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Research article

Measurement of expansin activity and plant cell wall creep by using a commercial texture analyzer

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ABSTRACT

Background: Expansins play an important role in cell wall metabolism and fruit softening. Determination of expansin activity is a challenging problem since it depends on measuring cell wall properties by using *ad hoc* extensometers, a fact that has strongly restricted its study. Then, the objective of the work was to adapt a methodology to measure cell wall creep and expansin activity using a commercial texture meter, equipped with miniature tensile grips and an *ad hoc* cuvette of easy construction.
Results: It was possible to measure hypocotyls acid growth and expansin activity in a reliable and reproducible way, using a commercial texture meter, common equipment found in laboratories of food science or postharvest technology. Expansin activity was detected in protein extracts from cucumber hypocotyls, tomato and strawberry fruits, and statistical differences in expansin activity were found in both fruit models at different ripening stages.
Conclusions: The possibility of measuring expansin activity following this adapted protocol with a commercial texture meter could contribute to ease and increase the analysis of expansin in different systems, leading to a better understanding of the properties of these proteins under different experimental conditions.

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1. Introduction

Expansins are non-hydrolytic proteins, first identified in studies of plant cell wall enlargement, and pointed out as responsible for the "acid growth" phenomenon [1]. This diverse protein family was proven to participate in different processes involving cell wall re-modelling, as cell enlargement, pollen tube and root hair growth, organ abscission, and fruit softening [2].

The role of expansins in fruit softening was first proven in tomato [3,4], and then a growing number of expansin genes were reported in other fruits such as strawberry [5,6,7], banana [8], apple [9], peach [10,11], and melon [12], among others. Texture changes in ripening fruits influence consumer preference, fruit storability, transportability, shelf-life, and response to pathogen attack [13]. Excessive softening is the main factor limiting fruit shelf life and postharvest storage [14]. The fruit cell wall disassembly is one of the main processes responsible for softening and textural changes during ripening, which is associated with biochemical changes in cell wall fractions resulting in breakdown of polymers such as cellulose, hemicelluloses and pectins [15,16]. This process involves hydrolytic reactions brought about by different hydrolytic enzymes, and also by the action of expansins [4,16,17,18]. Because of their particular action mechanism, expansins have been pointed out as potential accessory protein in biomass treatments to obtain fermentable sugars to be used in ethanol production, decreasing the crystallinity of cellulose and therefore increasing the modifiable surface area accessible to degrading enzymes [19].

Most studies performed on expansins were done at mRNA expression (Northern blots, RT-qPCR, microarrays) or protein expression (Western blots) levels. Expansin biological activity has been evaluated measuring its capacity to increase stress relaxation or creep of isolated cell walls, usually from cucumber or tomato hypocotyls [1,20]. This measurement has been performed using *ad hoc* built extensometers, and hardly a few attempts to use commercial equipment have been reported [9]. This fact has restricted its evaluation to a few laboratories around the world.

Therefore, the goal of this work was to adapt and validate a technique to measure cell wall creep and expansin activity using a commercial texture meter, a common equipment found in

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laboratories devoted to food science or postharvest technology. This optimized strategy was successfully assayed with extracts from cucumber hypocotyls, tomato and strawberry fruits. The availability of a simpler and reliable methodology to measure expansin activity could facilitate its evaluation and increase the knowledge of the properties of these relevant proteins.

2. Material and Methods

2.1. Plant Material

Cucumber and tomato hypocotyls sections were obtained as follows: cucumber (*Cucumis sativus* cv. Poinsett) or tomato (*Solanum lycopersicum* cv. Ailsa Craig) seeds were sterilized with 4% (*w*/*v*) NaClO, and washed five times with distilled water. Then, approximately 100 seeds were sown on soil:perlite (3:1) and the seedlings grown in the dark at 28°C for 4 or 6 d in the case of cucumber or tomato, respectively. Hypocotyls measuring 8–10 cm in length were selected to perform the measurements, and 3 cm from the apical section were excised to be frozen in liquid nitrogen and stored at -20°C, for no more than a week, until use.

Strawberry (*Fragaria* × *ananassa* Duch., cv Camarosa) fruit were obtained from local producers (La Plata, Buenos Aires, Argentine). The fruit were harvested at ripening stages white (W), 50%Red (50%R) and 100%Red (100%R), dissected, frozen in liquid nitrogen and then stored at -20°C, until use.

Tomato (*S. lycopersicum* cv. Ailsa Craig) plants were grown in a greenhouse under natural light conditions in La Plata (Buenos Aires, Argentine); fruit were harvested at Mature Green (MG), Breaker (BR), Pink (PI), Red Ripe (RR) and Over Red (OR) stage, the pericarp was dissected, frozen in liquid nitrogen and then stored at -20°C, until use.

2.2. Measurements of cell wall creep and expansin activity

2.2.1. Hypocotyls preparation

Frozen hypocotyls were slightly abraded with quartz sand and washed thoroughly with distilled water. Hypocotyls were put between two microscope slides and compressed with a 500 g weight for 5 min, and then stored at 4°C to be used along the day (labeled as native hypocotyls). To inactivate cell wall-bound enzyme activities, compressed hypocotyls were immersed in distilled water at 95°C for 20 s (labeled as heat-inactivated hypocotyls).

2.2.2. Cell wall creep assay

Measurements were performed using a Texture Analyzer device (TA.XTplus, Stable Micro Systems Texture Technologies®, Scarsdale, NY), equipped with miniature tensile grips, and a load cell PL/CEL/30, for 30 kg of maximum load. The measurement protocol was adapted from Cosgrove [21]. Cucumber or tomato native hypocotyls were fitted into an *ad-hoc* cuvette, and its extremes secured with the miniature tensile grips provided with the equipment (A/MTG). The cuvette was built from a 1 mL syringe plastic tube; its dimensions were 12 mm length, 4 mm external diameter, total volume 200 μL approximately. Near the bottom, a small orifice was done, to adapt a tubing (1 mm internal diameter; 30 cm length) connected in the other extreme to a T-shaped valve, which allowed the connection of two 1 mL syringes to exchange buffer composition (Fig. 1). The cuvette bottom was open to allow the hypocotyl fitting, being both

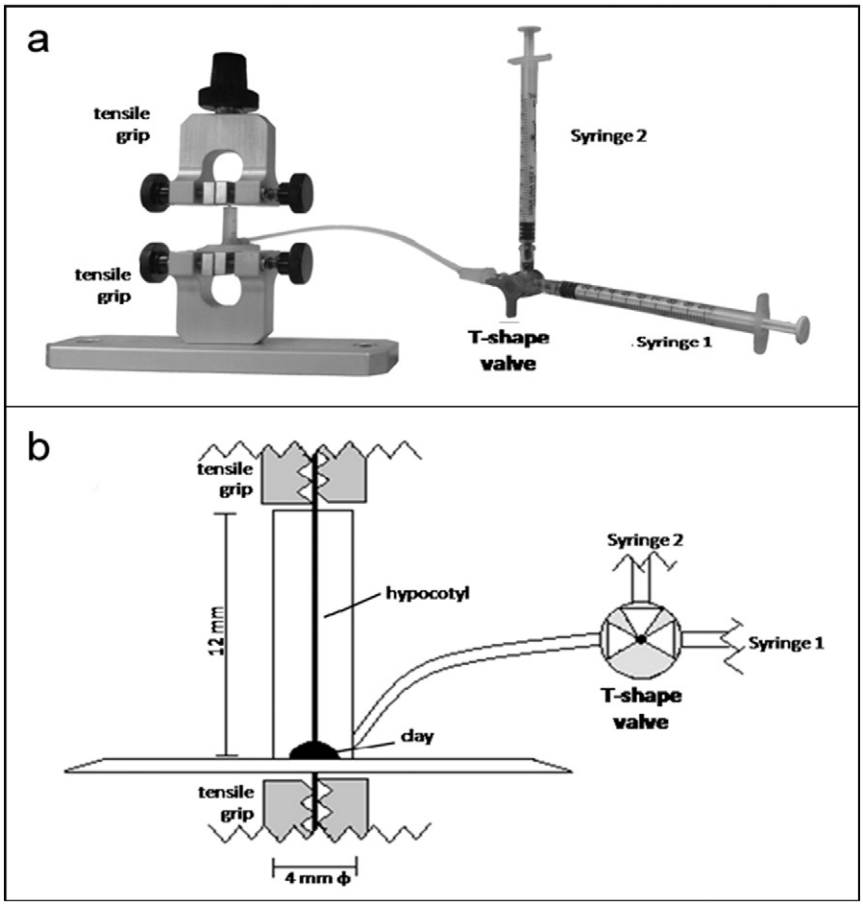

Fig. 1. (a) Picture of an *ad hoc* device used in acid creep and expansin activity measurements. (b) Schematic representation of the *ad hoc* cuvette. The solutions used in creep rate assay were: 50 mM HAc/NaAc buffer pH 4.5 (syringe 1) and 50 mM HEPES buffer pH 6.8 (syringe 2). In the case of expansin activity assay: 50 mM HAc/NaAc buffer pH 4.5 (syringe 1) and protein extract (syringe 2).

extremes protruding the cuvette. Once the hypocotyl was placed in the cuvette, the cuvette's bottom was sealed using a small amount of clay.

To begin the assay, the cuvette was filled with 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer, pH 6.8, and the texture meter was set in the tension mode to apply a constant force (20 or 10 g in the case of cucumber or tomato hypocotyls, respectively) during the whole test. The assay was started and continued for 900 s, while the elongation was recorded. After that, the buffer was rapidly exchanged to 50 mM HAc/NaAc [acetic acid/acetate] buffer pH 4.5 using the buffer exchange device, and the assay was reassumed for another 3100 s. Data were acquired with the software provided with the equipment (Exponent Lite Analysis Sofware®), at an acquisition rate of 1 point per second.

The creep rate was defined as the hypocotyl elongation corresponding to the step performed at pH 4.5, and was expressed in micrometers per minute (μm min^{-1}). Ten replicates were done for every condition.

In the case of assays aimed to analyze the effect of urea or NaCl on cell wall creep rate, 50 mM HAc/NaAc buffer, pH 4.5 was supplemented with 8 M urea or NaCl (0.1 M, 1 M, or 3 M), and the measurement was done as described above. All measurements were done by quintuplicate.

2.3. Plant extracts

The extracts to assay expansin activity were prepared as indicated below.

2.3.1. Cucumber hypocotyls

The following protocol was adapted from McQueen-Mason et al. [1]. Cucumber hypocotyls (5 g) were powdered in liquid nitrogen and re-suspended in 50 mL of buffer A (50 mM HAc/NaAc, pH 4.5; 2 mM EDTA [Ethylenediaminetetraacetic acid], 1 mM PMSF [phenylmethylsulfonyl fluoride]). The suspension was centrifuged at 10,000 × g for 30 min at 4°C and the supernatant was discarded. The pellet was washed with buffer A in a 1:1 (*v*/v) ratio and centrifuged at 10,000 × g for 30 min 4°C.

The pellet was re-suspended in buffer B (50 mM HEPES, pH 6.8; 1 M NaCl, 2 mM EDTA, 3 mM sodium metabisulfite), and extracted for 1.5 h at 4°C with shaking. Then, the extract was centrifuged at 10,000 × g for 10 min at 4°C, the supernatant collected and the total protein extract precipitated with cold-acetone in a 3:1 (*v*/v) ratio at -20°C for 15 min. Suspension was then centrifuged at 10,000 × g for 5 min, and the pellet washed once with cold acetone as before. The protein fraction was re-suspended in buffer C (50 mM HAc/NaAc pH 4.5) and stored at 4°C for up to a week.

2.3.2. Strawberry fruit

This protocol was adapted from Harrison et al. [7]. Five grams of frozen strawberry fruit were powdered in liquid nitrogen, re-suspended in 25 mL of buffer D (25 mM MOPS [3-(*N*-morpholino) propanesulfonic acid] pH 7.0, 0.5% *w*/*v* CTAB [cetyltrimethylammonium bromide], 30% *w*/*v* glycerol) and homogenized at room temperature. The suspension was centrifuged at 10,000 × g for 5 min at room temperature. The supernatant was collected, filtrated with filter paper and precipitated with cold-acetone in a 3:1 (*v*/*v*) ratio for 15 min at -20°C.

The protein fraction was centrifuged at 5000 × g for 10 min at 4°C, and the pellet was washed with the same volume of cold-acetone. Finally, the protein fraction was dried, re-suspended in 3 mL buffer C (50 mM HAc/NaAc, pH 4.5) and stored at 4°C for not more than a week.

2.3.3. Tomato fruit

This protocol was adapted from Rose et al. [22]. Frozen tomato fruit was powdered in liquid nitrogen and thawed in 100 mL of ice-cold buffer E (50 mM HEPES, pH 7.0, 5 mM DTT [dithiothreitol], 3 mM sodium metabisulfite, 2 mM EDTA, 0.1% [*w*/*v*] polyvinylpyrrolidone [Mr = 40,000], and 0.1% [*v*/*v*] Triton X-100) in 1 g of tissue/3 mL of buffer ratio. Samples were homogenized for 1 min on ice and then centrifuged at 10,000 × g for 30 min. Pellets were washed three times re-suspending them in 25 mL ice-cold distilled water, followed by a centrifugation step as before.

Pellets were subjected to protein extraction in 50 mL of buffer F (50 mM HEPES, pH 7.0, 5 mM DTT, 3 mM sodium metabisulfite, 2 mM EDTA, and 1.5 M NaCl). Suspensions were stirred at 4°C for 12 h, centrifuged as described above and supernatants stored at 4°C until use. Pellets were re-extracted in 10 mL of the same buffer for 2 h at 4°C and centrifuged in the same conditions as before. The two extracted fractions were mixed and protein precipitation was induced adding ammonium sulfate to a final concentration of 0.4 g mL^{-1}. The suspension was stirred at 4°C overnight, and then centrifuged at 10,000 x *g* for 30 min. Supernatant was discarded and protein pellets were re-suspended in 3 mL of buffer G (same as buffer F but without NaCl).

2.4. Expansin activity assays

Protein extracts from different plant sources, prepared as described above, were assayed for expansin activity using heat-inactivated cucumber or tomato hypocotyls clamped in the *ad hoc* cuvette, also described above, and fitted in a commercial texture meter. Once inactivated hypocotyls were clamped, the cuvette was filled with 50 mM HAc/NaAc buffer pH 4.5, the extension assay was started and maintained for 900 s. Then, the buffer was exchanged to a solution of total protein extract dissolved in the same buffer, and the assay was continued for 3100 s. The total creep rate (hypocotyl elongation corresponding to the step performed at pH 4.5 with the extract protein) was used to determine expansin activity, defined as the elongation expressed in micrometers per minute (μm min^{-1}).

Expansin net activity was calculated subtracting the mean of control (cell wall creep of heat-inactivated hypocotyls) from the total creep rate, and expressed as micrometers per minute (μm min^{-1}). Expansin specific activity was defined as expansin net activity per microgram of total protein extract, and expressed in micrometers per μg of total protein and minute of assay (μm min^{-1} μg^{-1}) [23]. All determinations were done by triplicate.

Additionally, in the case of strawberry, a dose–response expansin activity assay was performed using six different protein concentrations (5, 10, 25, 50, 100, 250 μg mL^{-1}) of a total protein extract obtained from 100% R fruit.

2.5. Protein quantification

All protein quantifications were performed by the Bradford assay using Bovine Serum Albumin (BSA) as standard [24].

2.6. Statistical analysis

Data from creep assays and expansin activity measurements were analyzed by ANOVA, and means were compared by Tukey or Dunnett tests ($P < 0.05$).

3. Results

3.1. Cell wall creep

Acid growth phenomenon is the principle on which expansin activity measurements are based. Taking that into account, the first step to adapt a commercial texture meter to an expansin activity measurement protocol was the setup of the acid growth phenomenon measurement using the commercial equipment.

The creep rate of cucumber and tomato native hypocotyls increased significantly with the buffer shift from pH 6.8 to pH 4.5 (Fig. 2).

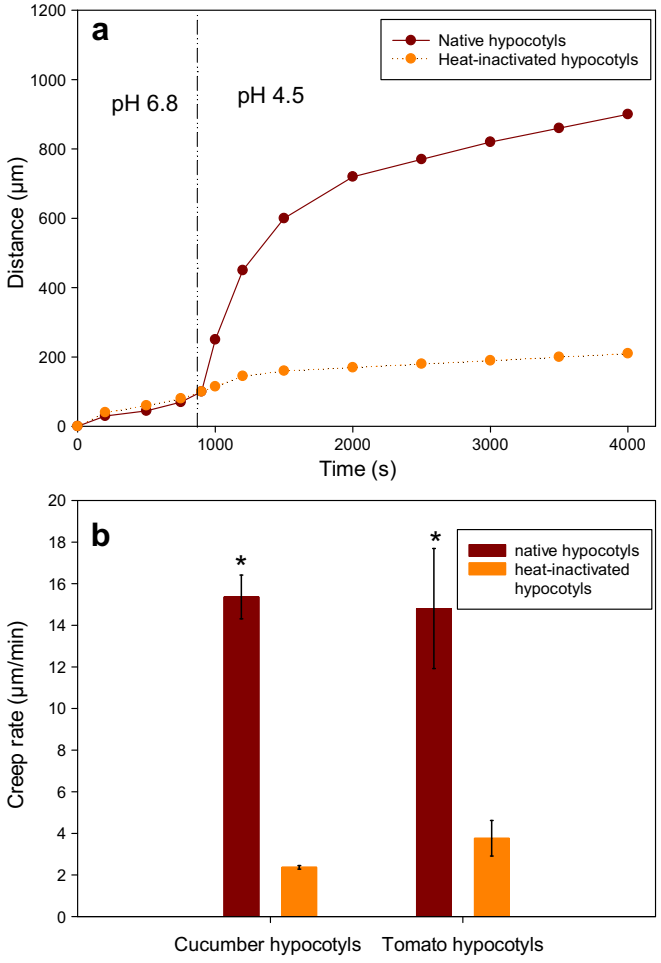

Fig. 2. Evaluation of the tissue acid growth. (a) Schematic representation of the curves of distance vs. time obtained for cucumber hypocotyls. (b) Creep rate of cucumber and tomato hypocotyls. Data correspond to creep rate means ± SE from ten independent replicates. Results were analyzed by ANOVA; means were compared by Dunnett test at $P < 0.05$. Asterisks indicate significant statistical differences between native and heat-inactivated hypocotyls from cucumber or tomato.

Although a slight elongation was detected on heat inactivated hypocotyls at pH 6.8, no response was observed after the buffer exchange (Fig. 2a). The creep rates at pH 4.5 was 7 fold higher in native cucumber hypocotyls than in heat inactivated controls, and 4 fold higher in native tomato hypocotyls than in the proper controls. The observed phenomenon is consistent with the fact that the "acid growth" is an enzyme dependent process [21].

3.2. Effect of chemicals agents on cell wall acid growth

The method sensibility was assessed evaluating the effect of chemical agents known to inhibit the acid growth phenomenon, such as urea and NaCl. Both agents decreased significantly native cucumber hypocotyls cell wall creep when compared with the control without chemical agents (Fig. 3). The treatments with 0.1 M NaCl, 1.0 NaCl and 8.0 M urea caused a similar decrease of creep rate. The cell wall creep rate at pH 4.5 was four times higher than the observed in the heat inactivated controls. The greater inhibitory effect was observed with 3.0 M NaCl (Fig. 3), being the elongation rate comparable with the observed in the controls. These results are in agreement with previous reports [21].

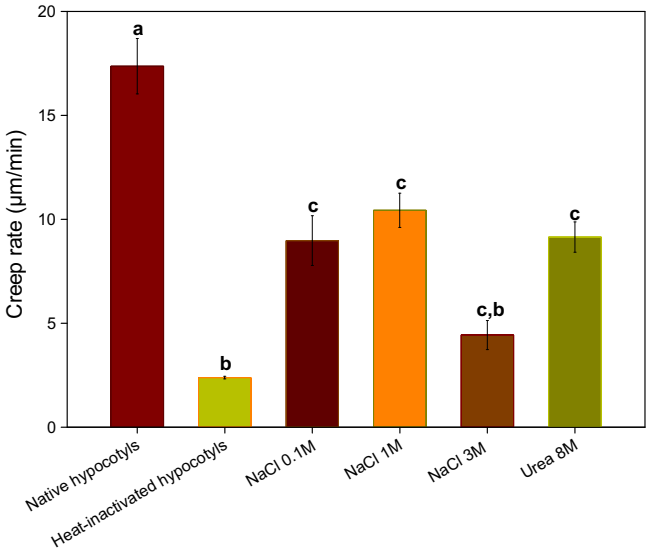

Fig. 3. The effect of Urea (8 M) and NaCl (0.1 M, 1 M and 3 M) on creep rate in native-cucumber-hypocotyl cell walls.(+): Native hypocotyls; (-): heat-inactivated hypocotyls. Data correspond to creep rate means ± SE of five independent determinations. An ANOVA analysis was performed and means were compared by Tukey test at $P < 0.05$; different letters indicate significant statistical differences.

3.3. Expansin activity assays

Expansin activity was assessed evaluating a protein extract capacity of reconstitute the acid growth phenomenon on heat inactivated hypocotyls.

As a start, total protein extracts from cucumber hypocotyls or strawberry fruits (100%R) were assayed using heat inactivated cucumber hypocotyls as cell wall matrix, and the results were compared with those reported by McQueen Mason et al. [1] and Harrison et al. [7], obtained using the "ad-hoc" extensometer. A similar approach was done with total protein extracts from tomato fruit and tomato heat inactivated hypocotyls; in this case, the results were compared with those from Rose et al. [22].

The cell wall creep rates of heat inactivated hypocotyls was significantly higher in all the cases where protein extracts were assayed, compared to those of the controls (Fig. 4a). Increments of 2.6-fold and 4-fold were observed on the creep rate of heat inactivated cucumber hypocotyls when protein extracts from cucumber hypocotyls or strawberry fruit (100%R) were assayed, respectively (Fig. 4a). For tomato heat inactivated hypocotyls, the creep rate increased 2.7-fold when the total tomato fruit protein extract (RR) was assayed (Fig. 4b). All the increments observed were attributed to presence of expansin proteins in the assayed extracts.

3.4. Expansin activity and fruit ripening

The variation of expansin activity during fruit ripening was analyzed, using strawberry (*Fragaria* × *ananassa* cv. Camarosa) and tomato (*S. lycopersicum* var. Ailsa Craig) as non-climacteric and climacteric fruit model, respectively.

3.4.1. Strawberry

Total protein extracts were prepared from strawberry fruit at three different ripening stages, white (W), 50%R and 100%R. Expansin activity was measured on each of them using heat inactivated cucumber hypocotyls as cell wall matrix (Fig. 5). Significant differences in expansin activity were observed between the stages 100%R and 50%R when compared to the controls without protein

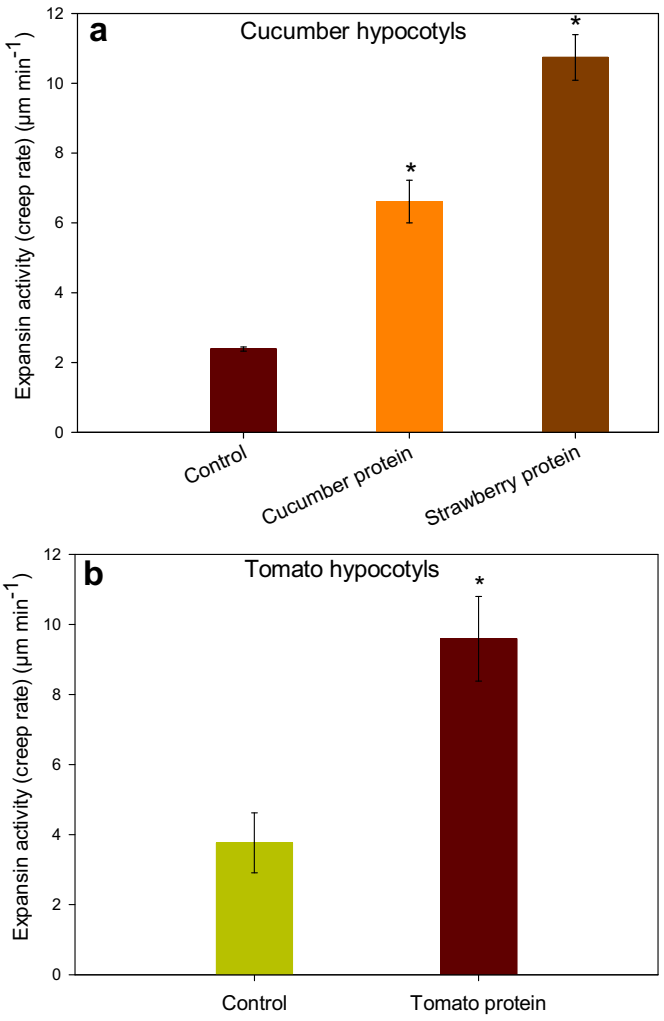

Fig. 4. Expansin activity assays using: (a) heat-inactivated cucumber hypocotyls, as cell wall matrix, with buffer pH 4.5 (Control), or total protein extracts from cucumber hypocotyls or strawberry fruit (100%R); (b) heat-inactivated tomato hypocotyls with buffer pH 4.5 (Control), or protein extract from red ripe tomato fruit (RR). Data correspond to expansin activity means ± SE of three independent determinations. Results were analyzed by ANOVA and means were compared by Dunnett test at $P < 0.05$. Asterisks indicate significant statistical difference in comparison with the control.

extract. No statistical difference was observed between the stages 100%R and 50%R or between the stage W and the control without protein extract. Although there are no statistical differences between the stages W and 50%R, there is a clear tendency of an increment in the expansin activity along the fruit ripening process.

3.4.2. Dose–response assay with 100% red strawberry fruit

To further characterize the sensitivity of the method described to measure expansin activity, a dose–response assay was performed on the 100% R strawberry fruit protein extract, using different concentrations and measuring the expansin activity (Fig. 6). The aim of this approach was to assess the minimum protein concentration necessary to perform a reproducible and statistically meaningful analysis. Under the experimental conditions used, this protein concentration was equal to or higher than 75 µg mL^{-1}, although we recommend protein concentrations above 100 µg mL^{-1}. To our knowledge, this is the first attempt to determine an optimum protein concentration to determine expansin activity on a strawberry fruit total protein extract.

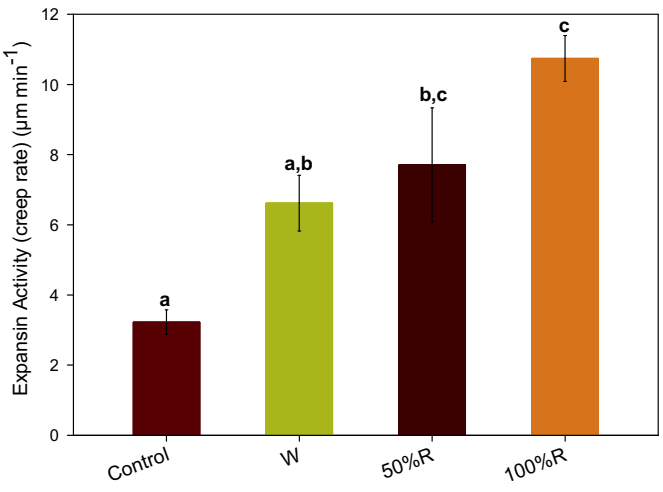

Fig. 5. Expansin activity during strawberry fruit ripening. Heat-inactivated cucumber hypocotyls, as cell wall matrix, with buffer pH 4.5 (Control), or total protein extracts from White stage (W); 50%R and 100%R were used. Protein concentration was the same in all the determinations (250 µg mL^{-1}). Data correspond to expansin activity means ± SE of three independent determinations. Results were analyzed by ANOVA and means were compared by Tukey test at $P < 0.05$; significant statistical differences are shown by different letters.

3.4.3. Tomato

Total protein extracts were prepared from tomato fruit at five different ripening stages: Mature Green (MG), Breaker (BR), Pink (PI), Red Ripe (RR) and Over Ripe (OR). Expansin activity was measured on each of them using heat inactivated tomato hypocotyls as cell wall matrix (Fig. 7).

Expansin activity was detected in protein extracts from fruit at BR, PI, RR and OR ripening stages, while no activity was found at MG stage (no significant differences were observed in the creep rate compared with the measured in the heat inactivated controls). The value of expansin activity in fruit at ripening stages ranging from BR to OR did not differ statistically each other (Fig. 7).

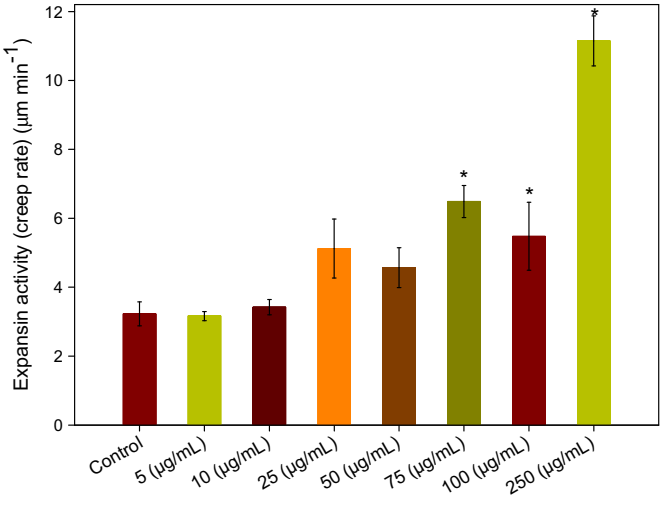

Fig. 6. Expansin activity dose–response curve. Heat-inactivated cucumber hypocotyls, as cell wall matrix, with buffer pH 4.5 (Control), or different amounts of total protein extract from 100%R strawberry fruit. Data correspond to expansin activity means ± SE of three independent determinations. Results were analyzed by ANOVA and means were compared by Dunnett test at $P < 0.05$. Asterisks indicate significant statistical differences from the control.

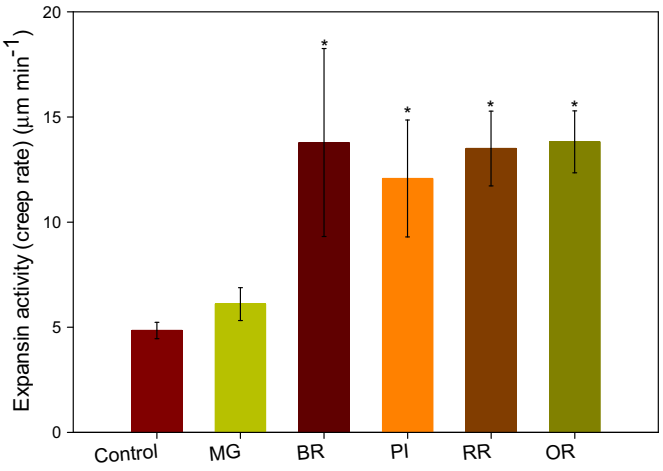

Fig. 7. Expansin activity during tomato fruit ripening. Heat-inactivated tomato hypocotyls with buffer pH 4.5 (Control), or protein extract from different ripening stages (Mature Green, MG; Breaker, BR; Pink, PI; Red Ripe, RR; and Over Ripe, OR) were used. Protein concentration was the same in all the determinations (500 µg mL^{-1}). Data correspond to expansin activity means ± SE of three independent determinations. Results were analyzed by ANOVA and means were compared by Dunnett test at $P < 0.05$; asterisk indicates significant statistical differences from the control.

4. Discussion

Originally, expansin activity was assessed qualitatively through a reconstitution assay of the acid growth phenomenon [1]. Through the years, several methods have been developed in order to evaluate different aspects of the cell wall mechanics. The most relevant methods using an extensometer are: (1) breaking strength, (2) elastic and plastic compliances, (3) stress relaxation, and (4) cell wall creep [25]. Some of these techniques, such as stress relaxation, have been adapted to the expansin activity measurement [9]. While the latter could be used to assess expansin activity, it lacks sensitivity being susceptible to small variations [25,26].

According to Cosgrove, the measurement of the cell wall creep mimics the *in vivo* wall extension process, and it readily distinguishes between growing and non-growing cell walls for many plant tissues. The difference between these two conditions is greater when the creep method is used, compared to any of the three other techniques. Moreover, creep assays are more sensitive to the activity of α- and β-expansins, making it an appropriate and attractive technique to be used in the evaluation of expansin activity [25]. Nonetheless, to the date, any experiment using this approach requires the *ad-hoc* extensometer, which has strongly restricted the study of expansins. Because of this difficulty, most expansin studies have been based on gene expression at both, mRNA and protein levels.

Our primary goal was to adapt the protocol to the use of a commercial texture analyzer, to bypass the construction of *ad hoc* extensometers and then expand the possibility to measure this activity in more laboratories around the world. Our results show that it is possible to assess both cell walls acid growth and expansin activity with a commercial texture meter and a cuvette of easy construction in a reproducible and trustworthy manner. The adapted protocol allowed us to reproduce the acid growth phenomenon, obtaining similar results than the ones obtained using the *ad-hoc* extensometer [21]. The inhibitory effect of chemical agents such as NaCl and urea on the creep rate could also be distinguished. In the case of NaCl, the salt is commonly used in protein extraction buffers to extract cell wall bound proteins. It is possible that the salt interferes with the intimate contact between expansins and the components of the cell wall, being this loss of interaction the responsible for the inhibitory effect. On the other hand, urea is a well-known chaotropic agent, producing the loss of protein structure at the concentration used. In this case, the denaturation of the hypocotyls native expansins is probably the cause of the inhibition. Buffer diffusion and incomplete protein denaturation in the cell wall matrix context could explain the fact that the creep rate inhibition is not complete.

The results obtained in the expansin activity measurements in protein extracts from cucumber hypocotyls, strawberry fruit and tomato fruit, are in agreement with previous reports where the *ad-hoc* extensometer was used [1,7,22]. The measurement included a statistical analysis to differentiate the activity observed on the protein extracts from the extension observed in the controls, which has not been commonly performed in previous reports.

Regarding expansin activity during strawberry fruit ripening, our results showed an increment in the proteins activity along the fruit ripening process. We were able to statistically differentiate the expansin activity between W and 100% R fruit stages, whereas no statistical difference was observed between W and 50% R stages.

There are previous reports showing expansins expression profiles in strawberry at both mRNA and protein levels. In 2006, Dotto et al. [6] analyzed mRNA expression of five different expansins (*FaEXP1, 2, 4, 5,* and *6*) in strawberry (*Fragaria* × *ananassa* Duch., cv Camarosa). According to their report, *FaEXP4* and *FaEXP6* expression profiles turned out to be not tissue specific, having a basal constitutive expression during fruit development and ripening, whereas *FaEXP2* and *FaEXP5* turned out to be fruit specific, increasing their expression during fruit ripening. Regarding *FaEXP1*, although it turned out to be not fruit specific, its expression was high in the fruit receptacle and it increased during fruit ripening [6]. Harrison et al. [7] also analyzed the mRNA expression of six expansins (*FaEXP2, 3, 4, 5, 6, 7*) in fruit of another strawberry cultivar (*Fragaria* × *ananassa* Duch., cv Brigthon). They found and defined three expression patterns during fruit development and ripening: up-down-up profile (*FaEXP3* and *FaEXP4*); down-up-down profile (*FaEXP6* and *FaEXP7*) and an increasing expression profile during fruit ripening (*FaEXP2, FaEXP5*).

At the protein expression level, Dotto et al. [6] reported the results of a western blot, analyzing total protein extracted from strawberries at different ripening stages using a heterologous expansin anti-body (raised against expansin 1 from tomato, LeEXP1). Their results showed that the higher expansin accumulation corresponded to 100% R stage, while in 50% R stage protein levels were very low and in W stage no protein could be detected. Harrison et al. [7] performed a similar analysis with a different heterologous anti-body (raised against expansin 1 from cucumber, Cs-EXP1), and found a similar protein expression profile.

Harrison et al. [7] also performed an expansin activity assay on strawberry fruit at different ripening stages using the *ad-hoc* extensometer. In that assay, 2 mm wide cellulose/xyloglucan strips matrixes were subjected to extension under a constant force (11 gr) in the absence or presence of protein extracts at concentrations of 150 µg mL^{-1}. They reported that expansin activity increased during fruit ripening, being the activity very low in W stage, and increasing exponentially to over ripe stage (20 µm min^{-1}), although they did not perform any statistical analysis on their results.

The results of this work, as well as Harrison's, describe a clear correlation between the protein expression profiles previously reported and the expansin activity measured at each fruit ripening stage.

Regarding expansin activity profile during tomato fruit ripening, expansin activity was first detected in BR stage, observing similar levels in all the samples corresponding to PI, RR and OR stages, a marked difference compared with the activity profile observed in strawberry fruit. Our results are in agreement with Rose et al. [22], who performed a qualitative analysis of the expansin activity in tomato using the *ad-hoc* extensometer and heat inactivated cucumber hypocotyls as matrix cell wall. Despite the fact that the assay can be carried out using another kind of cell wall matrix, we decided to use

tomato heat inactivated hypocotyls, for having a direct relationship with the nature of the protein extract.

In this line, Rose et al. [4] isolated and characterized the expression by northern blot of a tomato fruit specific expansin in *S. lycopersicum* cv. T5. They observed that the mRNA levels increased during the fruit ripening process. Brummell et al. [3] analyzed the expression of five tomato expansins (SlEXP1 to SlEXP5) in different tissues, and particularly during the fruit ripening process (*S. lycopersicum* cv. Best Bonnie), obtaining similar results than the reported by Rose et al. [4]. Of the five genes, only SlEXP1, SlEXP3 and SlEXP5 were expressed in fruit. SlEXP1 expression was fruit specific, being detected from Pi stage forward and increasing significantly during the fruit ripening. Although SlEXP3 and SlEXP5 were reported as not fruit specific genes, they were present at very low levels at early fruit ripening stages, and also presented detectable mRNA levels later during the fruit softening. Nonetheless, their expression was not comparable with the highly expressed SlEXP1. This suggests that SlEXP1 is the most relevant expansin during fruit ripening, and its accumulation correlates with the expansin activity profile observed along the tomato fruit ripening process.

One recurrent difficulty found when working with expansins is the lack of consensus in the definition of these proteins activity. Although most of the characterization attempts of expansins are based on the acid growth phenomenon, there is no agreement in the proteins activity definition [7,22,23]. It would be helpful to homologate the definition of expansin activity and its units, following a similar convention as used in regular enzymes. For this, in this report we show the results using the original definition of expansin activity, which is the creep rate at pH 4.5, but we also present them using two other expansin activity definitions (Table 1), aimed to facilitate activity comparisons in future reports.

Table 1
Expansin net activity and Expansin specific activity in extracts from different plant sources.

Vegetal matrix/ applied force	Protein extract	Exp. net act. ($\mu m \cdot min^{-1}$)[a]	Exp. specific act. ($\mu m \cdot min^{-1}\ \mu g^{-1}$)[b]
Cucumber hypocotyls/20 g	Cucumber hypocotyls (250 $\mu g \cdot mL^{-1}$)	4.2 ± 0.6	0.017 ± 0.002
	Strawberry W (250 $\mu g \cdot mL^{-1}$)	3.4 ± 0.8	0.013 ± 0.003
	Strawberry 50%R (250 $\mu g.mL^{-1}$)	4.5 ± 1.6	0.018 ± 0.006
	Strawberry 100%R (5 $\mu g \cdot mL^{-1}$)	NS	NS
	Strawberry 100%R (10 $\mu g \cdot mL^{-1}$)	NS	NS
	Strawberry 100%R (25 $\mu g \cdot mL^{-1}$)	1.9 ± 0.8	0.008 ± 0.003
	Strawberry 100%R (50 $\mu g \cdot mL^{-1}$)	1.3 ± 0.6	0.005 ± 0.002
	Strawberry 100%R (75 $\mu g \cdot mL^{-1}$)	3.3 ± 0.5	0.01 ± 0.002
	Strawberry 100%R (100 $\mu g \cdot mL^{-1}$)	2.2 ± 0.9	0.009 ± 0.004
	Strawberry 100%R (250 $\mu g \cdot mL^{-1}$)	8.0 ± 0.7	0.03 ± 0.003
	Tomato MG (500 $\mu g \cdot mL^{-1}$)	1.2 ± 0.8	0.002 ± 0.002
	Tomato Br (500 $\mu g \cdot mL^{-1}$)	6.2 ± 1.9	0.012 ± 0.004
Tomato hypocotyls/10 g	Tomato PI (500 $\mu g \cdot mL^{-1}$)	6.4 ± 2.1	0.012 ± 0.004
	Tomato RR (500 $\mu g \cdot mL^{-1}$)	7.3 ± 1.1	0.014 ± 0.002
	Tomato OR (500 $\mu g \cdot mL^{-1}$)	5.6 ± 0.9	0.011 ± 0.002

NS: not significant.

[a] Expansin net activity was calculated by subtracting the mean of negative control (cell wall creep of heat denatured hypocotyls) to expansin activity values. Data correspond to expansin net activity means ± SE of three independent determinations.

[b] Expansin specific activity was calculated as expansin net activity per microgram of total protein. Data correspond to expansin specific activity means ± SE of three independent determinations.

All the results presented in this report have been obtained with a texture meter (TA.XTplus, Stable Micro Systems Texture Technologies®, Scarsdale, NY) equipped with a load cell PL/CEL/30, for 30 kg of maximum load. This equipment offers the possibility to change the load cell for others such as PL/CEL/5 or PL/CEL/1 for 5 kg or 1 kg of maximum load, respectively. Whereas the device proved to be sensitive enough under the condition used, the force applied on the cell wall matrix is near the lower limit allowed by the equipment. Taking that into account, the use of a load cell of a lower capacity would improve the precision in the applied force on cell wall matrix, in the range necessary for this assay, increasing the methods sensitivity. The same considerations should be applicable to other texture meters available from other brands.

Here we describe the use of the adapted protocol in the characterization of a physiological process relevant in food science, using two different fruit models, but the protocol could easily be adapted to the characterization of expansins of different origin, and for different purposes, for example, the characterization of bacterial expansins for biotechnological uses. The use of different kinds of cell wall matrixes, such as synthetic cellulose strips, could also be adapted, giving more potential to the technique.

5. Conclusions

It is possible to adapt commercial equipment such as a texture meter to measure of both cell wall acid growth and expansin activity. It was possible to reproduce the measurements of acid growth phenomenon on different vegetative matrixes, and assay expansin activity in protein extracts of different sources, in a reproducible and trustworthy manner.

The adapted protocol was used to characterize the fruit ripening process in two different fruit models, strawberry for non-climacteric fruits, and tomato for climacteric fruits. The technique presented offers the possibility to extend the study of an important, but yet not fully characterized, protein family.

Conflict of interest

The authors declare that they have no conflict of interest.

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