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Effect of Pectic Oligomers on Physiological Responses of Chilling Injury in Discs Excised from Zucchini (*Cucurbita pepo* L.)

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The effect of pectic oligomers (OG) on ethylene biosynthesis, electrolyte leakage (EL), and CO_2 production was studied in discs excised from zucchini fruit (*Cucurbita pepo* L.) and stored at 20 or 2.5°C. At 20°C, OG enhanced ethylene biosynthesis and had a transient effect on decreasing EL, but showed little effect on respiratory rate; both the amount and size of the oligomer were important in changing both ethylene synthesis and EL. At 2.5°C, OG increased both ethylene biosynthesis and respiratory rate with a maximum effect at 100 μg of oligomer and peaking at 6 h; shorter oligomers demonstrated an even greater effect on ethylene biosynthesis, but differences were smaller in respiratory rate. EL at 2.5°C was affected most by 1 μg of OG and by monomeric galacturonic acid, with transient increases that peaked at 8 h. We suggest a signaling role for OG in the early steps of cold acclimation or chilling injury.

Key Words: **chilling injury; oligosaccharins; pectic oligomers; ethylene; electrolyte leakage; *Cucurbita pepo* L.; zucchini squash.**

Chilling injury (CI) is a physiological disorder induced by low, but not freezing, temperatures. Sensitive fruits show a variety of symptoms, often after being transferred to non-chilling temperatures (1, 2). Theories about CI have focused on the plasma membrane as the primary site for chill-induced membrane damage (3, 4). It is generally recognized that lateral phase transitions from the liquid crystal to the solid gel state occur in plant membranes as a result of the exposure of tissues to low temperatures (5). Membranes in the solid gel state become more permeable to solute molecules, causing cells to lose both compartmentalization and regulatory control. The result is a metabolic imbalance that leads to cell death provided that the temperature is under a critical minimum (2, 3). It is also known that the physical state of membranes depend on the degree of saturation of their lipids, and that fatty acid desaturases are expressed in response to low temperatures that re-establish the fluid state of membranes by introducing double bonds into the acyl chains (6, 7). However, the exact mechanisms by which plants perceive low temperature and by which the symptoms of CI are initiated remain unknown.

We theorize that the early steps in the CI phenomenon may be the same as those of the mechanisms of cold acclimation in plants. Studies have demonstrated that cold acclimation in plants is associated with gene expression that requires a transient influx of Ca^{2+} into the cytosol (8, 9). The calcium influx is known to occur through calcium channels localized in plasma membrane (10, 11). However, the nature and the sequence of the events leading to this Ca^{2+} influx are largely unknown.

Plant physiologists have been interested recently with novel oligosaccharides of both fungal and plant cell wall origin which posses biological activity as they act as signaling molecules eliciting plant defense responses (12, 13). These oligosaccharides, known as oligosaccharins, are released by enzymes and are thought to be involved in several plant developmental processes. The most studied plant oligosaccharins are those derived from pectin and xyloglucan. It is known that pectic oligosaccharins are involved in plant–pathogen interactions and they induce early responses in healthy vegetable tissues, e.g., ethylene biosynthesis, membrane depolymerization, and ion influx or ef-

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flux (14). At the present time oligosaccharins have not been correlated with abiotic stresses such as cold stress. It is our belief that such a relationship could occur.

A few studies relate changes in cell wall composition with CI (15–17). Wang and Adams (18) found significant changes in cell wall carbohydrate composition between chilled and nonchilled cucumbers; their results suggested that changes in composition could be due to cell wall-related enzymatic activities, but no enzymatic activity was evaluated. In recent work, Ramos-Clamont *et al.* (19) found increased polygalacturonase activity after storage of zucchini fruits at 2.5°C, peaking at 9 days; this trend in PG activity paralleled the production of ethylene. The results suggested a cascade of events, and prompted us to outline a model in which pectic oligosaccharins could be released from cell walls by PGs which were activated by low temperature. Pectic oligosaccharins in turn could serve as triggers for opening calcium channels, initiating thus the signal transduction pathway.

As a first approach, we investigated whether application of pectic oligosaccharides had an effect on ethylene production, respiratory rate, and electrolyte leakage in zucchini pericarp discs stored at 2.5 or 20°C to search for a possible role of pectic oligosaccharins in CI.

MATERIALS AND METHODS

Plant Material

Field-grown zucchini (*Cucurbita pepo* L.) fruit, cv. Raven were harvested on March–June 2001. Fruit for disc preparation were selected by size (17–20 cm) and quality attributes: turgor, dark-green color, free of wound and mechanical damage as well as free from insect infestation.

Disc Preparation

All procedures for preparation, handling and measurement of zucchini discs were done in a sterile hood as well as using sterile labware. From selected fruit to which both ends (2 cm) were removed with a knife, longitudinal strips approximately 3 mm in thickness were obtained. The strips were then randomly distributed on the work table. Ten discs (1 cm in diameter) were obtained with a cork borer, forming a cylinder within the barrel of the cork borer. The cylinder was then taken out by pushing in with a glass rod and the length of the cylinder was measured. From the diameter and length the volume of 10 discs was calculated.

Cut discs were briefly rinsed with a 0.1% sodium hypochlorite solution, followed by sterile deionized water. Drained discs were blotted on sterile filter paper to remove absorbed water and then placed, cut surface up, on sterile culture plates previously weighed. The weight of ten discs was recorded and each plate was assigned a number for further identification.

Immediately after being weighed, culture plates with the discs inside were placed in vacuum dessicator, which were stored in a chamber at 20°C. The HR inside the dessicator was 98%. Discs were kept at that condition until treatments were applied, usually no more than 18 h.

Preparation of Oligogalacturonides

Partial enzymatic hydrolysis of polygalacturonic acid, PGA. The pectic oligomers were obtained by the method described by Spiro *et al.* (20). A 2% solution of polygalacturonic acid (Na^+ salt) in 20 mM sodium acetate, pH 5, was treated for 8 h at 23°C with endo-α-1,4-polygalacturonase (EPG, 30 units: 1 unit of the EPG released 1 μM reducing sugar per min from a 1% solution of PGA at pH 5.2 and 30°C) purified from *Fusarium monoiliforme* (a gift from C. Bergmann of the CCRC). The solution was autoclaved for 15 min at 120°C to terminate the enzymatic hydrolysis. The autoclaved solution was cooled to 23°C.

Selective precipitation of oligogalacturonides by treatment of EPG-digested PGA with EtOH and NaOAc. The partial EPG digest of PGA was adjusted to contain 0.5% galacturonic acid residues (w/v), 50 mM NaOAc, and 11% EtOH (v/v). The mixture was kept overnight at 4°C. The precipitate that formed was collected by centrifugation (14,000*g* for 30 min at 4°C), dialyzed, and freeze dried. In this way, oligogalacturonides with a DP between 10 and 15 were obtained and were applied as a mixture to zucchini discs.

Q-Sepharose chromatography of the oligogalacturonides precipitated by EtOH and NaOAc. A solution of the NaOAc-EtOH-precipitated oligogalacturonides in 50 mM ammonium formate was adjusted to pH 6.5 with 10 mM ammonium hydroxide. Ammonium formate (1 M) was added until the conductivity of the solution was equal to that of 300 mM ammonium formate. The mixture was loaded onto a Q-Sepharose column (2.2 $\times$ 50 cm) that had been equilibrated with 300 mM ammonium formate, pH 6.5. The oligogalacturonides were eluted by a two-stage concentration gradient of ammonium formate, pH 6.5, at a flow rate of 5 ml/min, and fractions (5 ml) were collected after 120 min. Aliquots of every second fraction were assayed colorimetrically (21) for uronic acids. Fractions corresponding to uronic acid-containing peaks were pooled, dialyzed (2000 MWCO), and freeze dried. Aliquots of each of the pooled peaks were analyzed by HPAEC-PAD (Dionex, Sunnyvale, CA) to determine the DP of the major component.

Application of Treatments

Treatments consisted of the application of 100, 10, or 1 μg of a mixture of pectic oligomers to a single disc. Monomeric galacturonic acid (GA) and oligomer fractions with DP = 10–12 (F1) and DP = 13–15 (F2) were also applied in a single concentration of 100 μg. GA was applied to account for changes in pH of the unbuffered solutions. Treatments were done in triplicate with ten discs per replicate for CO_2 and ethylene production, and four discs for electrolyte leakage. Sterile unbuffered aqueous solutions of oligomers were made in such a way that 30 μl of solution was equal to the desired amount of oligomer to be applied. Treatments were applied by pipette in aliquots of 30 μl on the cut surface of the endocarp. Sterile deionized water was applied as control. Solutions were absorbed into the disc within 30 min after treatment. The discs were then carefully placed into 24.5-ml glass vials that were closed and stored at 20 or 2.5°C. Time was recorded and samples were taken every 2 h for analysis.

Electrolyte leakage (EL). Four discs from each treatment were stored at 2.5 and/or 20°C in glass vials. Every 2 h, three vials from each treatment were transferred from 2.5°C and kept at 20°C for 1 h. Then 15 ml of 20 mM mannitol were added, vials were mixed in an aliquot mixer (Thermolyne, Dubuque, IA) for 1 h, and electrical conductivity readings were taken using an electrical conductivity meter (Cole-Parmer, Chicago, IL) as a measure of electrolyte leakage from the discs. Vials were placed in a boiling bath for 30 min, cooled at room temperature, and a final conductivity reading was taken for total electrolytes. Vials with discs stored at 20°C were directly added to 15 ml of 20 mM mannitol every 2 h and handled as those at 2.5°C. All electrolyte leakage data were calculated as a percentage of the total electrolyte readings and the reported values are the mean of three replicates.

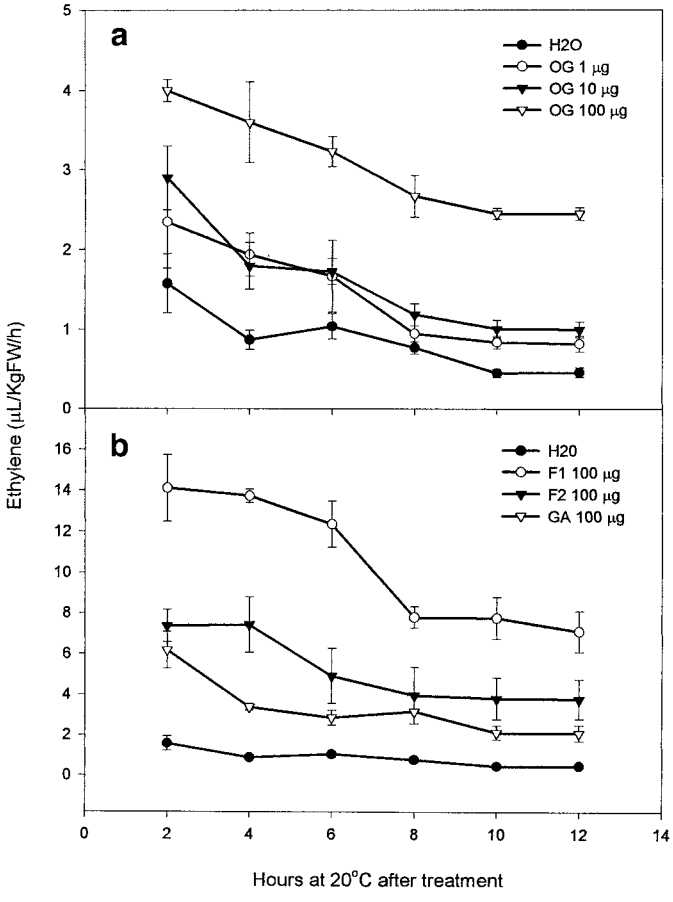

FIG. 1. Change in ethylene biosynthesis in response to pectic oligomers by zucchini pericarp discs when (a) three different amounts of a mixture of pectic oligomers (OG) and (b) 100 μg of shorter (F1) or larger (F2) oligomers and galacturonic acid (GA) were applied to individual discs before stored at 20°C. Bars indicate standard deviations for three replicates. See text for details.

Ethylene (C_2H_4) and CO_2 determination. Ten OG-treated discs were placed in 24.5-ml glass vials provided with an open-top cap and septa, and stored at 2.5 or 20°C. Vials at 20°C were capped immediately after placing the discs inside. For those samples at 2.5°C, three vials from each treatment were capped and transferred from 2.5°C to a room maintained at 20°C every 2 h and were allowed to warm to room temperature for 1 h. Gas samples were then taken for C_2H_4 and CO_2 determination by gas chromatography. From the vials stored at 20°C, gas samples were taken every 2 h and injected on the gas chromatograph (Varian Star 3400, MFG Corp., U.S.A.) using a TCD and FID detectors with a Haysep N column. Results were expressed as μl C_2H_4/kg.h^{-1} for ethylene, and as ml/kg.h^{-1} for CO_2, and were the average of three replicates.

RESULTS AND DISCUSSION

Ethylene Biosynthesis

A mixture of pectic oligomers (OG) induced an increase in the rate of ethylene synthesis when applied to zucchini pericarp discs stored at 20°C (Fig. 1a). The rate of ethylene production decreased with time for both control and oligomer treatments but increased with the amount of oligomer, reaching a maximum at 100 μg of oligomer mixture per disc (Fig. 1a). The levels of ethylene in response to control treatments could be a consequence of tissue damage; however, results show a clear enhancement of ethylene production by OG at 20°C. It is well known that OG induce ethylene biosynthesis in cultured pear cells (22, 23), orange albedo (24), whole tomato fruit and tomato pericarp discs (25–28); in all the studies above the ethylene increase was transient and peaked in a few hours which suggested a regulatory role for the oligomers in ethylene biosynthesis. Our results showed that in zucchini discs at 20°C ethylene production did not peak but decreased gradually with time in both control and oligomer treatments.

The size of OG also influenced the ethylene biosynthesis at 20°C (Fig. 1b). The general tendency was the same as for OG mixture concentration, but the amounts of ethylene produced were much higher. Shorter oligomers (F1) induced the major ethylene production at 20°C (Fig. 1b). The reason may be that in F1 are the most concentrated of the active sizes.

The physiological significance *in vivo* for the increase in ethylene levels by OGs in zucchini discs is unknown.

Pectic oligomers induced ethylene production in zucchini pericarp discs at 2.5°C, and the rate of biosynthesis increased with the amount of oligomer applied from 1 to 100 μg per disc (Fig. 2a). In response to both water and 1 μg oligomer treatments, ethylene production was not detected until 4 h (approximately 5 μl/kg fresh wt/h), after which its rate was almost constant in the course of the experiment. In response to 10 μg and 100 μg of oligomers the rate of ethylene production rose within 2 h, reached a maximum at 6 h, then declined slightly at 8 h and remained almost constant during the course of the next 4 h (Fig. 2a). In response to 100 μg of the oligomer mixture the rate of ethylene biosynthesis throughout the 12 h time period increased 100% or more from that at 10 μg of oligomers (Fig. 2a).

Ethylene production is a common event in CI (18, 29–31) but the ethylene-CI relationship is not clear. An increased production of ethylene in chilled cucumbers results from an enhanced capacity of the tissue for ACC synthesis (30), but the nature of its mechanism remain unknown. A prolonged exposure to cold seems to damage the system that converts ACC to ethylene, as ACC levels were higher in chilled cucumbers even when ethylene production started to decline (30). Figure 2a shows a transient increase in ethylene biosynthesis with a peak at 6 h for both 100 and 10 μg OG treatments. It is interesting that the transient increase seen in Fig. 2a was evoked by pectic oligomers which could act as an early signal to trigger a cascade of responses in order to either protect against or lead to CI, depending on both the size and amount of oligomer.

Figure 2b shows that treatment with F1 increased progressively the ethylene production during 10 h at 2.5°C in zucchini pericarp discs. At 6 h, the amount of

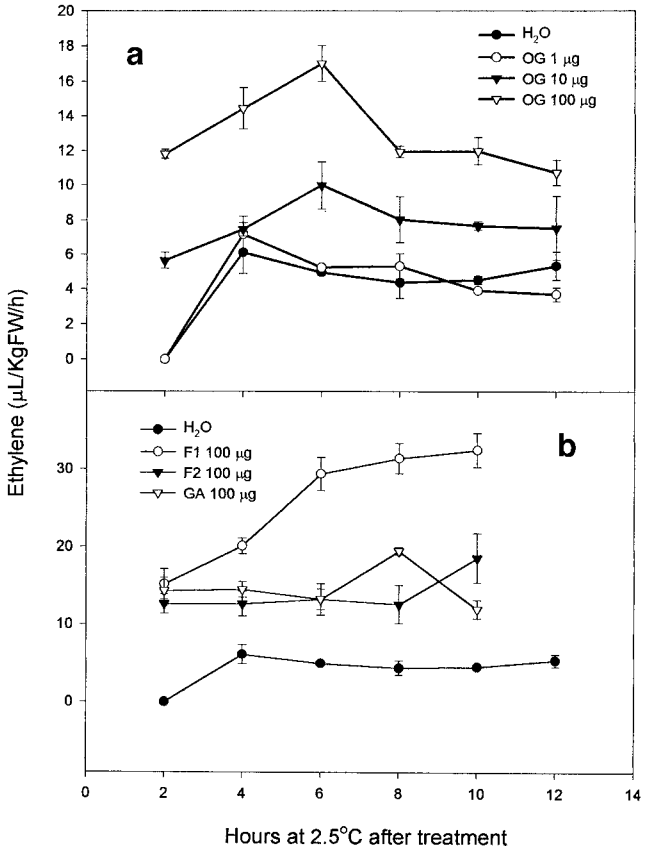

FIG. 2. Change in ethylene biosynthesis in response to pectic oligomers by zucchini pericarp discs when (a) three different amounts of a mixture of pectic oligomers (OG), and (b) 100 μg of shorter (F1) or larger (F2) oligomers and galacturonic acid (GA) were applied to individual discs before stored at 2.5°C. Bars indicate standard deviations for three replicates. See text for details.

ethylene was almost 100% of that induced at 2 h, then the increase was smaller and gradually tended to a constant value. This result support the assumption that the most active oligomers for ethylene production are in F1. GA monomer did not affect the ethylene production.

Electrolyte Leakage (EL)

Treatments with 1 and 10 μg of oligomer mixture had a transient effect on EL in zucchini pericarp discs stored at 20°C (Fig. 3a). The high EL values recorded at 2 h could be due to tissue damage which occurred while obtaining the discs. However, EL was much higher in treatments with 1 and 10 μg of OG than in the control, indicating an effect of oligomers on EL that can be noticed within the first 2 h. Electrolyte leakage is used as an indicator of damage to the plasma membrane in relation to environmental stresses (32). According to the traditional model, exposure to cold induces lateral phase transitions in membranes, which alters both their biophysical properties and physiological functions, including cell permeability (33, 34). An increased permeability results in an enhanced electrolyte flow, which is measured indirectly as electrolyte leakage (3, 34).

There are no reports about the effect of oligosaccharins on EL. However, although the mechanisms are not known, it has been shown that OG induced a number of rapid responses on the plant membrane surface. The effect of OG on ion flow through plasma membranes has been studied. For instance, tomato mesophyl cells are depolarized within 5 min of exposure to relatively high concentrations of OG (1 mg/ml) (35). Lower concentrations (10 μg/ml) of OG of specific size (DP = 12–15) induced a transient efflux of K^+, alkalinization of the medium, plasma membrane depolarization, and a decrease of the external Ca^{2+} in suspension cultured tobacco cells (36); this last effect has also been observed in carrot protoplasts (37).

In our study the reduced EL observed within the first 6 h in response to treatments with 1 and 10 μg of oligomer (Fig. 3a) can be due to an ion influx. Another

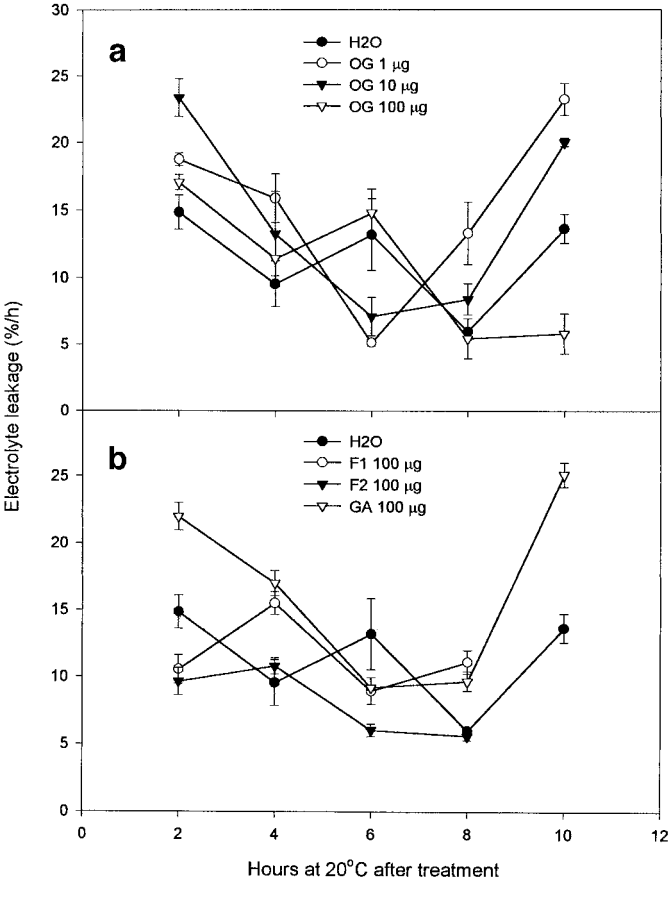

FIG. 3. Change in electrolyte leakage in response to pectic oligomers by zucchini pericarp discs when (a) three different amounts of a mixture of pectic oligomers (OG) and (b) 100 μg of shorter (F1) or larger (F2) oligomers and galacturonic acid (GA) were applied to individual discs before stored at 20°C. Bars indicate standard deviations for three replicates. See text for details.

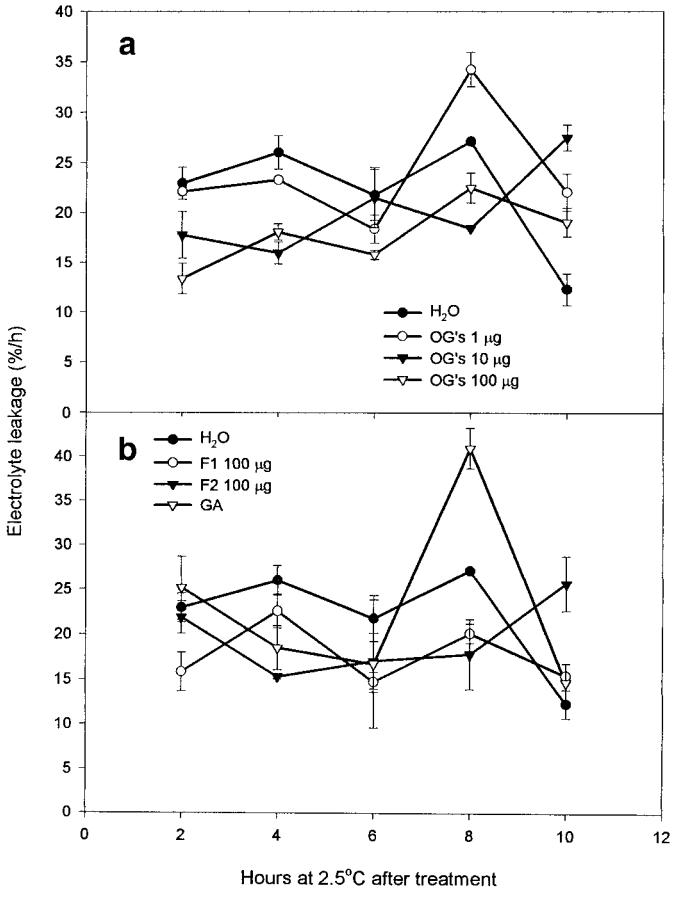

FIG. 4. Change in electrolyte leakage in response to pectic oligomers by zucchini pericarp discs when (a) three different amounts of a mixture of pectic oligomers (OG) and (b) 100 μg of shorter (F1) or larger (F2) oligomers and galacturonic acid (GA) were applied to individual discs before stored at 2.5°C. Bars indicate standard deviations for three replicates. See text for details.

explanation could be that oligomer–ion complexes were formed thus reducing the available ions in solution. However, the significant increases in EL after the minimum observed at 6 h suggests that the oligomer–ion forming process is reversible. This reversibility would be unlikely to occur in our system once the complex has been formed. It is therefore probable that EL fluctuations seen on Fig. 3a are due to an ion influx in response to OG, although it is difficult to suggest a role for such a mechanism. The effect of galacturonic acid monomer (GA) on EL at 20°C (Fig. 3b) is similar to the observed effect following treatment with 1 and 10 μg of OGs (Fig. 3a).

For those discs stored at 2.5°C the application of 1 μg of oligomer mixture increased EL which reached a maximum at 8 h (Fig. 4a). It is interesting to note that such effect was very similar to that induced by monomeric GA (Fig. 4b), so it is probable that GA was responsible for increasing the EL at 2.5°C.

Usually, in fruits stored under refrigeration, EL is measured in the course of several days and sometimes differences become distinguishable after a week. We observed differences in EL within a few hours. This early effect could be due to ion flow involved in signal transduction mechanisms rather than changes in membrane permeability related to its physical properties.

The appearance of a peak in EL with time suggests a transient cell permeability loss. Plant cold acclimation studies have shown that low temperature evokes an immediate and transient increase of free Ca^{2+} in the cytosol of plant cells (8–10, 38); such an increase is initiated by either an influx of Ca^{2+} through the plasmalemma or by releasing it from internal stores of calcium within the cell (e.g., vacuole) (9, 11). The calcium influx is strongly correlated with the expression of cold acclimation specific genes (*cas* genes) which confer freezing resistance to alfalfa (8). Blocking calcium influx prevents both the expression of *cas* genes and development of freezing tolerance in alfalfa cells (*Medicago sativa*) (39), whereas chemical agonists of the calcium channels induce the expression of *cas* genes in alfalfa cells at 25°C (8). The results above have shown that Ca^{2+} influx is an integral component of the signal transduction pathway in plant cold acclimation, but both nature and sequence of the events that lead to it are unknown (40).

Probably, the ups and downs in EL we found in zucchini pericarp discs treated with OG and then stored at 2.5°C are due to cell mechanisms in order to prepare the tissue for protecting itself against cold. If such is the case, OG may be the primer messenger of the signal cascade. It is worth noting that one of the most effective methods to reduce CI in zucchini fruits is thermal preconditioning, which consists of subjecting the fruits to 10 or 15°C for 2 days before their storage at 2.5°C; the mechanisms by which the CI is reduced are unknown, but an acclimation phenomenon has been suggested [see review in Lurie (4)].

Rate of Respiration

The rate of respiration in zucchini pericarp discs stored at 20°C decreased gradually from 130 to 170 mg CO_2/kgFW/h at 2 h to 50–70 mg CO_2/kgFW/h at 10 h among the different amounts of oligomer mixture tested (Fig. 5a). There was more CO_2 production when 100 μg of oligomer mixture was applied and the difference became more evident at 2 and 4 h.

Melotto *et al.* (26) showed that pectic OGs modulate ethylene biosynthesis in tomato and our results showed that at 20°C ethylene biosynthesis increased with the amount of oligomer mixture in zucchini discs (Fig. 1a). Ethylene is physiologically active at threshold levels and a small increase in its activity leads to important metabolic changes, especially in nonclimacteric fruits like zucchini in which ethylene production is very low (41). Usually, increases in ethylene biosynthesis are accompanied by changes in respiratory rate,

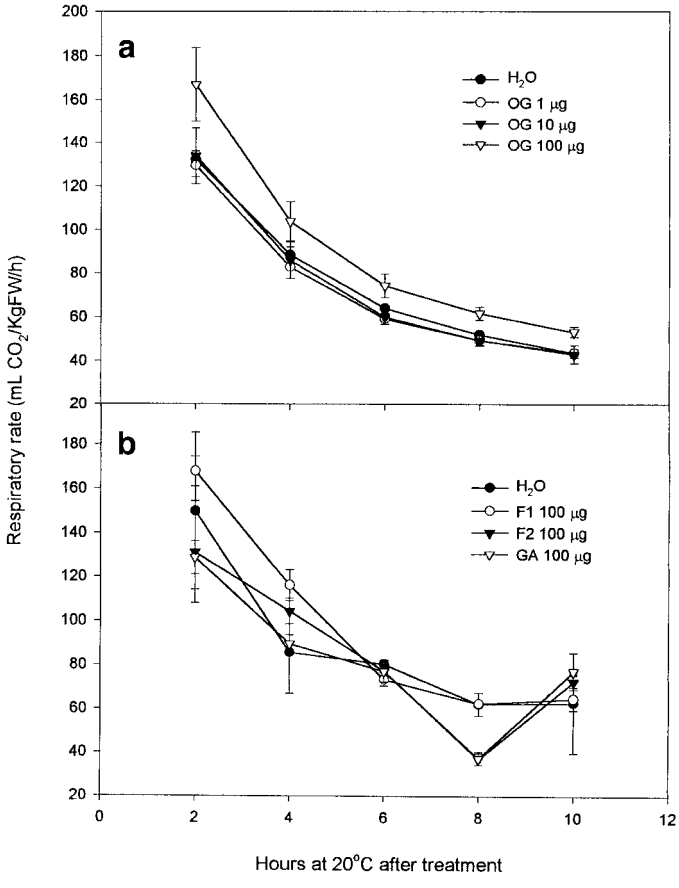

FIG. 5. Change in respiratory rate in response to pectic oligomers by zucchini pericarp discs when (a) three different amounts of a mixture of pectic oligomers (OG) and (b) 100 μg of shorter (F1) or larger (F2) oligomers and galacturonic acid (GA) were applied to individual discs before stored at 20°C. Bars indicate standard deviations for three replicates. See text for details.

so when either respiratory or metabolic activities of a plant tissue are characterized, both ethylene and CO_2 production are considered together (42). The higher CO_2 production in zucchini discs at 20°C in response to higher amounts of a mixture of OG (Fig. 5a) may be a function of either sensitivity or perception thresholds of the target ethylene-receptor cells. Cell sensitivity to ethylene varies even within a tissue (43, 44).

The rate of respiration in zucchini discs applied with 100 μg of oligomers of different size and stored at 20°C had a decreasing trend in the course of 10 h (Fig. 5b). There were no important differences among control and the treatments with GA, F1, and F2.

Temperature had a more outstanding effect than oligomer concentration on CO_2 production in zucchini discs. Figure 6a shows that the respiratory rate in discs stored at 2.5°C and further transferred to 20°C behaved the opposite to those stored at 20°C (Fig. 5a). The rate of respiration rose within 2 h with levels within a range of 200–300 mg CO_2/kgFW/h with the amount of oligomer applied, then increased in the course of 10 h until a maximum which was close to 500 mg/kgFW/h (Fig. 6a) was reached. Discs treated with 100 μg of oligomer mixture and stored at 2.5°C showed the highest rates of respiration, the same as with those stored at 20°C (Fig. 5a); however, the intensity of respiratory rate was lower in 20°C discs. These results suggest that the effect of low temperature on respiratory rate can be enhanced by pectic oligomers and that the effect is transient at higher concentrations of oligomer.

Generally, when a vegetable is stored at low temperature and then transferred to a warmer one its metabolism can increase up to 2 to 2.5 times with every 10°C. Because of the low temperature, the biochemical reactions are accelerated and therefore the rate of respiration increased; such a behavior is considered to be normal (45). In plant tissues that are susceptible to CI the metabolic changes are more exaggerated because the low temperature induces changes in both thermodynamic and kinetic properties of the respiratory enzymes, which then leads to an imbalance in glycolysis

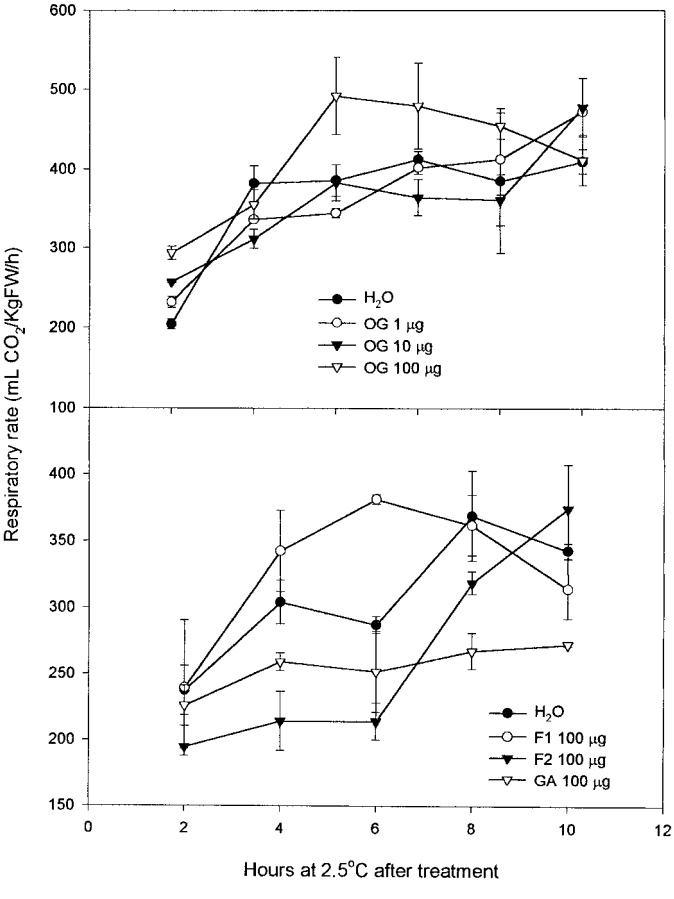

FIG. 6. Change in respiratory rate in response to pectic oligomers by zucchini pericarp discs when (a) three different amounts of a mixture of pectic oligomers (OG) and (b) 100 μg of shorter (F1) or larger (F2) oligomers and galacturonic acid (GA) were applied to individual discs before stored at 2.5°C. Bars indicate standard deviations for three replicates. See text for details.

and related reactions (46). Those changes are not inherent to respiratory enzymatic proteins but result from molecular rearrangements in mitochondrial membranes that are associated with the respiratory enzymes (46). In sensitive tissues the metabolic imbalance that results from the exposure to higher temperatures than the critical temperature is usually reversible as are the structural rearrangements of lipids and receptors at the membrane level, provided that the low temperature is maintained. However, when the tissue is transferred to a warmer temperature, the metabolic changes become irreversible. CI irreversibility is shown when respiration shows a permanent increase.

In our study, the permanent increase in respiratory rate at 2.5°C in response to water, 1 or 10 μg of oligomer mixture (Fig. 6a) and water, F2 or GA treatments suggest the probability of an irreversible chill-induced damage which could start within the first hours of exposure to cold. Also, the results show that at 2.5°C the increase in respiratory rate in response to 100 μg of oligomer mixture or to shorter oligomers (F1) was not permanent but peaked at about 6–8 h (Figs. 6a and 6b), and is coincident with the increase in ethylene production (Fig. 2a). These results suggest that shorter pectic oligosaccharides in high concentrations may act as early signals for protecting the tissue of zucchini against cold thus allowing the process to be reversible.

CONCLUSIONS

Pectic oligomers enhanced ethylene biosynthesis and had a transient effect on decreasing electrolyte leakage, but had little effect on the respiratory rate in zucchini pericarp discs at 20°C; both the amount and size of oligomer were important factors in changing both ethylene synthesis and electrolyte leakage.

At 2.5°C, OG increased both ethylene biosynthesis and respiratory rate with a maximum effect at 100 μg and peaking at 6 h; shorter oligomers had a more dramatic effect on both ethylene biosynthesis and respiratory rate. Electrolyte leakage at 2.5°C was affected more by the lowest amount of OG tested and by monomeric galacturonic acid with a transient increase which peaked at 8 h.

We suggest a signaling role for pectic oligosaccharides in the early steps of cold acclimation or CI symptoms.

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