Volcani Voice

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Agricultural Research Organization –Volcani Center Annual E-Newsletter

Message from the Editors

By Ada Rafaeli & Nirit Bernstein

In ARO – Volcani Center a staff of over 700 scientists and more than 300 graduate students is responsible for 70% of the research in the agricultural sciences in Israel and represents the research arm of the Ministry of Agriculture and Rural Development. With the growing awareness of global challenges in the production of sufficient food to feed our growing population and stabilizing our climate system, the Volcani Center has dedicated this past year to strategic planning for the next five years to enable the enhancement of innovative agricultural research activities for the benefit of Israel and the world. Translating this vision into clearly defined goals, the Volcani Center has set its objectives in the pursuit of its mission to:

1. **Developing knowledge, technologies and products** for food security, environmental protection and associated industries;

2. **Assimilating agricultural applications** and sustaining the environment in Israel and the world;

3. **Providing solutions for current and future crises**: Food, water, energy and climate;

4. **Educating future generations of scientists** in agricultural research and development and extension services to farmers.

These objectives will be achieved through basic and applied research and development of knowledge and technologies using state-of-the-art research tools for agriculture; the application of new and advanced technologies; the launching of centers of excellence; the recruitment of top grade researchers and associates; the establishment of focal themes; through international scientific activity and mobilizing financial resources. Moreover, these objectives will lead to significant collaboration with industry and farmers and the dissemination of information through scientific and public channels thereby enhancing awareness of our activities and achievements.

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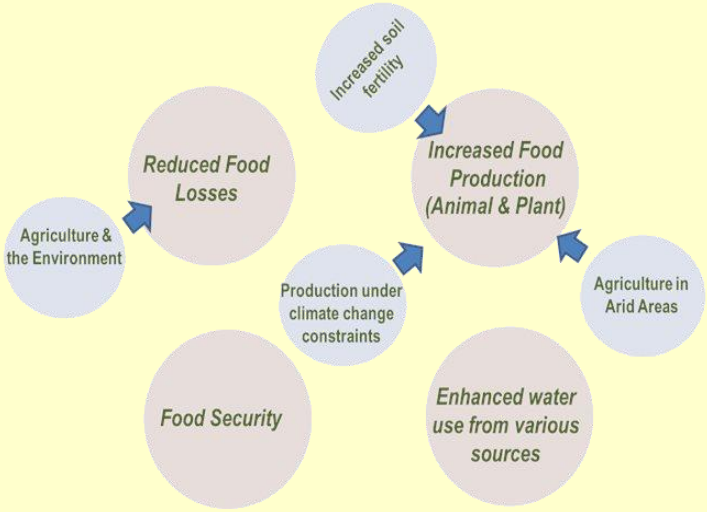
Focal Themes & Centers of Excellence



These objectives will be achieved through basic and applied research and development of knowledge and technologies using state-of-the-art research tools for agriculture; the application of new and advanced technologies; the launching of centers of excellence; the recruitment of top grade researchers and associates; the establishment of focal themes; through international scientific activity and mobilizing financial resources. Moreover, these objectives will lead to significant collaboration with industry and farmers and the dissemination of information through scientific and public channels thereby enhancing awareness of our activities and achievements.

Research for the next five years will be directed to the following broad focal themes: ***Increased Food Production – Enhanced Water Use – Reduced Food Losses – Food Security***. Recruitment of additional tenure-track scientists for advancing research on these themes will increase, thereby implementing the objectives as set forth by our new research strategy. Moreover, as part of the strategical planning, the Director of the Volcani Center, Prof. Yoram Kapulnik has allocated substantial funding this fiscal year (2013) to purchase advanced infrastructure for these selected Focal Themes.

Using Metabolomics in Basic & Applied Agricultural Research

By Mira Carmeli-Weissberg, Metabolomic Unit, Plant Sciences Institute

About first author

Dr. Mira Weissberg did her Ph.D in organic chemistry at Tel-Aviv University under the supervision of Prof. Shlomo Rozen, specializing in "Oxygen Transfer Reactions Using the HOF·CH3CN Complex". During this period she acquired extensive experience in Multistep Organic Synthesis, Multinuclear NMR Spectroscopy, HPLC, GC, UV-Vis, Fluorescence and IR spectroscopy. Mira joined the ARO in 2008 and established the Metabolomic Unit, specializing in identification and quantification of small molecules (metabolites) using a variety of state-of-the-art MS-based instruments and analysis software.

"Metabolomics is a discipline with a major impact on both basic and applied agricultural sciences."

Mira Weissberg heads the unit for separation and identification of Metabolites at the Agricultural Research Organization (ARO) Volcani Center. The repertoire of capabilities includes identification of known and unknown metabolites based on accurate mass (UPLC-QTOF-MS), quantification of metabolites, including plant hormones, using triple-quad based MS (UPLC-Triple Quadrupole-MS), as well as identification and quantification of volatile metabolites using HS-SPME/GC-MS. The unit functions in collaboration with many research groups in the ARO in diverse projects and is equipped to address most questions related to identification and quantification of small molecules.

Metabolite analysis, or metabolomics, is a discipline with a major impact on both basic and applied agricultural sciences. The technologies being developed for analysis of small molecules, metabolites present in organic extracts made from plant materials, are greatly changing our way of thinking about what is possible in plant biology.

A range of different separation and detection techniques are being refined and expanded, and their combination with advanced data management and data analysis approaches is already giving plant scientist's far deeper insights into the complexity of plant metabolism and plant metabolic composition than was imaginable just a few years ago. We recently applied metabolomic analyses to several different research projects. Here we present two examples; one for the use of metabolomics in applied research and the other to demonstrate the role of metabolomics in basic research.

Breeding for citrus fruit which can be consumed by the rising population dependent on Statin-prescriptions to counter high cholesterol blood levels

(Collaboration with Nir Carmi's lab)

Statins lower cholesterol levels in the body by inhibiting the rate-limiting enzyme of the pathway, HMG Co-A reductase. Statins are the most widely prescribed cholesterol-lowering medications, since they affect the cholesterol profile (LDL, HDL, and triglycerides) in a favorable way.

Administration of statin medications requires avoidance of negatively interacting drugs or compounds absorbed from food. Grapefruits, for instance, were found to contain the compound bergamottin, as well as other furanocoumarins, which are absorbed in the gut and inhibit the breakdown of statins in the human body by interacting with cytochrome P-450 and P-glycoprotein enzymes.

Thus, when grapefruit juice is consumed at or around the time statin drugs are ingested, the furanocoumarins absorbed in the gut prevent enzymatic breakdown of the statins, causing the drugs to accumulate in excess amounts in the body. This can be very dangerous and can cause a variety of health problems, such as liver damage or a rare condition called rhabdomyolysis (severe muscle and kidney damage).

Breeding for grapefruit varieties which are safe for consumption by the rising population, treated with statins, requires development of a screen based on sensitive analytical tools able to detect and quantify a variety of toxic furanocoumarins.

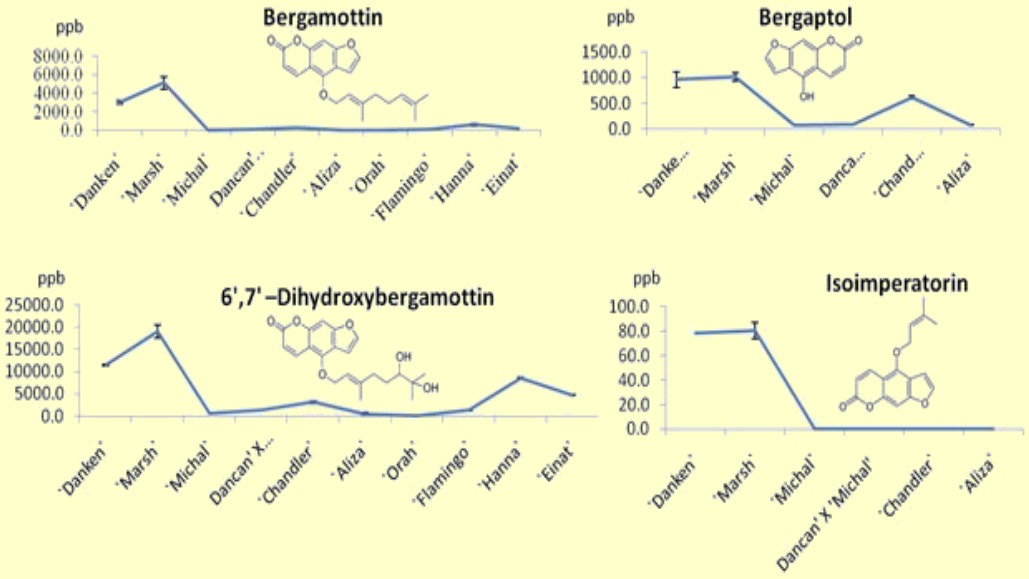


Figure 1 *Concentrations of furanocoumarins in some species of grapefruit, mandarins, oranges and pummelo detected by UPLC triple Quadropole MS.*

An extensive screen of varieties developed in the Israeli citrus breeding project (some results shown in Figure 1) shows that the grapefruit-like variety "Aliza" contains very low levels of furanocoumarins and therefore is of particular interest for commercialization as a grapefruit species, safe for consumption by statin users.

Understanding the molecular basis regulating bitterness of citrus fruit

(Collaboration with Yoram Eyal's Lab)

The suggested origin of citrus fruit trees is Southeast Asia and it is widely accepted that the ancestral citrus species, which gave rise through hybridization to all the edible citrus species/varieties known today, are pummelo, mandarin and citron. Domestication and breeding of citrus species/varieties for flavor aroma and other qualities, based on the ancestral species, has been an ongoing process during the last several thousand years, initially in Southeast Asia and China and much more recently in other countries around the globe. New varieties, boasting novel flavors and aromas are continuously generated that are mostly based on variability in the combinations and concentrations of secondary metabolites.

Thus, the composition of secondary metabolites in the citrus varieties known today is the result of selection under domestication, and often reflects human preferences for fruit flavors and aromas, and not necessarily physiological advantages for the tree. Bitterness is a common flavor characteristic in fruit of some species of the genus citrus and is determined by the quantity (concentration) and composition of branched-chain flavanone glycosides, the prevailing flavonoids in citrus

The bitter flavanone 7-O-neohesperidosides (e.g. neohesperidin, naringin) are the dominant flavanone glycosides in bitter citrus species (ie. pummelo, grapefruit, bitter orange) and are composed of the branched-chain disaccharide neohesperidose (rhamnose-2-*O*-glucose) *O*-linked to position-7 of the flavanone (see structure of flavanone glycosides in figure 2).

The tasteless 7-*O*-rutinosides (e.g. Hesperidin, Narirutin) are the exclusive flavanone glycosides in the non-bitter citrus species (ie. sweet oranges, mandarins, clementine, citron, lemon) and are composed of the branched-chain disaccharide rutinose (rhamnose-6-*O*-glucose) *O*-linked to position-7 of the flavanone[1]. Beyond the effect on fruit flavor, it is assumed that flavanone-glycosides have a role in protecting young citrus tissue against herbivore or disease, because they accumulate to very high concentrations in young tissue (mainly leaves and fruit) and are gradually diluted during continued development.

"….the composition of secondary metabolites in the citrus varieties known today is the result of selection under domestication, and often reflects human preferences for fruit flavors and aromas, and not necessarily physiological advantages for the tree."

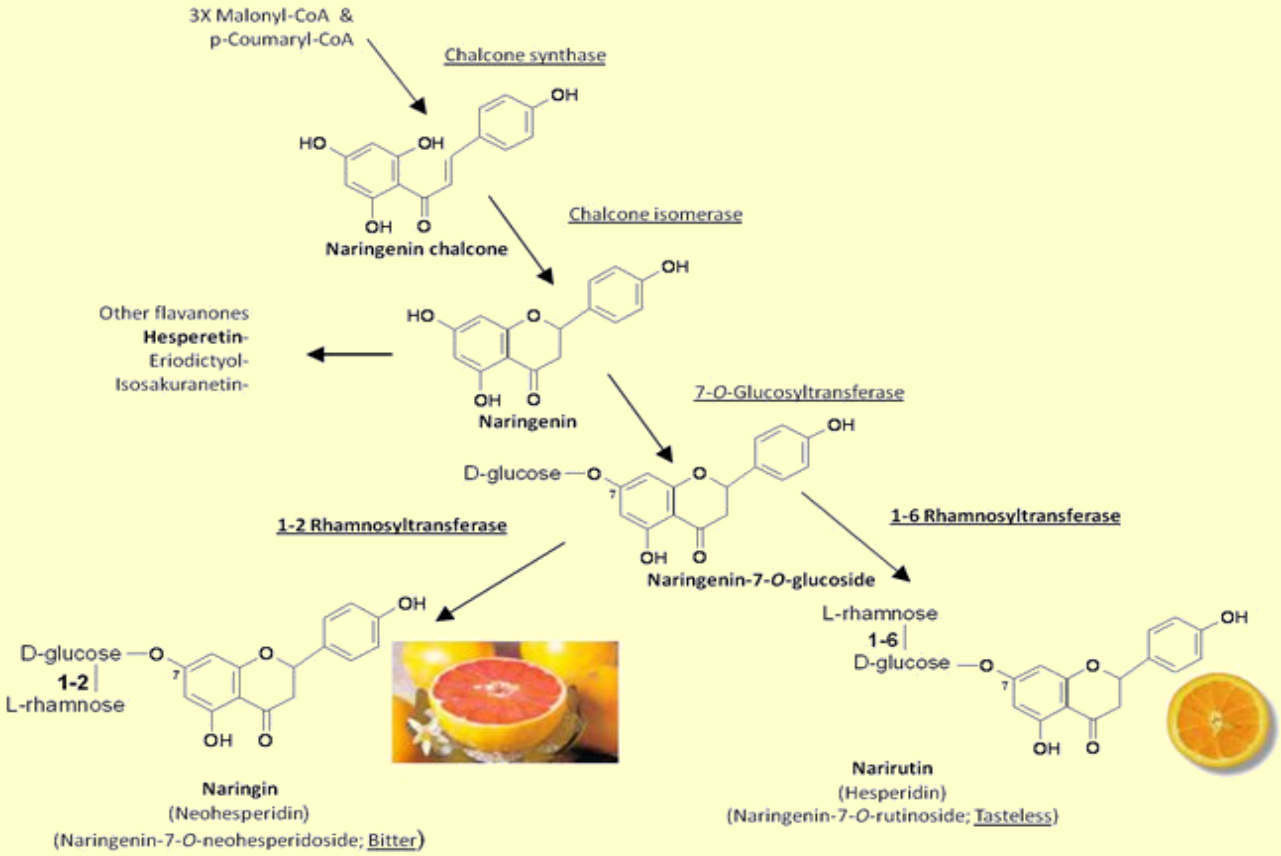


Figure 2 *Biosynthesis of flavonoid branched-chain glycosides in citrus. Three molecules of malonyl-CoA and one of p-coumaryl-CoA are condensed in a reaction catalyzed by chalcone synthase to create naringenin chalcone. A stereospecific ring closure isomeration step catalyzed by chalcone isomerase converts the chalcone to the flavanone naringenin. The latter is converted to other flavanones. Flavanones are glucosylated at position 7 to create flavanone-7-O-glucosides (e.g. naringenin-7-O-glucoside) by a 7GlcT (7-O-glucosyltransferase). The latter serves as a substrate for the bitterness determining step catalyzed by either a 1,6 rhamnosyltransferase (1,6RhaT) or a 1,2RhaT rhamnosyltransferase (1,2RhaT).*

The biosynthetic pathway leading to bitter neohesperidosides vs. tasteless rutinosides demonstrate that the bitter flavor determining enzymes in citrus are 1,2RhaT and 1,6RhaT. LC-MS (accurate mass) instrument was used for characterization of their activity. A functional analysis of corresponding candidate gene products based on accurate-mass metabolite analysis showed that the two branch-forming rhamnosyltransferases that utilize flavanone-7-*O*-glucose substrate are responsible for bitter/non-bitter flavor.

A 1, 2-rhamnosytransferase (1,2RhaT) leads to the bitter flavanone-7-*O*-neohesperidosides (neohesperidin and naringin), whereas a 1,6-rhamnosytransferase (1,6RhaT) leads to the tasteless flavanone-7-*O*-rutinosides (hesperidin and narirutin) that are common to non-bitter citrus species (Figure 3). Inheritance and expression of these two genes is the basis for bitterness in citrus.

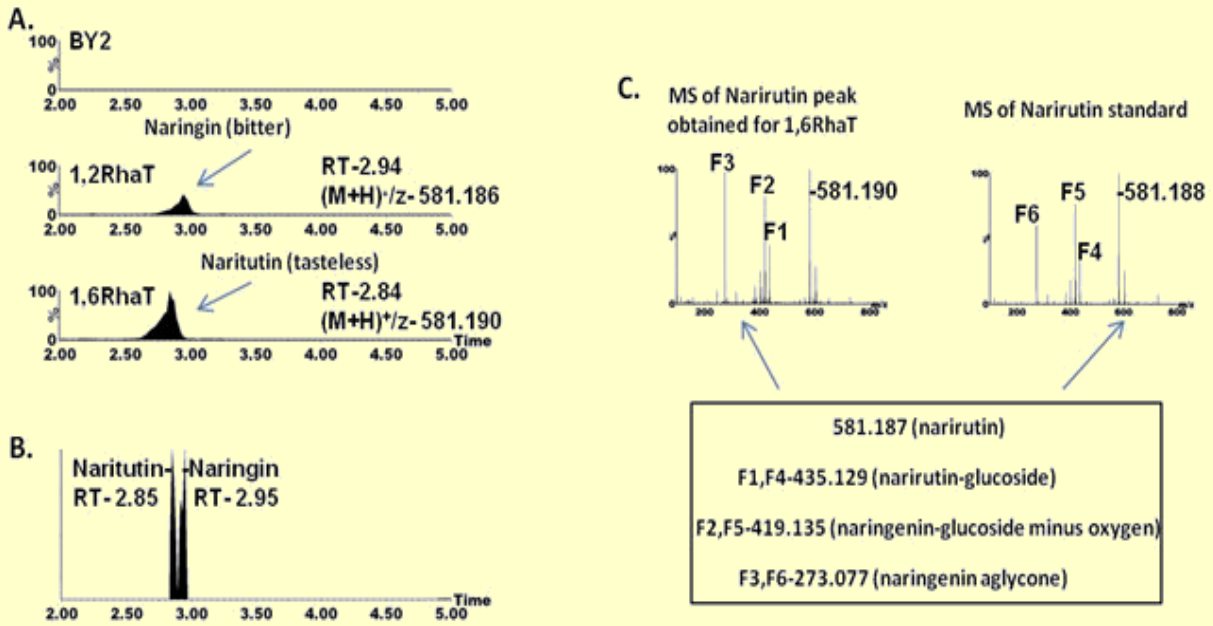


Figure 3 *(A) Chromatographic and MS data for the products of the citrus enzymes 1,2 rhamnosyltransferase (1,2 RhaT) and 1,6 rhamnosyltransferase (1,6RhaT) using naringenin substrate. Chromatographic peaks displayed are only those that conform to the calculated mass of narirutin/naringin ((M+H)+/z 581.187). Although the accurate masses of the branched-chain products obtained equally fit both narirutin/naringin ((M+H)+/z-581.187), the retention times clearly distinguish between the products of 1,2RhaT and the 1,6RhaT-candidate, as shown by chromatography of the standards (B). MS data of the product narirutin peak obtained for 1,6RhaT shows the expected breakdown products compared to the narirutin standard (C).*

The activity of 1,6RhaT on flavonoid substrates provoked the question of its ability to catalyze rhamnosylation on position 3 of flavonoids and specifically on anthocyanidin-3-glucosides. Anthocyanins are found in the cell vacuole, mostly in flowers and fruits but also in leaves, stems, and roots. They are odorless and nearly flavorless. Anthocyanin biosynthesis is of special interest to agriculture (ie. color of many fruit and flowers) as well as food science (potential natural food colorants). Anthocyanins are thought to be subject to physiochemical degradation. Temperature, pH and light are generally known to affect the color and stability of anthocyanins. Modifications, including glycosylations, affect the biological activity of the molecules, including possible effects on stability[1][2].

We studied activity of a transgenically expressed 1,6RhaT on anthocyanidin-3-glucose naturally produced in a red grape (*Vitis vinifera*) cell culture system. The red grape cells (RG) produce a variety of anthocyanins, including abundant peonidin-3-glcoside and cyanidin-3-glucoside, but no anthocyanidin-rutinosides. Anthocyanins were extracted from the wild type RG and from the transgenic RG-1,6RhaT cell line and analyzed by accurate-mass LC/MS (Figure 4A). The data demonstrates that peonidin-rutinoside (m/z 609.182; retention time 1.99) and cyanidin rutinoside (m/z 595.166; retention time 1.80) were produced in the transgenic cell line, but were absent from wild-type RG cells. It was concluded that 1,6RhaT can catalyze branched-chain rhamnosylation of anthocyanidin-glucosides.

The ability of 1,6RhaT to catalyze branched-chain rhamnosylation of anthocyanidin-glucosides in transgenic red grape cells raises the question whether anthocyanidin-rutinosides occur *in-vivo* in citrus (none have been described as yet to the best of our knowledge). Lemon (*Citrus limon*) accumulates dramatically visible levels of anthocyanins in newly emerging leaves, which were used for analysis of anthocyanin content, and specifically for the occurrence of the rutinoside of cyanidin, the dominant anthocyanidin in citrus. A peak of m/z 595.166 at retention time 1.80 was detected in Lemon anthocyanin extracts (Figure 4B) corresponding to cyanidin-rutinoside. The associated mass spectrum showing the molecular ion (595.166) and the expected fragments (cyanidin-glucoside, m/z 449.110; cyanidin, m/z 287.057) further support this identity. We concluded that 1,6RhaT is apparently involved in the biosynthesis of anthocyanidin-rutinosides in citrus leaves.

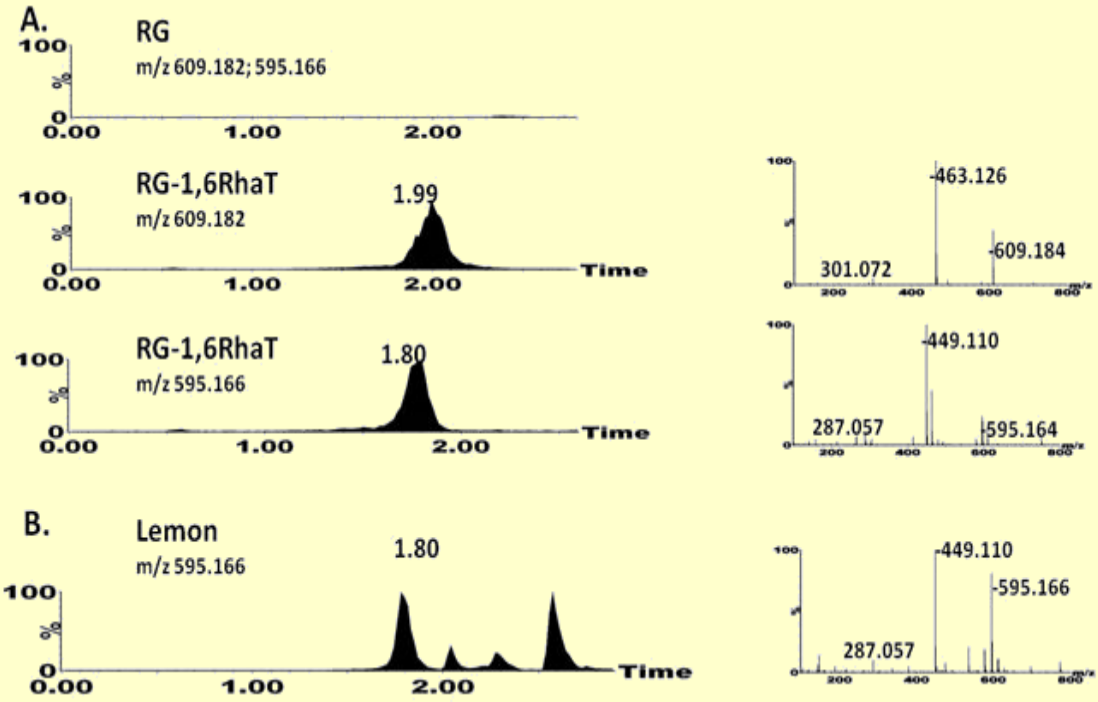


Figure 4 *(A) Anthocyanins were extracted from the transgenic cell line (RG-1,6RhaT) and the wild type cell line (RG) and were analyzed by accurate-mass LC/MS. Peaks displayed are only those that conform to the calculated mass of peonidin-rutinoside (m/z 609.182) or cyanidin-rutinoside (m/z 595.166). Mass (m/z) of the molecular ion and the resulting fragments are denoted. (B) Anthocyanins were extracted from young lemon leaves (ie. up to 1 cm length) and were analyzed by accurate-mass LC/MS. Peaks displayed are only those that conform to the calculated mass of cyanidin-rutinoside (m/z 595.166). Mass (m/z) of the molecular ion and the resulting fragments are denoted.*

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Yellow Shading Nets Impede Whiteflies Invasion and Decrease the Incidences of Whitefly-Transmitted Viral Diseases in Tomatoes

By David Ben-Yakir[1]*, Yehezkel Antignus[1], Yossi Offir[2] & Yosepha Shahak[3]
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About first author

Dr. David Ben-Yakir is Senior Research Scientist in the Department of Entomology, Nematology and Chemistry of the Institute of Plant Protection. His main research focuses on development of Integrated Pest Management (IPM) programs for protected and field crops.

Black shading nets are commonly used to protect agricultural crops from excessive solar radiation, wind, and for water saving. Recent studies have demonstrated that when black nets were replaced by either red, yellow, or pearl nets (ChromatiNets®), of equivalent shading capacity, it increased the tomatoes fruit yield and improved their quality. Ben-Yakir and co-workers studied the effects of colored shading nets on the infestation by the sweet potato whiteflies (*Bemisia tabaci*) and the incidence of the viral diseases transmitted by these insects. These studies were conducted in the semi-arid Besor region in southern Israel. Colored nets with 30-35% shading capacity were used. Whiteflies landed 40 times more often on the yellow shading net compared to the black or red nets. Although these shading nets permit free passage of whiteflies, the infestation levels of plants in boxes and tunnels covered by the yellow net were consistently 2-3 folds lower than in the same structures covered by the black or red nets. The incidences of the whitefly-borne tomato yellow leaf curl virus (TYLCV) in tomato plants grown under the black nets ranged between 15-50%. However, the disease rates in tomato plants grown under the yellow net were 2-4 folds lower than under the black or red nets. We propose that an arresting response of the whiteflies to the yellow net is responsible for the protection achieved.

Agricultural crops are often grown under protective nets for reducing damages caused by excessive solar radiation (e.g., sunburns), wind, hail, birds, and for saving irrigation water. In Israel, black nets that provide 30% shading are traditionally used as protective nets. Tomatoes are grown in Israel mostly under clear 50-mesh nets to protect them from the sweet potato whitefly, *Bemisia tabaci,* and the disease tomato yellow leaf curl virus (TYLCV) this pest transmits. The 50-mesh net covering has a negative effect on tomato productivity because it reduces ventilation and increases heat stress. Developing tomato varieties that are tolerant to TYLCV may permit to grow tomatoes under shading nets that do not cause the negative effects of the 50-mesh nets.

Colored shading nets have been developed for improving crop production by their optical properties, in addition to their physical protective properties. The colored nets modify the spectral composition of the transmitted and reflected sun light. These nets also transform direct light into scattered light. Studies have demonstrated that growing vegetables, fruit, and ornamental crops under certain colored shading nets can increase their yields and improve their quality [2]. One of these beneficial nets is yellow, a color that is known to attract whiteflies and other insect pests. Thus, crops grown under the yellow nets may be at a higher risk of pest infestation. We studied the risk of covering tomatoes with yellow shading nets on the infestation by whiteflies.

All studies were conducted in the semi-arid Besor region in southern Israel. We used colored shading nets (Chromatinet™, Polysac Plastics Industries, Israel) that have similar knitting and shading level (30-35%) (Figure 1). The landing preference of whiteflies was studied using nets attached to wooden frames (1x1 m) that were placed on the bare soil (Figure 2). Whiteflies landing were monitored using 4 clear sticky traps that were attached to each frame. As expected, whiteflies landed 40 times more often on the yellow shading net compared to the black or red nets (Figure 3). The penetration of whiteflies through the nets was studied in small cubed chambers (1x1x1 m) covered with the colored nets. The establishment of whiteflies on cotton plants was studied in similar chambers that were covered with either black or yellow nets (Figure 4).

"The protection from whiteflies is probably due to the induction of "an arrestment" response by the color and texture of the yellow net that mimics a plant leaf"

Whiteflies

Figure 1. ChromatiNets™ colored shading nets with knitting density that provides 30-35% shading.

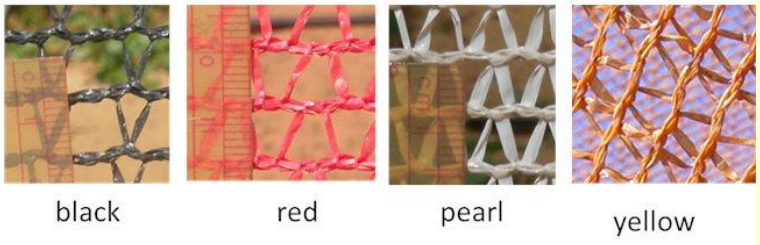

Figure 2. Colored nets stretched over wooden frames (1x1 m) used for studying the landing references of whiteflies.

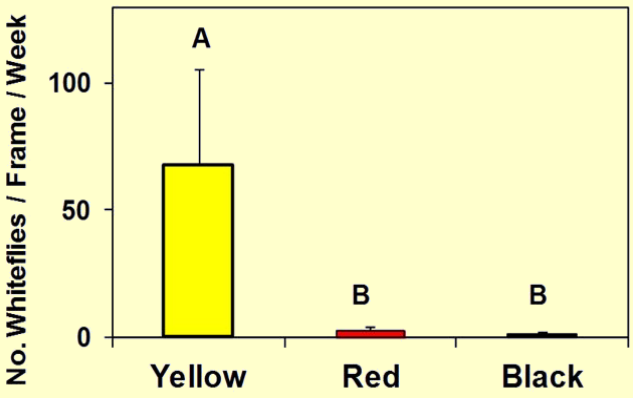

Figure 3 *The effect of net color on the landing preference of whiteflies.*

Figure 4. Small structures (about 1x1x1 m) covered with colored nets, with or without host plants inside, used for studying the risk of whitefly invasion.

Surprisingly, the number of whiteflies trapped inside the chambers covered by the yellow net was the lowest. Moreover, the number of whiteflies established on cotton plants grown inside the yellow net covered chambers was consistently lower than the number in the black net covered chambers.

To evaluate the effect of the colored nets on whiteflies and TSWV in the tomato crop we used 'walk-in' tunnels (6 × 6 × 2.5 m) that were covered by the colored shading nets.

Tomato plants were grown in these tunnels in 6 rows, each containing 10-12 plants. Every growing season the tomato plants were grown for 3-5 months. Plants were not treated with insecticides during the first month after planting to allow natural whitefly infestations. Plants were treated prophylactically against fungal diseases and mites with specific pesticides that do not affect whiteflies. Whiteflies were monitored using 2 yellow sticky traps that were placed at the two inner ends of each tunnel. Traps were changed and counted every week. Over the growing period we ensured 2-3 visual examinations of all plants to determine the rate of plants with TSWV disease symptoms.

Tomato plants had 15-40% higher fruit production under the red, yellow, and pearl nets compared to the black net [2]. During the first 3 weeks after planting the numbers of whiteflies that invaded and were trapped inside the tomato tunnels were 2-3 folds lower under the yellow and pearl nets compared to those numbers under the black or red nets.

A similar trend of trapping levels prevailed throughout the tomato growing period. About 10 weeks after planting the rate of viral infection with TSWV was also 2-4 folds lower under the yellow and pearl nets compared to those rates under the black or red nets (Figure 5).

The lack of correlation between the number of whiteflies landing on the yellow net and their penetrating through this net suggests that these pests are somehow stopped on this net. This is probably due to the induction of "arrestment" response by the color and texture of this net that mimics a plant leaf. Therefore, contrary to our expectation, whiteflies are less likely to infest plants that are grown under yellow netting.

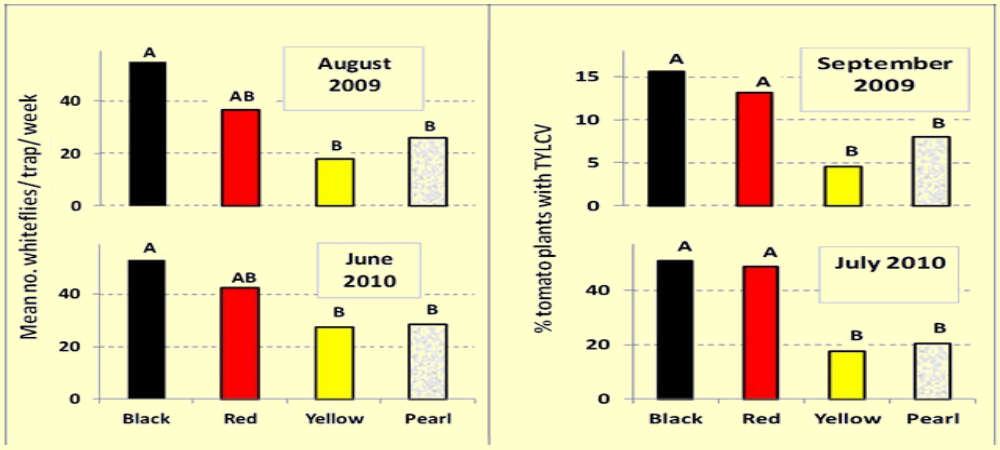

Figure 5 *The effect of net color on the infestation of tomato plants by whiteflies and the rate of plants with symptoms of the whitefly-borne TSWV*

Thanks to the workers of the Besor Experimental Station. This study was funded by grant no. 643-0039 from the Chief Scientist of the Israeli Ministry Agriculture and the Israeli Plants Production and Marketing Board.

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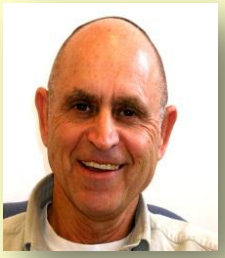
A New Role for Sugars in Stomatal Closure

By David Granot David Granot & Gilor Kelly, Plant Sciences Institute

About first author



David Granot is Senior Research Scientist in the Department of Vegetable Research, Institute of Plant Sciences. His main research focuses on sugar metabolism in plants and how we can use this knowledge to develop plants that save water. For a century, scientists have believed that sugars function as osmolytes that open stomata. This recent study undermines this paradigm, showing that sugars close stomata through hexokinase, a sugar sensor, thus allowing coordination of photosynthesis with water loss.

Water is the main factor limiting growth and development of many plants. Special pores on the surfaces of leaves, which are called stomata and are composed of two guard cells, open and close to allow the plant to balance the demand for carbon dioxide for photosynthesis with the loss of water through transpiration. At the mechanistic level, stomata open in response to increases in the osmolarity of the guard cells. These increases in osmolarity are followed by the movement of water into the guard cells. As the guard cells swell with water, the stomata open. Stomatal closure occurs in the opposite manner. About a century ago, it was proposed that sugars are the primary guard-cell osmolytes stimulating stomatal opening. This hypothesis was later modified by the discovery that potassium (K^+), chloride (Cl^-) and malate ions are the primary osmolytes that accumulate in guard cells and induce stomatal opening. However, it has been suggested that, over the course of the day, sugars replace the K^+ ions, keeping the stomata open. In contrast to this prevailing hypothesis, we discovered that sugars close stomata through hexokinases, which are enzymes that act as sugar sensors within guard cells. These findings indicate the existence of a feedback-inhibition mechanism that is mediated by a product of photosynthesis – sucrose. When the rate of sucrose production exceeds the rate at which sucrose is loaded into the phloem, the surplus sucrose is carried toward the stomata by the transpiration stream and stimulates stomatal closure via the enzyme hexokinase, thereby preventing the loss of precious water.

The need to develop crops that use water more efficiently

Plants need light, carbon dioxide (CO_2), water and minerals to grow. Of these, water is the major limiting factor for land plants. For this reason, terrestrial plants are coated with a waxy cuticle layer that prevents evaporation and water loss. Special pores in the cuticle, called stomata, open and close to allow CO_2 to enter the leaves. When these pores are open, water from inside the plant flows out to the atmosphere and CO_2 from the air enters the plant. The stomata are comprised of two guard cells and the pore they surround (Figures 1 & 2). The pores open at dawn to allow the entry of atmospheric CO_2, at the cost of extensive water loss (transpiration). When conditions are less favorable for the efficient fixation and utilization of carbon, the stomata close, to reduce the amount of water lost to the atmosphere. Most of the water taken up by plants (about 98%) is released to the atmosphere through the stomata (Figure 2). As a result, the ratio of carbon gain via photosynthesis to water loss via transpiration, the water use efficiency (WUE), is usually less than 2%. About 70% of the world's freshwater is used for agriculture. In light of the global water shortage, growing populations and mounting irrigation costs, we are faced with the challenge of reducing plants' water loss and increasing WUE.

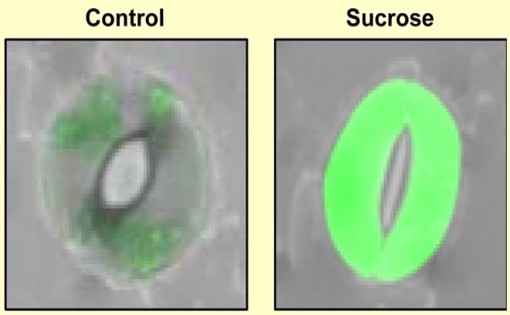


Figure 1 Sucrose stimulates stomatal closure. *Stomatal closure in response to sucrose is shown as elevated nitric oxide fluorescence (green coloration). Note that upon exposure to sucrose, the size of the stomatal aperture is reduced, in correlation with the intensity of the observed fluorescence.*

The prevailing understanding of the role of sugar in the regulation of stomata

Efforts are being made to discover ways to control stomatal behavior, to conserve water and increase WUE. At the mechanistic level, stomata open in response to increases in the osmolarity of the guard cells. These increases in osmolarity are followed by the movement of water into the guard cells which, in turn increases their volume and opens the stomata. Stomatal closure occurs in the opposite manner; as the osmolarity of guard cells decreases, their volume decreases [7].

About a century ago, it was proposed that sugars, generated from the degradation of starch in guard cells at dawn, are the primary guard-cell osmolytes stimulating stomatal opening [6]. This hypothesis was later modified by the discovery that potassium (K^+), chloride (Cl^-) and malate ions are the primary osmolytes that accumulate in guard cells and open stomata [7]. However, in addition to these ions, it has been suggested that the accumulation of sugar in guard cells also contributes to the osmotic state of the guard cells and the opening of the stomata [8].

The hypothesis that sugar induces stomatal opening was based primarily on correlations between the sugar content of guard cells and stomatal aperture. The accumulation of sugar in guard cells of open stomata over the course of the day and a concomitant decrease in K^+ concentration was observed [8]. Therefore, the prevailing hypothesis was that from midday on, sugar replaces K^+ as the major osmolyte, keeping the stomata open. Yet, this hypothesis has never been tested experimentally.

".....the surplus sucrose is carried toward the stomata by the transpiration stream and stimulates stomatal closure ...."

New hypothesis regarding the role of sugar in the regulation of stomata

Despite the prevailing hypothesis, we and others [3] suspected that sugar might induce stomatal closure rather than stomatal opening for the following theoretical reasons. In order for plants to use water efficiently, there must be an appropriate balance between the demand for CO_2 for photosynthesis and water loss through transpiration. The primary product of photosynthesis is sugar, which is exported out of the mesophyll cells, where it is produced, and moves into the phloem. As stated above, most (98%) of the water coming up from roots into the xylem vessels located adjacent to the phloem is transpired to the atmosphere. The stream of water and the stream of sugars intersect each other in the intercellular space (apoplast) and, as a result, some of the sugar mixes with and is carried along by the water flowing toward the stomata (Figure 2). At the stomata, the water evaporates into the air while the sugar accumulates in the vicinity of the guard cells [3]. When the rate of photosynthesis is high and the mesophyll cells produce more sugar than can be uploaded into the phloem, more surplus sucrose is carried toward the stomata. We hypothesized that the accumulation of sugar in the vicinity of the guard cells might reduce stomatal aperture, thereby coordinating photosynthesis with transpiration.

The new discovery regarding the role of sugar in the regulation of stomata

We reached the hypothesis described above following an unexpected observation while studying the role of hexokinase (HXK) in plants. HXK is an essential hexose (sugar) -phosphorylating enzyme that mediates sugar sensing, in addition to catalyzing the addition of a phosphate group to a molecule of sugar (see details in BOX p 14). Over the last two and a half decades, the role of HXK in plants has been the subject of intense study.

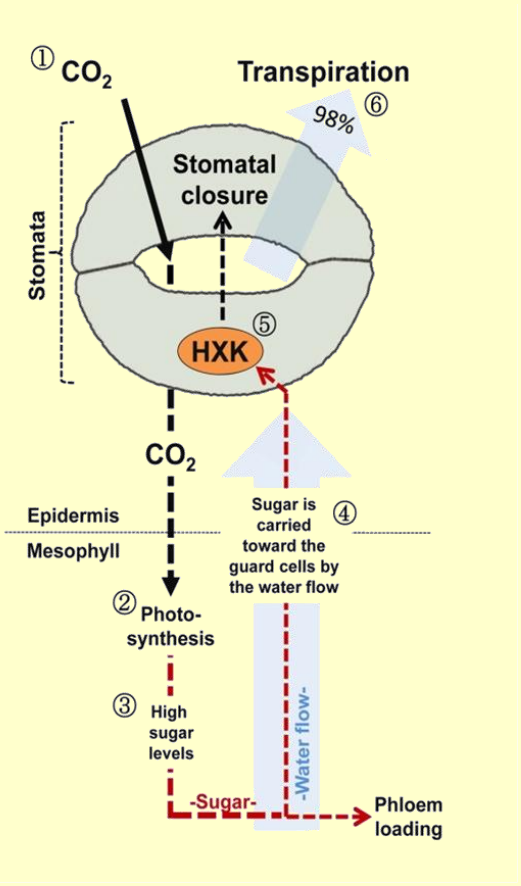


Figure 2 The feedback-inhibition mechanism of sugar. *Stomata open to allow entrance of atmospheric CO_2 into the leaves. (1) This CO_2 is then fixed through photosynthesis in the mesophyll cells (2) where sucrose is produced. The sucrose is exported to the apoplast and loaded into the phloem vessels. As the rate of photosynthesis increases, the amount of sucrose in the apoplast increases (3). When the rate of sucrose production exceeds the rate at which sucrose is loaded into the phloem, the surplus sucrose is carried toward the stomata by the transpiration stream (4) and stimulates stomatal closure via HXK (5), thereby reducing transpiration (6), preventing unnecessary water loss when sugar levels are sufficient. Closing the stomata will reduce the flow of CO_2 into the leaf, the rate of photosynthesis and the production of sugars. As a result, less sucrose will be carried toward the stomata, relieving the HXK closing effect and causing the stomata to re-open. The red dashed line represents sugar (sucrose). The blue arrow represents the flow of water*

The main conclusion of this work has been that HXK monitors glucose levels in photosynthetic tissues and inhibits photosynthesis when sugar levels are sufficiently high [2, 1]. A central approach in the study of HXK has been the analysis of plants that overexpress HXK. We generated tomato and Arabidopsis plants that overexpress HXK and noticed that HXK reduced stomatal aperture [4, 5]. This new observation prompted us to come up with our hypothesis regarding the role of sugar and HXK in the regulation of stomata.

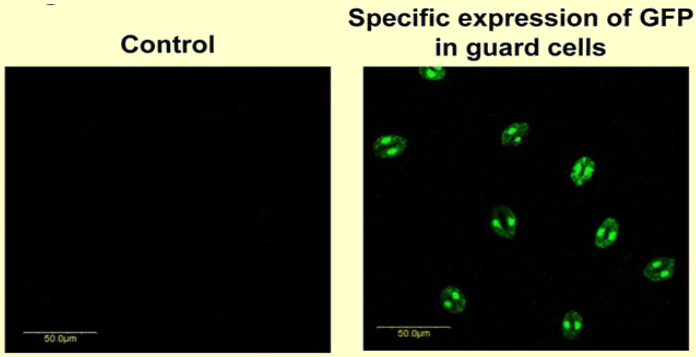


Figure 3 GFP expression under the control of the guard cell specific promoter. Green fluorescent protein (GFP) was used to verify that the guard cell-specific promoter is indeed expressed exclusively in guard cells.

Sugar stimulates stomatal closure via HXK

A functional approach has been taken to examine the role of sugar and HXK in the regulation of stomata. Exposure of leaves or epidermal peels (composed of only guard cells and epidermis) to sugar stimulates stomatal closure [5]. HXK inhibitor blocks the effect of sugar on stomata, indicating that HXK is an essential component of the sugar effect. Finally, the role of HXK in stomatal closure was examined following specific expression of HXK in guard cells, under the control of a guard-cell specific promoter. To verify that the promoter was indeed expressed exclusively in guard cells, we expressed green fluorescent protein under this promoter and found that its expression was indeed specific to guard cells and was not detected in any other tissue or cell type (Figure 3). Exclusive expression of HXK in guard cells stimulates stomatal colsure [5].

These observations show that, in contrast to the century-old model, sugar stimulates stomatal closure and does so via the sugar-sensing enzyme HXK. These findings support the existence of a feedback-inhibition mechanism that is mediated by sugar (the product of photosynthesis) and HXK. According to this hypothesis, when the rate of sucrose production exceeds the rate at which sucrose is loaded into the phloem, the surplus sucrose is carried toward the stomata by the transpiration stream and stimulates stomatal closure via HXK, thereby preventing the loss of precious water (Figure 2). This feedback system may represent a rapid mechanism that coordinates CO_2 uptake, sugar production and sugar utilization with water loss.

Hexokinase in Plants

The primary end product of photosynthesis in many plants is sucrose (a glucose-fructose disaccharide known as the common sweetener white sugar). Sucrose can be metabolized in photosynthetic (source) tissues or exported to non-photosynthetic (sink) tissues, where it serves as an initial substrate for all organic metabolic pathways/ However, before it can be metabolized, sucrose must be cleaved by either invertase (INV) or sucrose synthase (SUS), the only two families of sucrose-cleaving enzyme that have been identified in plants. INV cleaves sucrose into glucose and fructose; whereas SUS cleaves sucrose in the presence of UDP to yield UDP-glucose (UDP-G) and fructose. Both glucose and fructose must be phosphorylated before they can be used in any metabolic process. Hence, hexose-phosphorylating enzymes are essential for all aspects of plant metabolism and development. Only two types of hexose-phosphorylating enzymes exist in plants, HXK and fructokinase (FRK). HXK is the only enzyme that can phosphorylate glucose in plants.

Using HXK to increase WUE

The fact that HXK acts as a sugar sensor in guard cells that coordinates photosynthesis with transpiration suggests that it may be possible to use this enzyme to help plants conserve water without negatively affecting photosynthesis, that is, to increase WUE while maintaining high crop yields. To examine this possibility, we used transgenic tomato plants that expressed high levels of HXK only in their guard cells (referred to as SPV lines for reasons described below). The exclusive expression of HXK in guard cells was achieved through the use of the guard cell-specific promoter (Figure 3). We analyzed the WUE of the transformed (SPV) plants, using the LI-COR gas-exchange system (LI-COR; Lincoln, NE, USA), and discovered an increase in WUE in those plants (Figure 4). We found that while net photosynthesis remained unaffected, transpiration (Figure 4A) was reduced by up to 25% relative to the wild type (Figure 4B). This improved the instantaneous water use efficiency (IWUE) of the transformed plants by 30-35% (Figure 4C). Our results demonstrate that exclusive expression of HXK in guard cells is an efficient way to improve the WUE of plants. This technology (named SPV for Smart Plant Valves) has been patented (patent no. 61/569,251) and is currently being implemented in various crops.

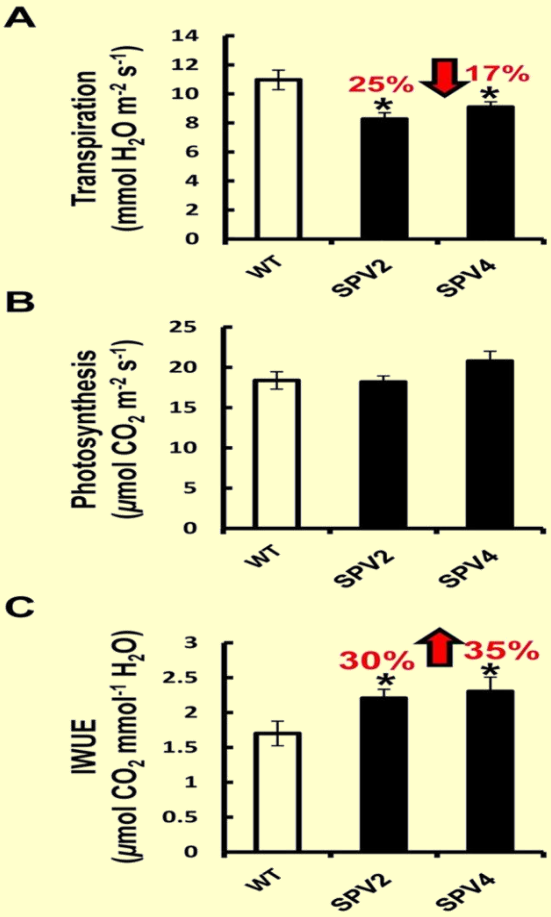


Figure 4 Elevated expression of hexokinase in guard cells reduces transpiration and improves water use efficiency. *A gas-exchange analysis of wild-type (control) and two independent transgenic tomato lines expressing HXK specifically in their guard cells (SPV) was performed using a Li-6400 portable gas-exchange system (LI-COR). Transpiration (A), photosynthesis (B) and instantaneous water use efficiency (IWUE; C) were measured under regular growth conditions. Asterisks indicate significant difference relative to the wild type. The red arrows and percentage numbers indicate the amount of the effects*

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Focus on Microbial Food Safety: is it really worth washing leafy greens?

By Shlomo Sela (Saldinger), Postharvest & Food Sciences Institute

About first author

Shlomo Sela (Saldinger) is Head of the Department of Food Quality and Safety of the Institute of Postharvest and Food Sciences. His research focuses on microbial food safety. Prof. Sela was awarded Scientist of the Year, 2012 on his exceptional break-through research in the field of food safety and sanitation from microbe contaminants. His research findings have significant practical repercussions for agriculture and food safety.

Fresh or minimally processed ready to eat fruits and vegetables are perceived by consumers as being healthy, tasty, and convenient. Nevertheless, the last few decades have witnessed increased illness following consumption of contaminated fresh- or minimally processed produce. Unlike other types of foods which can undergo thermal process, such as pasteurization or cooking, Ready-to-eat minimally processed fruits and vegetables pose a challenge to the food industry, since current disinfection technologies can not completely eliminate foodborne pathogens. Consequently, the agro-food sector is now searching for alternative means to minimize produce contamination. As part of this effort, studies in Prof. Sela laboratory are aimed at elucidating potential mechanisms of produce contamination. During a study on the attachment of Salmonella (a food-borne bacterial pathogen) to leaf surface of iceberg lettuce, Dr. Yulia Krouptiski (at that time a PhD student at Prof. Sela lab) who led the project, together with Eddy Belausov, a confocal microscopy specialist, have noticed that fluorescent-tagged bacteria were moving on the leaf surface toward special openings called stomata. These openings participate in gas exchange between the interior and exterior of the plant and enables respiration and photosynthesis. *Salmonella* did not only aggregated near stomata but also penetrated into the interior of the leaf, a protected environment, rich in water and nutrient ingredients that favor bacterial survival. Further studies have revealed that *Salmonella* internalization is regulated by both plant- and bacterial factors. It seems that the pathogen is attracted to sugars produce by chlorophyll-containing cells at the interior of the leaf tissue, which serve as a delicious food for bacteria, as well. Genetic analysis revealed that the pathogen perceive the presence of sugars within the leaf tissue by a unique chemotaxis system, which directs its movement toward the sugars through the open stomata. Movement of the bacteria is mediated by specific motility organelles called flagella. Additional experiments showed that *Salmonella* internalization vary greatly between different leafy vegetables and even between cultivars of the same plant.

These findings shed a new light on a new contamination route through leaf internalization, which provides the human pathogen with a rich and physical-protected micro-environment. The discovery has important implication to the safety of leafy vegetables as internalized bacteria are much more persistent and resist washing and disinfection compared to surface-attached bacteria. Further studies are conducted to search for new intervening approaches to enhance the microbial safety of leafy vegetables.

Fresh fruits and vegetables are perceived by consumers as being healthy, tasty, and convenient foods. Ready-to-eat (RTE) minimally processed fruits and vegetables market has grown in recent years due to increase in consumption. These food products are especially convenient to a busy and health-conscious consumer. However, with the increase production of RTE food, the fresh produce industry is facing a new challenge, e.g. the protection of consumers against associate microbiological hazards. Fresh produce and minimally processed fruits and vegetables were implicated in the last three decades in several large outbreaks of *Salmonella* and *E. coli*. One such outbreak occurred in Germany and other European countries in 2011, where a highly virulent *E. coli* strain associated with fresh sprouts sickened thousands of people and killed 53 after eating contaminated salads.

Foodborne illnesses were traditionally linked to consumption of contaminated foods of animal origin, such as meat, eggs and dairy products. This has been attributed to the presence of these pathogens in the intestine of livestock animals. However, the last few decades have witnessed a dramatic increase in the frequency of foodborne illnesses associated with consumption of fresh produce. This has been attributed to a number of reasons, amongst them are increased consumption of fresh produce and the introduction of a new food category of minimally processed (cut/washed) ready-to-eat vegetables and fruits, which provides fast and convenient healthy food, centralization of fresh produce processing, and globalization of trade.

Produce contamination may occur along the food-production chain from farm to fork. On-farm contamination may result from contaminated irrigation water, or fertilization with raw- or partially treated animal manure, which frequently contains zoonotic pathogens, such as *Salmonella* and toxin-producing *E. coli*.

"……to minimize fresh produce contamination is through better understanding of the potential contamination routes …."

Among the vegetables, lettuce has been linked to numerous outbreaks of enteric bacterial pathogens, *E. coli* and *Salmonella*.

Currently, the fresh produce industry does not have an effective method to completely eliminate foodborne pathogens from fresh produce. One approach to minimize fresh produce contamination is through better understanding of the potential contamination routes, which could be used for risk assessment.

In a recent study performed at the Department of Food Quality and Safety and supported by the U.S.A - Israel Bi-national Agricultural Research & Development Fund in collaboration with Dr. M. Brandl (ARS, USDA), Prof. Shlomo Sela and co-workers focused on the attachment of *Salmonella* to lettuce leaf. In order to visualize the pathogen on the leaf surface, the researchers have utilized electron scanning microscopy (Figure 1). During the study it was observed that live Salmonella cells were aggregated at the rim and within stomata (Figure 2).

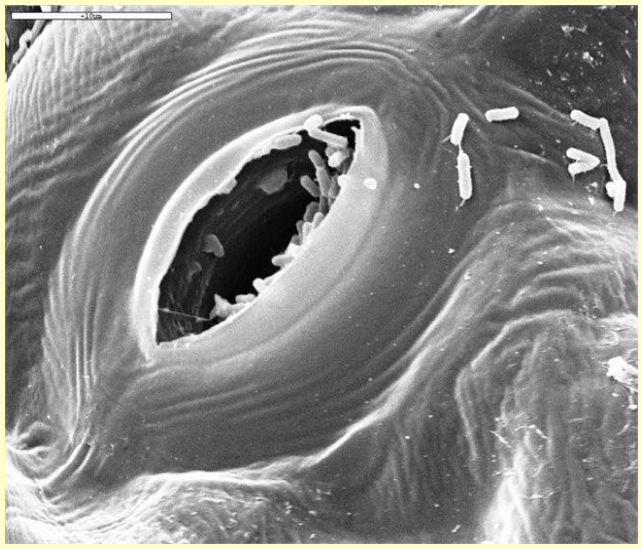

Figure 1 *Scanning microscopy image showing bacteria, possibly Salmonella, within and nearby stoma on iceberg lettuce leaf. However, this technique requires special fixation steps and does not distinguish between Salmonella and other plant-associated bacteria, which might also reside on the leaf surface. Consequently, they have specifically labeled Salmonella cells with a green-fluorescent protein. This kind of labeling facilitates visualization of the live pathogen and enables precise localization of the fluorescent bacteria on the lettuce leaf under a special fluorescence microscope.*

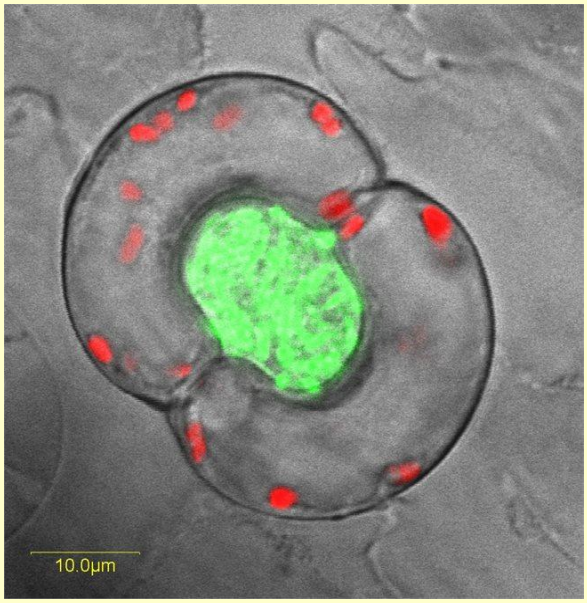

***Figure* 2** Fluorescence microscopy image showing a single fully-opened stoma on iceberg lettuce leaf, surrounded by a pair of guard cells. The green color represents fluorescently-labeled *Salmonella*. The red color is autofluorescence emitted from chlorophyll in the guard cells.

Stomata are microscopic pores on the surface of plants that enable exchange of carbon dioxide and oxygen, for photosynthesis and respiration. Each stoma is surrounded by a pair of specialized guard cells, which open and close the pores in response to environmental conditions. It should be mentioned that the majority of the leaf surface is coated by a wax layer that protect the plant cells from dehydration. During the study [1], Dr. Yulia Kroupitski (at that time a PhD student in Prof. Sela's lab), together with Eddy Belausov, a confocal microscopy specialist, were amazed to observe that in some cases, fluorescent bacteria were actually moving on the leaf surface toward the stomata and entered the sub-stomatal space. This was the first time ever that *Salmonella* cells were visualized entering stomata in real-time.

In order to distinguish between passive and active internalization, the *Salmonella* cells were killed and then tested for movement and internalization. Interestingly, dead bacteria were not able to aggregate near or enter stomata, suggesting that this feature is in fact an active process mediated by the live bacteria. Further experiments have demonstrated that internalization was stimulated in the light and was inhibited in the dark, raising the notion that the human pathogen is attracted to the nutrient-rich environment within the leaf interior. Many bacteria possess chemotaxis systems, which allow them to move toward nutrients or away from toxic compounds. *Salmonella* is also known to possess, such a system, which includes specific chemo-receptors to sense chemicals in the environment, and specialized motility organelles called flagella. To gain further insight into the mechanisms underlying *Salmonella* internalization, the researchers have utilized genetic tools in order knock-out some key *Salmonella* genes involved in chemotaxis and motility. Indeed, mutants in chemotaxis and motility were impaired in stomata internalization. To further support the idea that *Salmonella* were penetrating stomata in an effort to search for nutrients (food), internalization experiments were repeated in the presence of various sugars that are naturally synthesized during photosynthesis and in the presence of sap derived from un-infected lettuce leaves. As anticipated, *Salmonella* internalization was inhibited in the presence of these additives.

Based on these results, it is concluded that *Salmonella*, and possibly other enteric pathogens may contaminate leafy vegetables also by internalization through stomata. These finding have important food-safety implications since internalized bacteria are considered to resist current washing and disinfection technologies. Whether the identified route of contamination occurs in real life, in the field or during storage or processing of leafy vegetable, remains to be explored.

During a continuing study, in laboratory experiments, it was found that internalization of *Salmonella* varies greatly from plant to plant and even among cultivars. These findings raise the possibility that breeding programs for leafy vegetables with lower internalization capacity might be one of the future directions to mitigate leaf contamination through stomata internalization

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