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Pre-heated blades for harvesting baby-leaves reduce the risk of *Escherichia coli* internalization in leaves

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Abstract

Background. Pathogenic enterobacteria can travel through the plant vascular bundles, by penetrating from cuts and persist into ready-to-eat leafy greens. As the cutting site is the main point of entrance and uptake, we tested how different cutting strategies can reduce bacterial internalization in leaves. Horizontal cuts at the base of the leaves were performed with two different types of tools, the first with a scalpel (by pulling the blade) and the second with a scissor-action that has blades that cuts by gliding against a thicker blade. Scissor-action generally makes closer border cuts. Blades of both types of tools have worked at 25 °C and 200 °C. The goal was to study how these different types of cuts and temperatures affected

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jsfa.12335

bacterial uptake in leaves. Experiments were repeated on different plant genotypes and at different wilting stages.

Results. Our finding showed that cutting baby-leaves with a scissor at 200 °C significantly reduced the bacterial uptake when compared with the not heated (which simulates a mechanized lettuce harvester). The most effective cutting treatments to reduce bacterial uptake were in order: scissor 200 °C > scissor 25 °C > scalpel 200 °C > scalpel 25 °C. The scissor heated at 200 °C was also preventing bacterial uptake on wilted baby-leaves.

Conclusions. This finding could provide further contribution in terms of safety during harvest and suggest that a pre-heated blade supports safety during harvest of leafy greens.

Keywords: baby leaves, bacterial internalization, bacterial uptake, produce safety, ready-to-eat produce, produce harvest

Introduction

Internalization of enterobacteria in fresh ready-to-eat leaves is important from a food safety perspective [1]. A number of evidences has shown that microbes such as *Escherichia coli* can infiltrate in xylem vessel in plant tissues [2–4], including tomato [5], rocket and Swiss chard baby-leaves [6] and baby-spinach [7]. Enterobacteria can penetrate in vessel from roots [3,8], flowers [9], wounded fruits [10–12]. Ready-to-eat leaves at the cut interphase are therefore exposed to bacterial uptake and internalization of pathogens [13,14]. When bacteria are penetrated and internalized, washing steps of fresh leaves are less effective, neither by using UV, heat or disinfectants [15]. Internalization of food-borne bacteria represent a serious risk for health [16], for example

Salmonella internalized in leafy green survives after 75 min at pH 2.7, therefore once internalized the pathogen can survive to the gastrointestinal digestion [17].

Ready-to-eat salads is a class of food having certain degree of risk of contamination [18,19]. Contamination can occur at any stage: primary production, harvest, washing, packaging, distribution, display and retail [1]. In addition, poor consumer awareness about food safety is a main issue with bacteria uptake in leaves due to poor storage and cross-contaminations [18]. Poor preservation and handling practices at consumption level support risk for bacterial proliferation and internalization in leaves.

The cutting site may also be responsible for the bacterial internalization in leaves, in addition to wounds and open stomata, for example [20,21]. In industry, salads and baby-leaves can be cut by using a mechanized harvester (Figure 1, A). The cutting site could also contribute for water retention and uptake. For example, in the industry of cut flowers, to support water absorption, cutting strategies may help in some extension to maintain the flowers a little fresher [22]. Different types of cut produce less damage at the cross section supporting higher water uptake and retention [22]. In fact, no metabolic energy is used to generate the driving force for fluid flow in the main vessel [23], therefore bacteria can be transported by water through the vascular bundles via cell wall capillary forces. Xylem conduits, for example, present pits membrane separating the conduit lumens [23]. Such pits have sizes compatible with the passage of phytopathogens [23] and therefore human pathogen as well.

In this paper we hypothesize that different types of cuts affect bacterial uptake in leafy greens at the baby-leaves stage. We aim to identify a type of cut that better contribute to suturing/closing the main vessels leading to reduced bacterial uptake.

Materials and Methods

Bacterial strain. Escherichia coli ATCC 35218 has been used to assess the uptake through the mid vein of the leaves. The strain of *E. coli* 35218, although is classified as biosafety level 1, holds specific virulence genes associated with different *E. coli* pathotypes related to human and animal infections, such as *fimH*, *papA*, *papC*, *papG*, *papE*, *sfaS*, *hlyA* (gene for α-hemolysin), *kpsM*, *fyuA*, *ompT* [24].

Plant material. Ready-to-eat baby-leaves were purchased at the same grocery store. The leaves were obtained from ready-to-eat packages and used within 48 h from the cutting date reported on the package. We used ready-to-eat leaves because we found the water uptake was very high in these leaves, representing a good control to observe uptake. Four ready-to-eat varieties were used: *Lactuca sativa* (green lettuce), *Spinacia oleracea* (baby spinach), *Lactuca sativa var. crispa* (red Lettuce), *Eruca sativa* (rucola). Leaves of all varieties were chosen with an average length of 6.5 cm.

Additional experiments were performed on lettuce and spinach grown in hydroponics in our growth chamber and in the field to test additional growth conditions. Lettuce and spinach were hydroponically grown in a floating system in a growth chamber at 14±2 °C (night) and 21±2 °C (day) with a photoperiod of 16 h under fluorescent lighting units Osram L36W/77 (36 W, 120 cm in length, 26 mm in diameter) up to the baby-leaves stage. Plants were fertilized by using half-strength Hoagland's nutrient solution (macroelements expressed in mM and microelements in μM: N 7.5, P 0.5, K 3.0, Ca 2.5, Mg 1.0, Fe 25.0, B 23.1, Mn 4.6, Zn 0.39, Cu 0.16, Mo 0.06; pH: 5.56; CE: 1.12 mS/cm). Instead, for the lettuce grown in soil, it was grown a greenhouse according with a summer fertilization regime: fertilization before and after seeing with manure (100 kg / 1000m^2) and ternary fertilizer (NKP) (70 kg / 1000^2). For the bacterial uptake test with the in-house grown leaves were cut with different cutting strategy

and stored at 4°C for 48 h into a closed plastic bag before exposure to the bacteria suspension, simulating ready-to-eat packaging.

Bacterial uptake test. Petiole were cut starting from the basal portion, and all leaves had similar length by measuring them with a ruler. Cutting was performed by using the following methodologies: a) metal scalpel, by pulling the blade forwards slightly until the blade slices the tissue (and not by cutting by putting pressure from the top, to avoid compression of the vessels). b) A metal scissor with a scissor-action, that cuts by gliding against a thicker sharp blade. Sharpness in scissors-action is not the same as sharpness in a scalpel. A scalpel is so designed that the faces of the blade are nearly parallel and meet at an acute angle. Scissor-action blades have edges as sharp, but do not meet at a very acute angle, in fact commonly the angle at the cutting edge is nearly a right angle. That is so that the pinching action at the point of cutting is strong and concentrated. The scalpel and the scissor were used at 25 °C or heated at 200 °C on a heating plate (VELP Scientifica, Italy) and the correct temperature of the metal was measured with an infrared thermometer with a laser pointer (Helect, China). To prevent rapid lose in temperature when cutting the leaves, the metals was re-heated before each cut. The cut performed with a scalpel at 25 °C was used as a control.

For each replica, a glycerol stock of the *E. coli* strain was used to start an overnight culture in Lysogeny Broth (LB, Oxoid, UK) at 37°C in fast shaking of 130 r.p.m. After incubation, 1 mL from the overnight culture was pelleted via centrifugation at 8000 g for 1 min, then washed in 1 mL of sterile physiological solution (PS, NaCl 0.85% w/v in H_2O). The washing step was repeated three times. Washed cells were finally diluted in PS containing approximately 10^8 cells/mL. The bacterial working suspension was prepared by adding 500µL of the washed cells (from the tube with 10^8 cells/mL) into 50mL of sterile PS. To assure that the working solution

started any replica with the same titre of cells, 1:10 dilution of the washed cells was measured at the spectrophotometer resulting in a OD_{600}=0.2±0.05. The initial cell concentration used for the bacterial uptake was in the range of 10^6 cell/mL.

Once the suspension was prepared, leaves were placed on a sterile tips rack in vertical position, with the petiole submerged 0.5 cm into the bacterial suspension. Incubation was completed in 1.5 h at 25 °C. After incubation, to assess bacterial uptake, leaves were cut with a sterile scissor in segments (about 6 segments) starting from the top (Figure 1 B). The mid vein of each cut section was then streaked on McConkey agar plates (Oxoid, UK) to check presence/absence of *E. coli*. The uptake index was calculated by using the following methodology: When *E. coli* was present a score of 1 was assigned, and 0 in case of absence. The uptake index (*ui*) was calculated by the mean of the 0 and 1 values starting from the top of the leave until the lowest 30 % (Figure 1, B). A *ui* value near 0 means that *E. coli* was absent in any segment (no uptake above 30 % of the leaf), intermediate values reflected how far *E. coli* moved upward, while the value 1 indicates the top of the leaf (but this never happened). The lowest 30 % of the leaves was excluded since *E. coli* cells were always present being the petiole submerged in the bacterial suspension. To confirm the goodness of the *ui* index, a significant regression effect ($p<0.0001$) showed that the uptake index *ui* decreased inversely proportionally with the top of the leave, as expected (Figure 1, C). The linear regression was performed by plotting all the values from Figure 2.

Controls leaves were used by assessing presence of *E. coli* without artificial contamination from each batch used for the experiments. Briefly, control leaves not artificially contaminated were grinded in PS and 100µL of this suspension were plated onto McConkey. In addition, the strain *E. coli* 35218 contains a genetic cassette for the resistance to ampicillin (100 µg/mL) for easier discrimination from other *E. coli* when needed. At least 6 replicas per cutting type was performed from at least 3 different packages purchased in different days.

Treatment of leaves with NaOCl. Uptake of *E. coli* in spinach was achieved as described in the bacterial uptake test. Leaves were incubated for uptake for 1.5 h with the cutting side submerged about 0.5 cm in a *E. coli* suspension of Log 6 (CFU/mL). After uptake, entire leaves were washed once for 10 min in 0.5 L of distilled water with or without 0.04 % NaOCl (20mL of a solution 1% NaClO). After the washing a portion of leaves between 2 and 4 cm from the cutting site was grinded in 0.5 mL of sterile PS, then 20 µL were plated onto MacConkey. As a control the 4.5 Log(CFU/mL) bacterial suspension was also treated with 0.04 %. NaOCl. Up to 15 replicas were done.

Water uptake assay in vascular bundles. Vascular bundles water uptake was observed by using safranine 40 % w/v (Merck, Darmstadt, Germany) in water by incubating the first 0.5 cm of the leaves with different cuts obtained by a scalpel and a scissor 200 °C for 1.5 h into the staining solution.

Wilting index. Wilting was assessed by using the wilting index as proposed in [25]. Briefly, the rating scale for the wilting of green vegetables was: 0 for no sign of wilting to 9 (Extreme, not acceptable under normal conditions). To associate uptake with wilting, leaves were left in a box on a bench up to the wilting stages 3 (Slight, not objectionable) to 5 (Moderate, becoming objectionable). At stage 5 leaves appears still edible in terms visual appreciation. We further introduced a score 4 when leaves were classified in between 3 and 5. Wilting stages beyond level 5 were not assessed because leaves appeared objectionable and not edible by a potential consumer.

Statistical analysis. Significancy was tested by using the proportions test (with confidence intervals [CI]) within the software Statistics 4 data Analysis e Statistics AMOS - SPSS. PRISM software was used to produce the graphs.

Results

Vascular bundles protect the internalized *Escherichia coli* cells against common disinfectants. A suspension of planktonic 4.5 CFU/mL *E. coli* in PS treated with NaOCl 4 % for 10 min is reduced by about 4 logarithms (Figure 2), conversely, if the cells are internalized into the vessels of baby-leaves spinach the reduction is not significant (Figure 2).

To measure the bacterial uptake in different genotypes, cuts were performed with a scissor or a scalpel at both 25 °C and 200 °C (Figure 3). When the four varieties were combined for each treatment, the scissor heated at 200°C showed the lowest *ui* value (*ui* 0.08, when compared with the scalpel at 25 °C *ui* 0.40). Interestingly, the scalpel at 200 °C and the scissor at 25 °C showed similar performance (Figure 3, A). Considering the different varieties separately: Heating of the scissor (Figure 3, B) showed a significant drop in uptake for the green lettuce (from *ui* 0.40 to 0.12 for the scissor at 25 °C and 200 °C, respectively) and baby spinach (from *ui* 0.25 to *ui* 0.08 for the scissor at 25 °C and 200 °C, respectively) (Figure 3 B). Reduction of the uptake was also obtained by heating the scalpel to 200 °C, where three varieties reduced their uptake when compared with the scalpel at 25 °C: Red lettuce (from 0.45 to 0.27), rucola (from 0.36 to 0.18) and baby spinach (from 0.31 to 0.14) (Figure 3, B). With reference to the genotype, the Baby spinach and rucola were the varieties in which the uptake was overall reduced (Supplementary material S1).

After the experiments performed on purchased ready-to-eat baby-leaves, we further tested if the preheated scalpel and scissor were effective also on our own grown plants produced both hydroponically and on soil at the baby-leaves stage. For these experiments we tested the most

effective methods for each type of cut, with 200 °C blades (Figure 3 A), and as previously shown, also in this case the scissor and scalpel at 200°C were able to significantly reduce the bacterial uptake when compared with the control (the scalpel 25 °C) (Supplementary materials S2).

We further assessed the correlation between bacterial uptake and wilting (lower water content). The four purchased varieties were brought to different wilting stages and then bacterial uptake was measured. As wilting is a process where water is depleted, we expected higher uptake in wilted leaves due to increased suction. The hypothesis was confirmed, as leaves of all genotypes were cut with scalpel and scissor at 25 °C showed that advanced wilting stage 4 and 5 corresponds to higher *ui* when compared with the stage wilting stage 3 (increasing from *ui* 0.23 to *ui* 0.42 for wilting stages 3 and 5, respectively, for the cut performed with the scalpel, and from "no uptake" to 0.30 for the scissor) (Figure 4 A, B). The experiment repeated with the scissor at 25 °C showed similar trend as for the scalpel. The experiments were repeated with both the scalpel and scissor heated at 200 °C. The cut performed with the scalpel at 200 °C did not showed better performance when compared with the cut performed on 25 °C (Figure 4 C). Interestingly, the cut performed with the scissor heated at 200 °C before wilting the leaves strongly reduced the uptake in advanced wilting stages (4 and 5) (Figure 4, D).

We finally visualized how different cuts affect uptake by using safranin as a color indicator. We tested the cut with a scalpel and the cut with scissor heated at 200 °C (Figure 5). The scissor at 200 °C shows a clear decrease in safranin uptake (red colour, Figure 5 A) by closing the main veins (Figure 5 B).

Discussion

This study aims to evaluate the effect of different pre-heated blades for harvesting baby-leaves in order to reduce the risk of *E. coli* internalization in leaves. We showed that *E. coli* cells internalized in spinach leaves were recovered after 10 min of NaClO 0.04% treatment, showing protection of *E. coli* cells by the plant internal tissues (bundles) (Figure 2). The ability of coliform cells to be internalized and protected into plant tissue has been showed also by others: for example, *Salmonella* cells internalized in lettuce and amaranth survived up to 2.0 log CFU/g after 75 min treatment at pH 2.7 [17,26]. The plant tissue can therefore protect cells escaping decontamination procedures. Beside the fact the cells can enter in leaves from wounds and open stomata [20,21], the harvest cutting site may also be responsible for the bacterial internalization in leaves.

Here we present a set of experiments aiming to determine to what extent different cutting strategies may reduce bacterial uptake in ready-to-eat baby-leaves. As showed in Figure 3A, which encompass the most general result, the scissor action with blades heated at 200 °C had the strongest effect preventing bacterial uptake, followed by a cut with scissor at 25 °C. This effect is due to the suturing of leaf petiole by the heated blades, as showed by colorant safranin uptake (Figure 5). Anyway, we noticed that bacterial uptake and the response to treatment was to some extent vegetable dependent (Figure 3 B, C). In our specific case this could depend by bundles sizes, softness of the plant tissue and transpiration. This was particularly observed with the cut performed with the scissor at 25 °C, where red lettuce and rucola genotypes were the less contaminated. Other studies about coliform internalization (and contamination) in plant have found that plant genotype (including cultivars) and consequent plant tissue anatomy (internalization in tomato fruits wounds, trichomes, phyllosphere) were responsible for different *Salmonella* contamination [26–28]. *E. coli* or *Salmonella*, for example, can differently persist in different lettuce genotype due to different callose deposition [29].

We also compared the cutting methods on different leaves: ready-to-eat baby-leaves purchased at the grocery store (we used ready-to-eat because we found the water uptake was very high in these leaves, being a good control), and in-house produced in hydroponically and in soil. Hydroponic and soil grown lettuce were also compared because other studies showed that growth conditions of the same genotype may affect morphological differences in roots, antioxidant capacity, including lignin accumulation and moisture [30]. Nevertheless, we showed that in any different growth condition the heated blades were more efficient in reducing bacterial uptake when compared with the control (scalpel at 25 °C).

In this study we also proposed also that heated blades protect from bacterial uptake in wilted plants. Wilted plants gradually recover water if exposed again to it [31]. A recent work conducted to study chlorate (a disinfectant) uptake in bundle demonstrated that baby-leaves with lower leaf water content increased chlorate uptake significantly as effect of the water recovering [32]. As bacteria are transported in the water flow, this posed the foundation of the hypothesis that the bacterial uptake was higher in wilted leaves, as confirmed in Figure 4 panel A. When the wilted score 5 (the highest wilting stage at which consumers would eat the leave, Figure 5) are compared with less wilted stages, we see that only the cut performed with the scissor at 200 °C strongly decreased the bacterial uptake. This indicate that this type of cut could potentially protect those leaves with a lower water content.

Concluding, the results showed that the gliding action simulated by a scissor-action with preheating at 200 °C had the better chance to suture the cutting site. Can this action be applied to modern mechanized harvesters? This aspect needs to be further explored. Modern mechanized harvesters use rotating or oscillating stainless steel blades, with a cutting unit with a band blade running at environmental temperature. By proper modifications of cutting system

it would be possible to implement a heated pulley. In this case, the steel cutting unit could constantly reach the required temperature. Heating blades may result in higher cost especially in the winter season. Nevertheless, this information shows an additional trajectory to counteract produce contamination. In addition, blade heating can also have a positive effect in killing potential pathogens already during the cut or dispersed on the blade itself.

Heated blades may also contribute to the optimization of the harvesting system and shelf life: further studies should measure to what extent a harvest performed with a heated blade reduces leaf wilting during transport from the field to the retailer, maintains high quality of the vegetables, including organoleptic properties [33–35].

Acknowledgments

The authors thank Dr. Tania Henriquez for helping with the presentation of data.

CAPTIONS

Figure 1. Experimental setup. A) The panel shows a head lettuce harvester. The machine has a rotating blade that moves at moderate speed, cutting the salad heads with care and precision. The current state-of-the-art does not have a heated blade (red rectangle). B) Picture describing how the cut were performed starting from the top of the leaves. C) Linear regression ($p<0.001$) of the *ui* versus the length of the leaf. 100 % represents the top section of the leaf.

Figure 2. Total and internalized *E. coli* populations (log (CFU/mL)) in spinach. First two columns represent the controls, *E. coli* is resuspended in PS. Third and fourth columns represent the *E. coli* internalized. Error bars represent the standard deviation. Horizontal bars indicate significant pairwise and the significance is reported above the bar. $p<0.05$.

Figure 3. *E. coli* uptake in different genotypes and according with different types of cuts. A) Different types of cut averaged on all genotypes. B) Cuts performed with a scissor (25 °C and 200 °C). C) Cuts performed with a scalpel (25 °C and 200 °C). Error bars represent the standard deviation. Horizontal bars indicate significant pairwise and the significance is reported above the bar. $p<0.05$.

Figure 4. *E. coli* uptake on different wilting stages of baby-leaves and different cut types. *E. coli* uptake at different wilting stages tested on all varieties: A) cut with a scalpel 25 °C, B) cut by a scissor at 25°C, C) cut by a scalpel 200 °C, D) cut by a scissor at 200 °C. Wilting stages 3: Slight, not objectionable, 5: Moderate, becoming objectionable. We further introduced a score 4 when leaves were classified in between 3 and 5. Error bars represent the standard deviation. Horizontal bars indicate significant pairwise and the significance is reported above the bar. $p<0.05$.

Figure 5. Visualization of water uptake by safranin staining in lattuce baby-leaves. Panel A) uptake of safranin 40 %. Panel B) example of best cuts, showing suturing of lettuce petiole.

Supplementary Material S1. *E. coli* uptake in different genotypes and according with different types of cuts.

Supplementary Material S2. *E. coli* uptake according with different type of cuts on lettuce growth hydroponically and on soil. A) Cuts performed on baby-leaves produced hydroponically; B) Cuts performed on baby-leaves produced on soil. Error bars represent the standard deviation. Horizontal bars indicate significant pairwise and the significance is reported above the bar. $p<0.05$.

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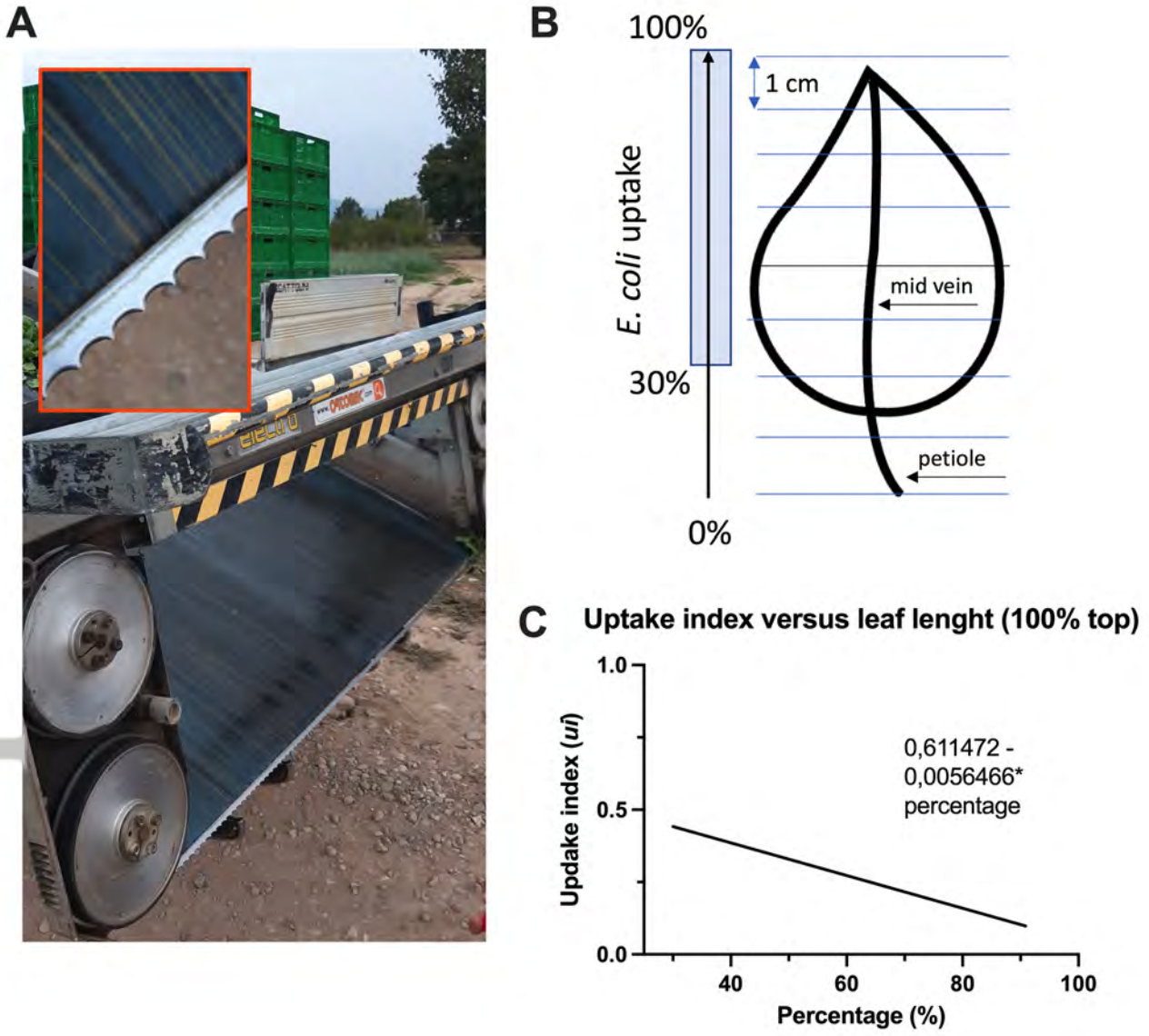

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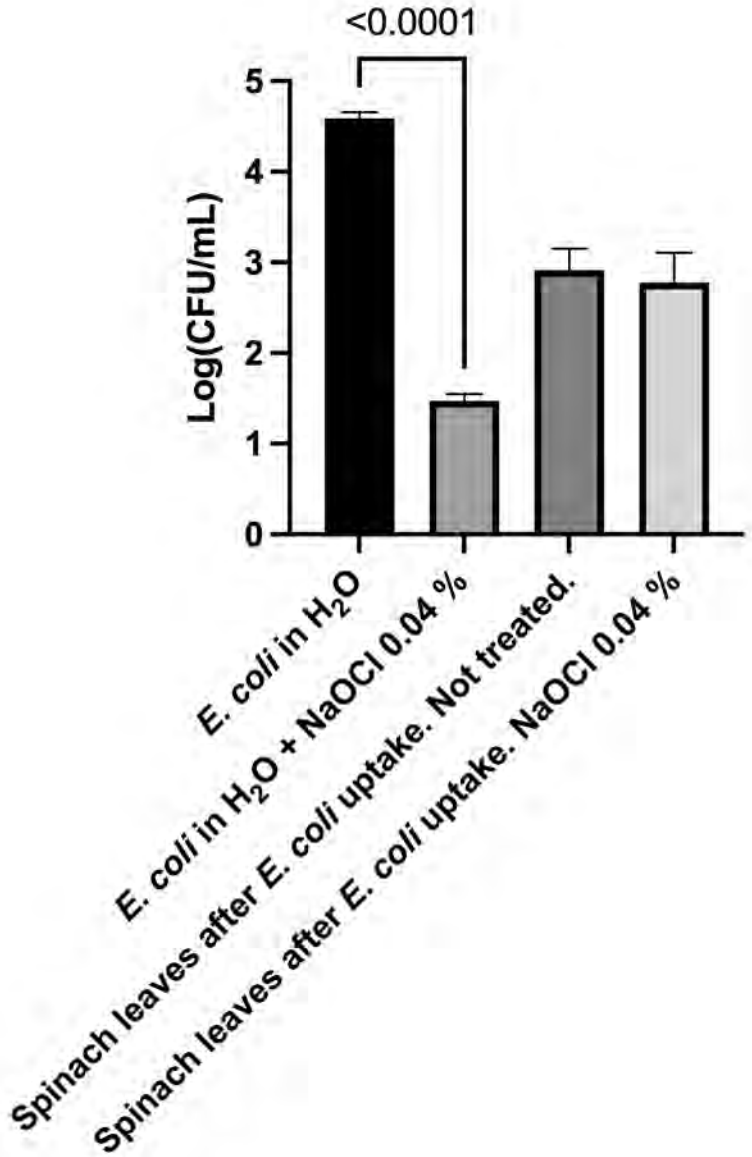

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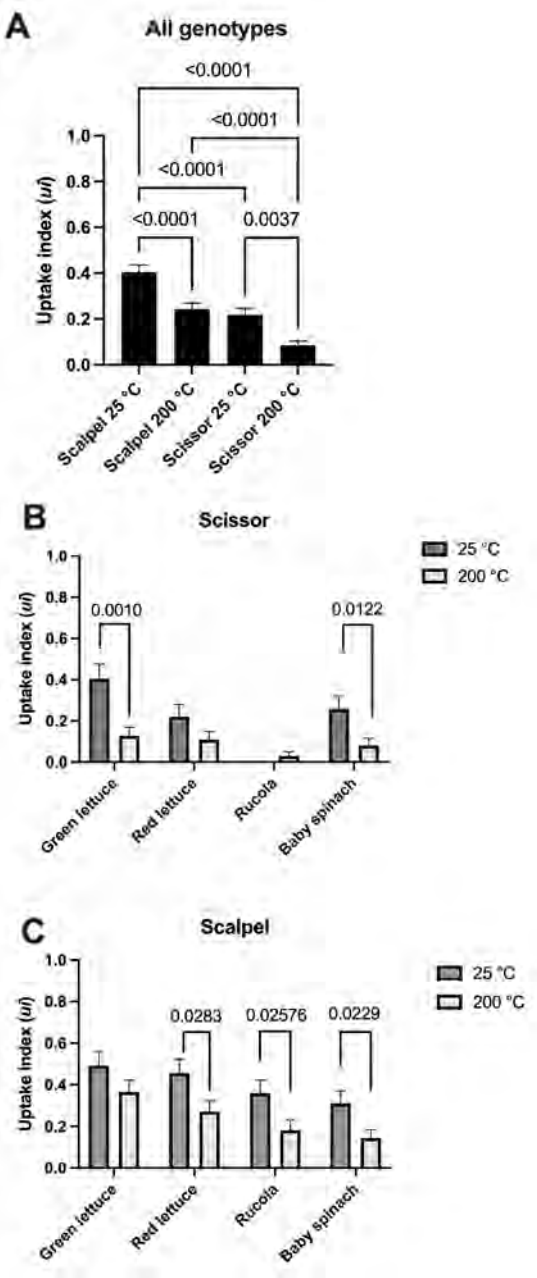

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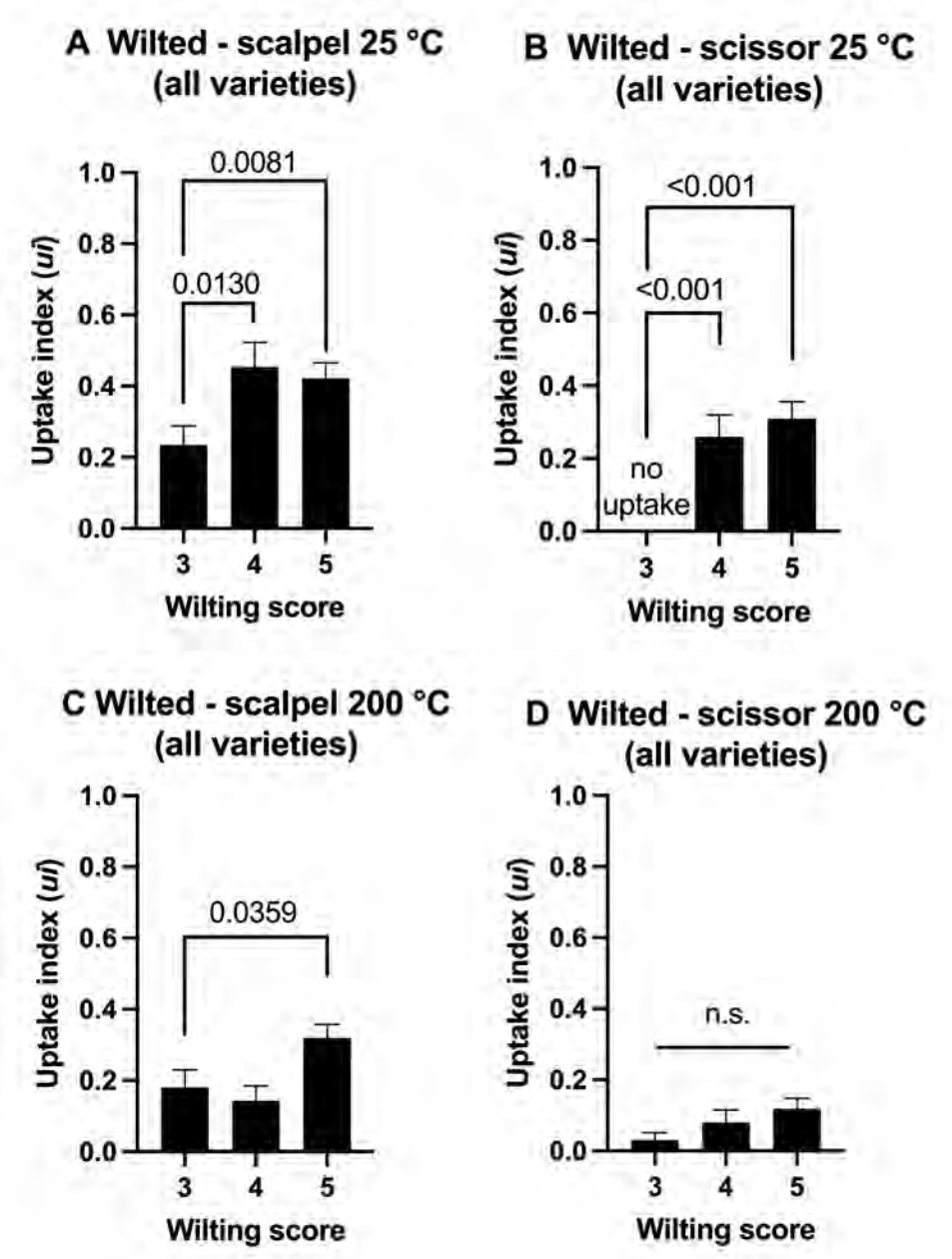

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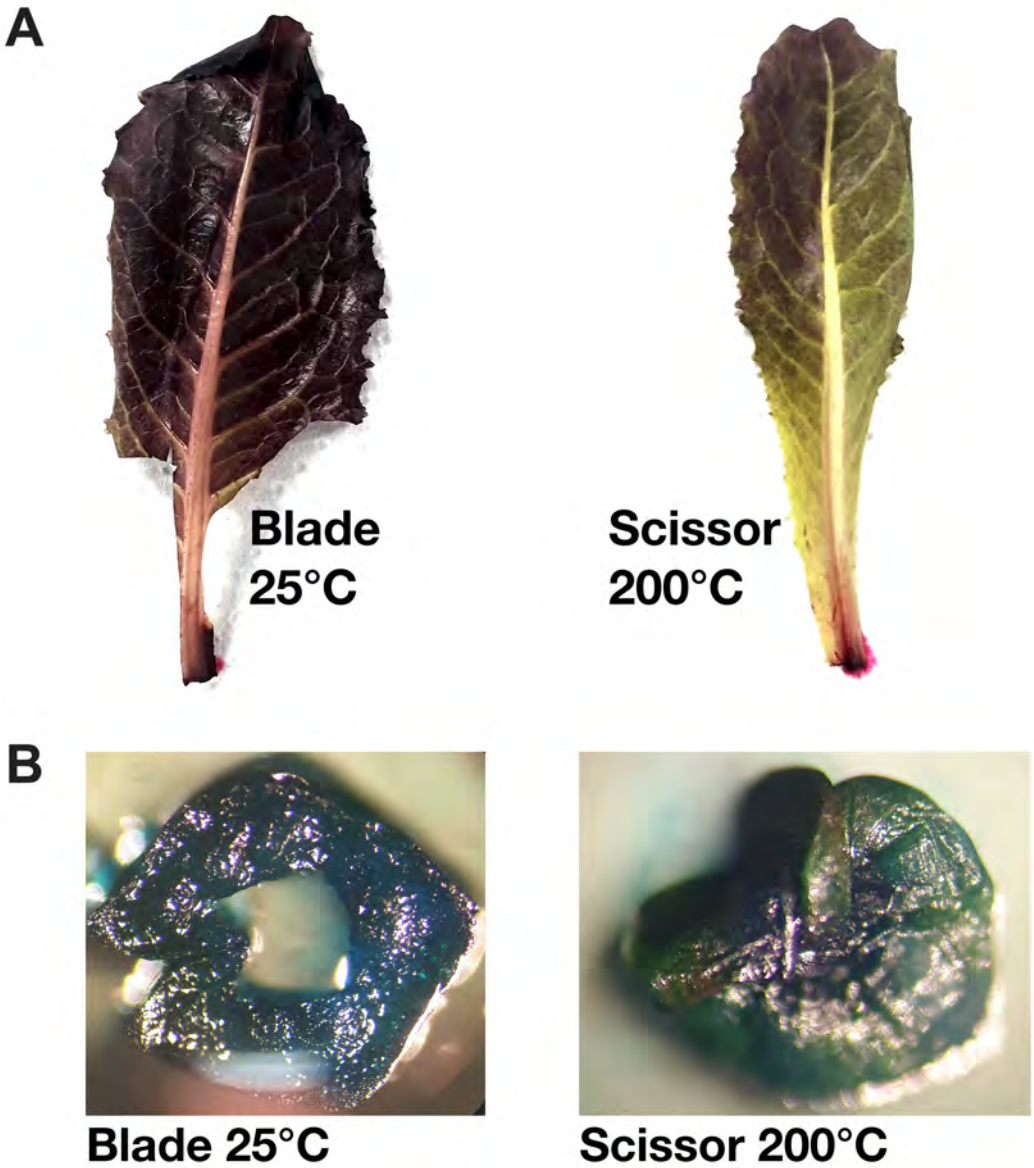

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