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Influence of Cold Acclimation on the Mechanical Strength of Carrot (*Daucus carota* L.) Tissue

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Summary

We have investigated the influence of cold acclimation on the mechanical strength of carrot (*Daucus carota* L.) taproots. Changes in the mechanical strength were monitored when cold acclimation was induced in carrot plants cultivated in a growth chamber under strict climate control and in taproots harvested from field cultivation, where the plants had been exposed to the natural variations in climate. The appearance and accumulation of an antifreeze protein in the cell wall isolated from cold-stored taproots showed that a cold acclimation process is in progress in the harvested taproot derived from carrot plants grown in the field. The force needed to slice the taproots significantly increased during the first 12 weeks of storage, where the higher concentration of the antifreeze protein indicated the highest development of cold acclimation during that period of time. The increase in tissue rigidity during cold acclimation was also shown by the increase of the Young's modulus in taproot tissue from carrot plants acclimated 11 weeks under controlled temperature conditions. After 24 weeks of storage there was a significant increase in slicing force that was accompanied by signs of cell membrane deterioration, as measured by relative electrolyte leakage. Thus, the later increase in tissue strength might be related with a senescence process.

Zusammenfassung

Einfluss von Kälteanpassung auf die Festigkeit von Möhren (*Daucus carota* L.). Wir haben den Einfluss von Kälteanpassung auf die Festigkeitseigenschaften von Möhrenrüben (*Daucus carota* L.) untersucht. Sowohl bei Möhren, die in Pflanzenwuchskammern mit exakter Klimaführung angezogen worden waren, als auch bei solchen, die im Freiland unter natürlichen Klimabedingungen kultiviert wurden, konnten Veränderungen der Festigkeitseigenschaften als Folge einer Kälteanpassung nachgewiesen werden. Das Auftreten und die Akkumulation eines Gefrierschutzproteins in den Zellwänden von kühlgelagerten Möhrenrüben deutet darauf hin, dass die Prozesse der Kälteanpassung bei den Freilandmöhren zum Zeitpunkt der Ernte bereits induziert waren. Die Kraft, die zum Schneiden der Möhren aufgebracht werden musste, stieg in den ersten zwölf Lagerwochen signifikant an. Zum gleichen Zeitpunkt konnte in den Möhren auch eine höhere Konzentration an Gefrierschutzprotein nachgewiesen werden, was auf die höchste Kälteanpassungsrate hinweist. Im Wurzelgewebe von elf Wochen unter kontrollierten Bedingungen akklimatisierten Möhren zeigte sich die Zunahme der Gewebefestigkeit auch im Anstieg des ermittelten Elastizitätsmoduls (Young's modulus). Nach 24 Wochen Lagerung erhöhte sich die Schneidkraft signifikant. Dies ging mit Anzeichen von Zellmembranzerstörung, gemessen als relativer Elektrolytverlust, einher. Der später auftretende Anstieg der Gewebefestigkeit könnte demnach Folge einer beginnenden Seneszenz sein.

Key words. tissue strength – antifreeze protein – relative electrolyte leakage

Introduction

Texture is a complex quality attribute and knowledge concerning the relationship between texture and structure in fruits and vegetables is a key to understand and control specific textural characteristics (JACKMAN and STANLEY 1995; ALVAREZ et al. 2000).

Much of the texture of plant foods can be attributed to the structure and the chemical composition of the cell wall, the middle lamella and to the turgor generated within living cells by osmosis (JACKMAN and STANLEY 1995). Metabolic changes involving modifications of these structural elements will influence tissue mechanical properties. Thus, a most basic understanding of the generation of mechanical properties is needed at the biochemical and metabolic level (VINCENT 1993).

Metabolic and structural changes at the end of the growth period and during cold storage have been at-

tributed to the stress response to low temperature known as cold acclimation (FUCHIGAMI et al. 1996; SMALLWOOD et al. 1999; HERPPICH et al. 2001). Cold acclimation is a very complex process involving anatomical and histological adjustments and, in particular, numerous physiological alterations that are regulated at the gene expression level (For reviews see GRAY et al. 1997; KACPERSKA 1999; PEARCE 1999; THOMASHOW 2001). The accumulation of large amounts of an antifreeze protein (AFP) in the cell wall of cold-acclimated taproots has recently been described and used as a biological marker in studies of cold acclimation (SMALLWOOD et al. 1999).

The purpose of this investigation has been to get new insights into metabolic changes that can influence the texture of the carrot taproots. Changes in the mechanical strength of taproots are described when cold acclimation was induced in both carrot plants grown in a controlled growth chamber and in the harvested taproots from plants grown in the field.

Materials and Methods

Carrot plants grown in controlled environment

Plant material, growth conditions, and induction of cold acclimation. Carrot plants (*Daucus carota* L. cv. 'Autumn King') were cultivated and cold-treated as described by SMALLWOOD et al. (1999). Carrot plants were sown in pots and cultivated for 12 weeks in a greenhouse at 21 °C. Cold acclimation was induced by transferring the plants to a Conviron growth chamber set at a cycle of 6 °C for 8 h in light and 2 °C for 16 h in dark conditions for 6 and 11 weeks. The induction of cold acclimation under these conditions has been demonstrated by SMALLWOOD et al. (1999). Experimentation was chosen to be carried out on 6 weeks-acclimated taproots based on preliminary experiments on carrot cell cultures incubated at 4 °C showing a reduction in cell wall pore size. Preliminary experiments also showed an increase in freezing tolerance in whole plants after 6 weeks under cold conditions; an increase that was further developed after 11 weeks.

Mechanical strength analysed by compression tests. The bulk tissue compression tests were based on the method described by THIEL and DONALD (1998). Taproots were harvested from carrot plants, which were cold acclimated (CA) under the controlled conditions described above and from carrot plants cultivated under greenhouse conditions (NA). Tissue cores, 4 mm diameter, were immediately obtained from the phloem parenchyma of both CA and NA carrot taproots and cut to a length of approximately 4 mm with a scalpel. The cylinders were placed in 5 mM potassium chloride to prevent dehydration prior to the experiments. The tissue was only in the solution for about two minutes. The solution helped preventing the loss of potassium ions from the tissue (S. McQueen-Mason, pers. comm.). Tests were performed on five cylinders taken from the same taproot; four taproots were used.

The compression was accomplished using a Universal Instron Machine (series 1122, UK) equipped with a 1 KN load cell. The tissue cylinder was placed in vertical position and compressed with a plate attached to the crosshead, which was lowered at a rate of 2 mm min^{-1}. The data was recorded on a chart at a speed of 100 mm min^{-1}.

In each test the tissue was compressed to failure, signalling the end of the test. The data for the load and deformation were taken from chart measurements. The load being the height of the curve on the Y-axis measured from the base line to the peak of the curve (i.e. point of failure). The deformation was measured as the distance along the X-axis from the start of the experiment to the point of failure divided by the magnification, which is the chart speed over crosshead speed.

The tissue length was measured accurately using a micrometer before and after the test. From the tissue dimensions (fractional change in length) and the data on the chart, values of stress (load/cross sectional area of the tissue in mm^2), strain (deformation/initial length in mm), and Young's modulus (stress/strain) were calculated according to NOBEL (1991).

Carrot plants grown under field conditions

Plant material. Carrot plants (*Daucus carota* L. cv. 'Nerac'), sown the second week of May by growers in southern Sweden were used. Carrot taproots were harvested the last week of October 2000. The carrots, 400 kg, were washed, hydro-cooled to 3 °C, packed in one huge polyethylene plastic bag and stored in a cold room at 0 °C and a relative humidity close to saturation, being the producer's routine procedure. Over a period of six months, batches of medium-sized carrots (19.5±1.0 cm in length, 3.0±0.2 cm diameter) free from defects, were selected at indicated times at the farm storage facility and transported to our laboratory in plastic bags kept on ice in insulated boxes. The carrots were then taken out of the bags and placed in a closed, refrigerated chamber in a water-saturated atmosphere at –0.2 °C, and were used for experiments within one week.

Chemical analyses (electrolyte leakage). The dry matter content was determined after drying the sample (ca 3 g fresh weight) for 16 hours in a vacuum-oven (Forma Scientific, Germany) at 70 °C and 100 Pa of absolute pressure.

Damage to cell membranes during storage at 0°C was tested by measurements of Relative Electrolyte Leakage (REL), based on the principle that damage to cell membranes results in enhanced leakage of solutes into the apoplastic water (LINDÉN et al. 2000). Taproot cores (50 mm long and 11 mm in diameter) were excised longitudinally from the phloem parenchyma of the middle part of the taproot using a cork borer. The cores were cut into 1 mm-thick discs that were rinsed (3x) with deionised distilled water. The discs (20) were incubated in 20 ml deionised distilled water in 50 ml plastic bottles at 20 °C. After 20 h, the conductance of the incubation medium was measured using a conductivity meter (CDM210, Radiometer, Copenhagen) to determine electrolyte leakage. Then tissue integrity was destroyed by freezing at –80 °C. After thawing at 4°C overnight, the conductivity of the bathing medium was measured to determine the total electrolyte content of the tissue. REL was calculated as the ratio of conductivity before and after freezing.

Both dry matter and REL were measured on 54 taproots every time they were sampled from the farm storage facility as indicated above.

Preparation of cell wall fractions and extraction of cell wall proteins. Cell wall fractions were prepared from harvested taproots during long-term storage. Cell wall proteins were extracted as described by GÓMEZ et al. (2004), based on the method published by CORKE and ROBERTS (1997) with certain modifications (N. Carpita, pers. commun.).

Unless otherwise indicated, all steps below were performed at 4 °C. Taproot cores, 50 mm long and 11 mm in diameter, were excised longitudinally from the phloem parenchyma of the middle part of the taproot using a cork borer. Two cores of approximately 7 g each were taken from each carrot. The cores were cut in small pieces and ground to a fine powder using liquid nitrogen and a mortar and pestle. The powder was homogenized in ice-cold 0.5 M KCl, 40 mM glycylglycine (pH 7.5), 1 % (w/v) Triton-X 100, 0.15 mM spermine, 0.5 mM spermidine and a few drops of octanol, using a tissue homogeniser (Ultraturrax) for 5 min (ratio g powder : ml buffer, 1:3). The homogenate was sonicated using an ultrasonic disintegrator equipped with a titanium probe with a diameter of 9.5 mm (100 W model, MSE, USA) in a 50 ml tube for 12 min at intervals of 5 sec, following by centrifugation at 1000 x g for 10 min at 4 °C. The pelleted cell walls were transferred to a nylon filter (50-μm pore size), and taken through four washing steps: a) 250 ml of 40 mM glycylglycine pH 7.5, b) 200 ml of H_2O, c) 250 ml of acetone, and d) 200 ml of H_2O. The cell wall preparations were stored under liquid nitrogen until the time of analyses.

For the extraction of proteins, 100 mg of the cell wall preparation was mixed with 400 μl of water and sonicated for 5 min using a sonicator bath (Bransonic 220, Kebo-Lab, Stockholm). 400 μl of Laemmli buffer without mercaptoethanol (2.2 % (w/v) SDS, 0.07 M Tris HCl) were added to the preparation and boiled for 5 min. Samples were centrifuged at 8000 rpm for 5 min and the supernatant was collected.

Sample concentration and quantification. The proteins extracted were concentrated by using centrifugal filter devices (Microcon YM-3, Millipore), with which 500 μl of the sample were reduced to 30 μl after centrifugation at 14000 x g for 100 min at room temperature.

The proteins in the concentrated samples were quantified prior to SDS-PAGE using a modified Lowry method (ALAM 1991) so that the same amount of protein (5 μg) could be loaded into each slot of the polyacrylamide gel. Prior to electrophoresis the sample was supplied with mercaptoethanol to a final concentration of 10 % (v/v).

Western blotting. Cell wall proteins were separated by SDS-page followed by electroblotting to a polyvinylidene fluoride transfer membrane (Immobilon-P, Millipore) before being probed with a polyclonal, affinity-purified carrot AFP antibody previously described by WORRALL et al. (1998). Bound primary antibody was detected using anti-rabbit secondary antibody and "ECL plus" western blotting reagent (Amersham Pharmacia Biotech) was used according to the manufacturer's protocol for immunodetection.

Mechanical strength analysed by slicing tests. The method described by GÓMEZ and SJÖHOLM (2001) was used. The carrots were sliced using stainless steel microtome blades (Feather S35, 0.26 mm total thickness) adapted to fit a Universal Instron Machine, (series 442H1004, UK) with a 100 N load cell. The carrot was placed perpendicular to the blade and sliced at a crosshead speed of 1016 mm min^{-1}. A new blade was used on every sixth carrot.

Carrots were removed from the refrigerated chamber, peeled manually, and immediately sliced in the centre. A computer connected to the Instron, using a "Lab View" program recorded the force-penetration data. From the force-penetration curves, the peak force (maximum cutting force, measured in Newtons) was obtained.

Statistical analyses. Statistical significance was tested by means of ANOVA analyses using MINITAB software (Minitab Inc., USA).

Results

Carrot plants grown in controlled environment

A significant increase in the values of stress and Young's modulus ($P<0.05$) was found in taproots of carrot plants kept 11 weeks under cold conditions when compared with the control (Table 1).

Table 1. Compression tests on non-acclimated (NA), 6 and 11 weeks cold-acclimated (CA) carrot phloem parenchyma tissue. Taproots were harvested from carrot plants grown under controlled temperature conditions as described in the materials and methods section. Mean values of four roots replicated five times are reported.

Time of acclimation	Stress (MPa)		Strain		Youngs Modulus (MPa)	
	NA	CA[1]	NA	CA[1]	NA	CA[1]
6 weeks	1.000	0.906	1.000	0.811	1.000	1.117
11 weeks	1.000	1.298[a]	1.000	1.060	1.000	1.225[a]

[1]Values are expressed as relative to NA

[a]Value significantly different ($P<0.05$) from control (NA) in each time of acclimation

Carrot plants grown under field conditions

The dry matter, initially 10.7 g 100 g^{-1}, decreased slightly during the storage period, being statistically significantly lower after 18 weeks of storage (n=54, P<0.05). Although postharvest water losses can realistically not be prevented, the high humidity conditions at the farm storage facility, however, prevented a dehydration that would result in an increase in dry matter. During the storage period, REL values remained practically unchanged during the first 18 weeks of storage, increasing significantly (P<0.05) after the 20th week of storage (Table 2).

Western blots of the extracted cell wall proteins showed that harvested taproots accumulated AFP in their cell walls reaching a maximum level after 12 weeks of storage at 0 °C, followed by a gradual decrease (Fig. 1).

The force necessary to slice the taproots changed during the storage period (Fig. 2). There is a slight decrease in the slicing force during the first 7 weeks of storage followed by a significant increase (P<0.05) in the 12th week. After the 12th week the slicing force tends to decrease up to the 18th week followed by a significant increase (P<0.05) up to the 24th week. The latter increase was accompanied by a significant increase in REL, as reported in Table 2 (indicated with an asterisk in Fig. 2).

Discussion

The results presented here show that texture of carrot taproots is a dynamic property and suggest that it is influenced by environmental conditions. HERPPICH et al. (2001), have previously suggested that tissue strength of carrot taproots might be strongly influenced by biochemical and physiological changes associated with the cold acclimation process.

In our experiments, the appearance and accumulation of AFP in the cell wall isolated from cold-stored taproots shows that a cold acclimation process is in progress in the harvested taproot derived from carrot plants grown in the field. Interestingly, the force needed to slice the taproots significantly increased during the first 12 weeks of storage, where the higher concentration of AFP indicates the highest development of cold acclimation during that period of time. The increase in tissue rigidity during cold acclimation was also shown by the increase of the Young's modulus in taproot tissue from carrot plants acclimated 11 weeks under strictly controlled temperature conditions. Both methods, compression and slicing tests, have been reported to produce reliable results for the toughness of plant tissue and to reflect the mechanical properties of the cell wall (ALVAREZ et al. 2000; THIEL and DONALD 1998; NOBEL 1991).

The increase in tissue mechanical strength of other plant species during cold acclimation has been described (STEFANOWSKA et al. 1999). It is an effect attributed to chemical changes in the cell wall (GÓMEZ et al. 2004). The consequences of cold-dependent modifications on cell wall structure on the tissue resistance to freezing are not fully understood. Increased cell wall rigidity and reduction of pore size during cold acclimation is thought to protect cells against intracellular freezing (RAJASHEKAR and BURKE 1996; RAJASHEKAR and LAFTA 1996). Small pores, acting as microcapillaries within cell wall structure, are likely to significantly depress the freezing point of water contained within the pores and present a barrier for the propagation of ice (WISNIEWSKI 1995).

After 24 weeks of storage, a sign of cell membrane dysfunction was detected with the significant increase in the REL value. Interestingly, this increase was accompanied by a significant increase in the slicing force. This result suggests that this increase of tissue strength might be a consequence of metabolic processes associated with stress responses related with membrane dysfunction.

Conclusions and future research

The results presented here give new insights into the possible biological principles involved in the dynamics of mechanical properties of carrot taproots. Knowledge of cell wall dynamics during cold acclimation

Table 2. Changes in Relative Electrolyte Leakage (REL) of field grown taproot tissue during long-term storage (n = 54). Means within columns sharing the same letter are not significantly different (P<0.05).

Storage time (weeks)	REL (x 10^{-2})
1	$9.74^{a} \pm 2.43$
4	$9.26^{a} \pm 1.64$
7	$8.74^{a} \pm 1.15$
12	$9.40^{a} \pm 2.61$
14	$8.43^{a} \pm 1.52$
18	$9.06^{a} \pm 2.36$
20	$12.00^{a} \pm 5.70$
24	$14.52^{b} \pm 6.62$

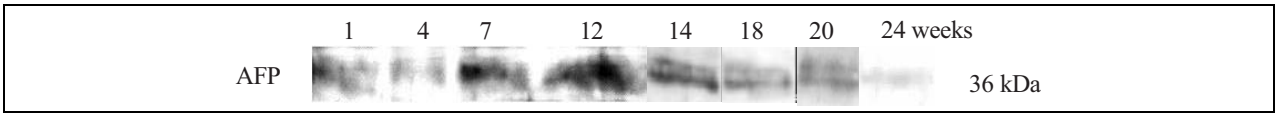


Fig. 1. Typical experimental result of a Western blot analysis showing the accumulation of a 36 kDa antifreeze protein (AFP) in carrot cell walls isolated from phloem parenchyma tissue of taproots, as described in the Materials and methods section, during long-term storage. Taproots were harvested from a field cultivation where the plants had been exposed to the natural variations in climate. Storage time in weeks at 0°C is indicated above each lane. Cell wall proteins from three carrots were analysed each week.

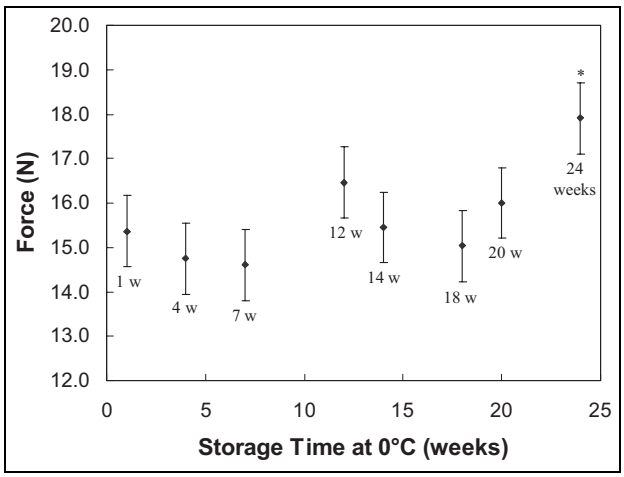


Fig. 2. Changes in the mechanical strength of field grown taproots during long-term storage at 0 °C as measured by slicing tests, mean values of 40 measurements are reported. Error bars represent 95 % confidence intervals of the mean based on a pooled standard deviation.

would give more information about the actual mechanisms involved in changes of the mechanical strength of the taproots. The understanding of biologically induced changes in vegetables such as carrots continues to be a research goal as a key knowledge that can be used to predict quality parameters in fresh vegetables.

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