergillus niger N25 by directed evolution and site-directed mutagenesis. Enzyme and Microbial Technology 108:74-81.
Teniza GO (2008). Study of the milk serum of cow's milk and proposal for the reuse of it. National Polytechnic Institute.
Tonon RV, Brabet C, Hubinger MD (2010). Anthocyanin stability and antioxidant activity of spray-dried açai (Euterpe oleracea Mart.) juice produced with different carrier agents. Food Research International 43(3):907-914.
Tsakali E, Petrotos K, Allessandro AD (2010). A review on whey composition and the methods used for its utilization for food and pharmaceutical products. 6th International Conference on Simulation and Modelling in the Food and Bio-Industry. FOODSIM 8 p.
Turki IY, Marium SA, Ahmed DE, Omer ME (2011). The effect of additional graded levels of guar germ (Cyamopsis tetragonoloba) on broilers diet. Journal of Science and Technology 12(3):33-37.

Van Boekel MJS (1998). Effect of Heating on Maillard Reaction in Milk. Food Chemistry 62(4):403-414.
Van der Ven C, Muresan S, Gruppen H, De Bont DBA, Merck KB, Voragen AGJ (2002). FTIR spectra of whey and casein hydrolysates in relation to their functional properties. Journal of Agricultural and Food Chemistry 50(24):6943-6950.
Zhao J, Whistler RL (1994). Spherical aggregates of starch granules as flavor carriers. Food technology 48(7):104-105
Zin El-Din M, Aoki T (1993). Polymerization of casein on heating milk. International Dairy Journal 3(7):581-588.

Related Journals: