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Anaerobic exposure before or after wounding reduces the production of wound-induced phenolic compounds in fresh-cut lettuce

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ABSTRACT

Wounding lettuce (*Lactuca sativa* L., Longifolia) leaves by excising 5-mm thick mid-rib segments increased phenylpropanoid metabolism with the synthesis and accumulation of wound-induced phenolic compounds (WIPC). Immersing freshly excised segments for 1 h in 20 °C water agitated with air or N_2 produced a 45% or 65% reduction in wound-induced phenolic content, respectively, compared to non-immersed segments when measured after incubation for 48 h at 10 °C in air. In contrast, agitating the water with O_2 produced a 23% increase in WIPC over the non-immersed controls. The enhanced reduction of WIPC in N_2 versus air agitated water, and the increase in WIPC in O_2 agitated water suggests that anaerobiosis, and not dilution of the wound signal, was the cause of the reduction in WIPC. Holding 5-mm segments in an anaerobic N_2 atmosphere produced a similar reduction in WIPC as did holding the segments in water. Delaying the 1 h anaerobic treatment for up to 3 h had no significant effect on the ability of the anaerobic treatment to reduce WIPC. Exposing 8-cm long mid-rib sections to anaerobiosis for 2 h before excision of the 5-mm segments reduced subsequent WIPC from the 5-mm segments. The previous anaerobic treatment of the 8-cm sections predisposed the tissue to have a reduced response to subsequent wounding. After a 2 d lag in WIPC accumulation, the rates of accumulation were similar for the air and 2 h anaerobic treated 5-mm segments. Using vacuum treatments to facilitate the loss of volatile products of anaerobic metabolism (e.g., acetaldehyde and ethanol) did not have a significant effect on the accumulation of WIPC. Ion leakage from the symplastic volume of the tissue (i.e., across the cell membrane) was unaffected by the anaerobic treatments, but leakage from the apoplastic volume increased with increasing duration of the anaerobic treatment. Immersing fresh-cut lettuce in an aqueous solutions did not reduce the wound response because of dilution of the wound signal, but because of the anaerobic environment created within the tissue. Some remnant of the anaerobic treatment seems to persist in the tissue and delay the accumulation of WIPC.

1. Introduction

Wounding lettuce (*Lactuca sativa* L., Longifolia) leaves stimulates phenylpropanoid metabolism with the subsequent synthesis and accumulation of soluble o-diphenol compounds (e.g., chlorogenic acid) (Degl'Innocenti et al., 2005; Mai and Glomb, 2013; Tomás-Barberán et al., 1997). At 10 °C, the activity of phenylalanine ammonia-lyase, the initial step in phenolic metabolism, reaches a peak after 24 h, while the accumulation of newly synthesized phenolic compounds (Abs 320 nm g^{-1}) plateaus at 48 h (Tomás-Barberán et al., 1997). These soluble wound-induced phenolic compounds (WIPC) are sequestered in the vacuole and participate in browning reactions when membranes become disrupted (e.g., depolarization following wounding or during senescence) which allows the mixing of substrates and enzymes (e.g., polyphenol oxidase, peroxidase) in injured and adjacent non-injured tissue (See Hunter et al., 2017 for a review of the biochemistry of phenolic metabolism in wounded lettuce tissue). The undesirable development of tissue browning following the accumulation of the newly synthesized WIPC causes a significant loss of quality in minimally processed lettuce (Lopez-Galvez et al., 1996).

The signal that transduces the physical wound into a physiological response (e.g., the *de novo* synthesis of PAL and accumulation of WIPC) is unknown in lettuce (Campos-Vargas and Saltveit, 2002), but it may involve components of the phospholipid signaling pathway (Saltveit et al., 2005). Recent research has identified a number of possible components of the signal (Carlos et al., 2017). It appears that a chemical signal is produced at the site of injury and migrates or propagates into adjacent non-injured tissue at a rate of ca. 5 mm h^{-1} where it induces an increased synthesis and accumulation of WIPC in tissue up to several cm from the site of injury (Ke and Saltveit, 1989). A kinetic analysis of wound induction of ethylene, and of PAL induction by ethylene and wounding indicated that the increase in wound-induced

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PAL in lettuce did not proceed through the induced synthesis and action of ethylene (Ke and Saltveit, 1989; Campos-Vargas and Saltveit, 2002).

While research continues to elucidate the components of the wound signal that induces the browning process (García et al., 2017), various treatment have been identified that reduce the wound response by interfering with the synthesis, propagation, or activity of the wound signal. For example, immersing freshly excised lettuce tissue in an aqueous solution reduces the wound response, possibly because a portion of the wound signal leaches from the tissue into the surrounding solution. The remaining signal is diluted and therefore less active. Research reported in this paper was undertaken to see if the reduction in the wound response that follows immersing fresh-cut lettuce in an aqueous solutions was the result of a dilution of the wound signal, or of an anaerobic environment created within the tissue.

2. Materials and methods

2.1. Plant material

Romaine lettuce (*Lactuca sativa* L., Longifolia) was purchased from local vendors and stored at 2.5 °C until used. Outer damaged leaves were discarded and the next 5 non-damaged leaves were carefully detached from the stem and used in all subsequent experiments. The leaf blade was excised from the leaf, leaving achlorophyllous mid-rib tissue that was trimmed from the base to produce sections 8 cm in length. These 8 cm long mid-rib sections were cut into 5-mm thick segments. The 8 cm sections or 5 mm segments were held for varying lengths of time in water, or atmospheres consisting of air (20.9% O_2), or 100% N_2 or O_2 at 20 °C.

In some anaerobic experiments, 12 g of 5-mm segments were held in 35-mL plastic syringes through which flowed humidified gases at 200 mL min^{-1}. A one-hole rubber stopper was inserted in the plunger end of the syringe and the end of a flexible tube inserted in the rubber stopper was immersed in a beaker of water to monitor the flow of N_2 and to prevent any air from entering the syringe. In other experiments, the sections were held in a plastic bag through which flowed humidified 100% N_2 at 200 mL min^{-1} before, during and/or after the sections were segmented. The flexibility of the bag allowed the 8-cm sections to be cut into 5-mm segments with a razor blade in the bag while still under an anaerobic atmosphere.

After treatment, about 12 g of segments were put into 10 × 100 mm diameter plastic Petri dishes. The dishes were placed in 20 × 15 × 10 cm plastic tubs lined with wet paper towels. The tubs were loosely covered with aluminum foil and held at 10 °C in ethylene-free air. Initial samples of 3 g of tissue were placed in 50-mL plastic centrifuge tubes and frozen at −20 °C.

2.2. Measurement of phenolic content

Three samples of 3 g from each treatment were placed in 50-mL plastic centrifuge tubes along with 20 mL methanol. The tissue was homogenized and the absorbance of a clarified extract read at 320 nm as previously described (Ke and Saltveit, 1989; Loaiza-Velarde et al., 1997; Campos-Vargas and Saltveit, 2002) and expressed as Abs 320 nm per gram fresh weight (Abs 320 g^{-1}). This measure is highly correlated with the chlorogenic acid content of wounded lettuce leaf tissue and subsequent tissue browning (Tomás-Barberán et al., 1997).

2.3. Measurement of respiration

Carbon dioxide production was calculated from changes in the head space concentration in a static setup. Two whole non-damaged leaves were enclosed in 1.2 L glass jars. Twelve g of freshly excised 5-mm segments were enclosed in 375 mL glass jars. The jars were capped, and gas samples were periodically withdrawn through a rubber serum stopper inserted in the jar's lid and analyzed using an infrared CO_2 analyzer. The rate of respiration was calculated as mL CO_2 $(kg\ h)^{-1}$

2.4. Immersion in water

12 g of freshly excised 5-mm mid-rib segments were immersed in 1 L of water for 60 min through which a turbulent flow of air or 100% N_2 or O_2 bubbled at 200 mL min^{-1}. Prior to the addition of the segments, air, N_2 or O_2 had bubbled through the 1 L of water for 120 min. The gases flowed through an "aquarium air stone" positioned at the bottom of a 1.5-L glass jar. The 'air stone' produced many small bubbles that hasten saturating the water with the gases.

2.5. Application of vacuum to tissue segments

12 g of 5-mm mid-rib segments were put into a 60 mL plastic syringe immediately after spending 120 min in a 200 mL min^{-1} flow of humidified N_2. The plunger was inserted and set to 22 mL. The syringe tip was capped with a valve in the closed position and the plunger pulled back to the 62 mL mark. After 15 s, the valve was moved to the open position allowing humidified air to enter the syringe. The syringe plunged was again set to 22 mL, and the process was repeated 10 times. Since the 12 g of tissue would occupy about 12 mL, the initial void volume was 10 mL (22-12). Pulling the plunger to 62 mL lowered the pressure within the syringe from 101 to 20 kPa; [101x (22-12)/(62-12)]. After 10 cycles of this vacuum treatment, the segments were handled as previously described to allow for accumulation of WIPC. The fresh weight of the 12 g samples was not significantly changed by the vacuum treatments (data not shown).

2.6. Measurement of ion leakage

Ten freshly excised 5-mm segments were immersed in 100 mL of 0–400 mM aqueous mannitol solutions in a 250 mL glass beaker. The change in fresh weight of the segments over time was used to calculate the isotonic 250 mM concentration for the 5-mm mid-rib segments.

Four 5-mm segments were put into 20 mL of an isotonic 250 mM mannitol solution in a 50 mL plastic centrifuge tube. The conductivity of the solution was periodically measured for 180 min. The tubes were gently shaken between readings. The tubes were then capped, and frozen and thawed twice with shaking. The total conductivity of the solution was read after the tubes had come to 20 °C with shaking and the rate of leakage expressed as percent total conductivity per h.

2.7. Statistics

The Romaine lettuce was obtained from local sources. The excised tissue exhibited significant variability in the magnitude of the wound-induced increase in phenolic content. Experiments were done over 18 months, so some variability was expected since different cultivars of lettuce would have been grown under different field conditions. However, although the magnitude of the responses differed among experiments, the variability within any experiment was small, and the response itself was consistent among all experiments for identical treatments. That is, there was no experiment in which wounding did not increase phenolic content, and in which an anaerobic treatment did not reduce this increase. Because of the variability among experiments, some experiments were repeated up to five times with similar results. There were three replicates of each treatment in each experiment. Means and standard deviations (n = 9 to 15) were calculated from similar treatments.

3. Results and discussion

3.1. Wound-induced phenolic metabolism

As previously described, the level of extractable WIPC (i.e., Abs