



Proprietary elicitor affects seed germination and delays fruit senescence

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Abstract

The proprietary elicitor, BEYOND All Natural Plant Amendment, improves seed germination rates with tomato, bean, corn and many other vegetable and flower seeds. Compared to controls, treated plants emerge sooner and are more vigorous, which results in statistically increased crop performance and higher yields. This elicitor does not appear to be a systemic agent in plants, but to impact receptors on the cell surface and initiate molecular level signal transduction processes. For example, induction of β -1,3-glucanase in treated seeds is associated with improvement in germination rates. This elicitor does not utilize ethylene as a secondary messenger, but appears to reduce ethylene biosynthesis. Application of this elicitor either by foliar spray or root feeding prior to harvest delays fruit senescence by inhibiting ethylene biogenesis. Presently, ripening of harvested citrus is initiated with ethylene at the start of storage. Since fruit from BEYOND treated trees exhibit better storage characteristics, it may be concluded that ethylene biosynthesis is reduced by application of the elicitor to citrus. This phenomenon is also studied using triple response assays on etiolated *Arabidopsis thaliana* seedlings. BEYOND treated seeds show no stimulation of ethylene biosynthesis, as do some elicitors. Since this elicitor reduces ethylene formation, physiological factors controlling plant development are no longer negatively impacted by ethylene, resulting in improved growth at all stages. BEYOND All Natural Plant Amendment is manufactured by AgriHouse Inc., Berthoud, Colorado USA (US Patent No. 6,193,988).

Key words: elicitor, germination, β -1,3-glucanase, ethylene, senescence, triple response, signal transduction, tomato, adzuki bean, mung bean, maize, Arabidopsis, metabolites.

Introduction

Plants produce various secondary metabolites that allow interaction with the environment. Elicitors can enhance secondary metabolite production and/or second messenger development, known as signal transduction ¹. The interplay of these elicitors and secondary metabolites enables the now-alerted plant to better overcome biotic and abiotic (environmental) stresses. While some elicitors stimulate defense responses in the plant, others induce plant growth responses that result in increased dry weight biomass, root size and stem caliper, bloom and harvest ².

As an organic patented material derived from exoskeletons of crustaceans, BEYOND All Natural Plant Amendment is considered an elicitor ³. Elicitors provide external stimuli that trigger the changes in the plant cells, which lead to cascades of reactions and production of secondary metabolites, ultimately helping the plant overcome stress factors ⁴. Elicitors are stimuli of biotic and abiotic types. For example, the latter are represented by natural stresses to the plant from touch, shear forces (wind), temperature shocks and osmotic stresses. Biotic elicitors include glucan polymers, glycoproteins, low molecular weight organic acids, fungal xylanases and cell wall materials and segments of bacterial flagella. High affinity binding sites have been characterized for oligo- β -glucosides, such as oligochitins, oligochitosans, yeast N-glycan and β -1, 4-linked galacturonate oligomers ⁵. The stimuli are perceived by receptors on the plant cells, which lead to activation of secondary messengers that transmit signals into the cell through signal transduction pathways that ultimately results in gene expression and the biochemical changes that

benefit the plant. Interplay of the signaling molecules also regulates the entire pathway by factors, which influence signal transduction pathways. These factors include polyamines, calcium, jasmonates, salicylates, nitric oxide and ethylene ⁵.

Materials and Methods

Seed germination: Adzuki bean (*Phaseolus angularis*), mung bean (*Phaseolus aureus*) and tomato (*Lycopersicon esculentum*) seeds were germinated in trays containing fifty seeds each. Twelve layers of paper towels were placed in each tray, and the tray was covered with aluminum foil. Three identically treated trays represented replicates for each experiment that were repeated at least two times. These, along with one flask per tray containing 200 mL distilled water, were autoclaved for 20 min at 121°C. The dried seeds were surface sterilized in 1.0 (v/v) percent sodium hypochlorite and then thoroughly rinsed in sterile distilled water before being placed between the paper towels. For treatments the elicitor was added in a concentration of 1 mg/mL; controls were without elicitor. The solution was then poured over the paper towels, and the trays were re-covered with aluminum foil. The seeds were allowed to germinate at room temperature in a dark environment for indicated periods of time.

Alternatively, single seed germinations were conducted in 1.5 cm x 15 cm pyrex test tubes. Cylindrical rockwool plugs, 2.0 cm in length and 1.5 cm in diameter each with a hole 0.5 cm diameter and 1.0 cm in depth, served as the growing matrix for adzuki and mung beans. Aseptically, 1.0 mL of elicitor (at various

concentrations from 0 to 2.0 mg/mL) was pipetted into the hole at the center of each plug. The rockwool plugs were then dried in a laminar flow hood. After surface-sterilizing the beans in a 1.0 (v/v) percent bleach solution for 30 seconds, the beans were rinsed three times using the same elicitor concentration solution as added to the matrix. Finally, adzuki beans were loaded into the hole in the plugs, white crown up. The plugs were then placed aseptically into individual sterile test tubes and at the appropriate time, 1.5 mL of distilled water moistened the rockwool, which served to germinate the seed and to present the elicitor to the growing sprout. Beans generally germinated after 5 days of darkness at 22°C and allowed to grow for appropriate periods of time, as indicated.

The laboratory sweet corn (*Zea mays* var. *Rugosa*) seed (Frontier Seed Sweetie 82, Glendale, AZ) were soaked for three minutes in BEYOND concentrate and subsequently sprayed with 1% solutions at seven day intervals. The testing consisted of seven day pre-chill followed by seven day standard germination using Association of Official Seed Analysts (AOSA) methods at STA Labs in Longmont, CO. The seedlings treated with BEYOND had slightly more vigor, longer root and shoot lengths and higher chlorophyll levels at the end of testing.

Laminarinase assay: The enzyme solution for the laminarinase assay was prepared by placing 0.1 g of adzuki bean root tissue in 10 ml of 0.05 M sodium phosphate buffer (pH 5.5) and homogenized for 30 seconds. Homogenization was conducted using a Kinematic Ag Model PT 1200 Polytron electronic homogenizer, set on power level 3 in 1.5 cm x 15 cm pyrex test tubes. The homogenate was centrifuged at 10,000 rpm for 10 min. The supernatant was then filtered using Sephadex G25 to remove low molecular weight sugars and peptides and allow the desired enzymes to pass through.

β -1,3-glucanase assay: A colorimetric assay was used to determine enzyme levels in the solution. All assays were done in duplicate. The soluble β -1,3-glucan laminarin polysaccharide substrate is cleaved by laminarinase, leaving reducing ends. Dinitrosalicylic acid (DNSA) reagent reacts with these ends and leads to a visible color change that was quantified using a double beam spectrophotometer at 540 nm. The reaction tubes were prepared using 1.0 mL laminarin (10 mg/ml) solution and 1.0 mL enzyme solution, and incubated for 30 minutes at 37°C. DNSA reagent (3.0 ml) was then added to each tube and the tubes were placed in a boiling water bath for 10 min. The absorbance after the reaction was compared to a standard curve of known concentrations of glucose. Background reactions were calculated by preparing blanks with the sodium phosphate buffer replacing either the enzyme solution or the substrate. The absorbance of the blanks was subtracted from the absorbance of the reaction to get a corrected absorbance. The amount of laminarinase in the enzyme solution was calculated using the slope of glucose standard curves, prepared on the day of the assay.

Protein content: Protein levels were calculated using Pierce bicinchoninic acid (BCA) protein assay. Bovine serum albumin concentrations from 0.1 to 0.4 mg/mL were used to generate a standard curve. Aliquots of 100 μ L of each standard or unknown were placed in a test tube. 2.0 mL working reagent [50 parts reagent

A (sodium carbonate, sodium bicarbonate, BCA, and sodium tartrate in 0.2 N sodium hydroxide) to 1 part reagent B (4% cupric sulfate)] was added and the tubes were incubated in a water bath set at 37°C for 30 minutes. The absorbance readings of each tube taken at 562 nm were then used to determine the unknown protein levels.

Triple response assay: Using 47 mm Petri dishes, 15 to 30 seeds of *Arabidopsis thaliana* WT Col were placed in a row across agar (1.0%) containing 1x Murashige Skoog basal salts medium (no sucrose) and ½ x B5 vitamins. To the agar for treatments was added 1.0 mL /litre of BEYOND; controls were both with and without ethylene and without BEYOND. The plates were held in a vertical position in a styrofoam box using double stick tape and kept at 4°C for 72 hours. The plates were then exposed to constant fluorescent lighting at room temperature for 18-20 hours. Two Petri dishes could then be placed vertically in 230 mL (pint) Mason jars, the lids for which had a 5 mm (¼ inch) hole drilled and fitted in place with a small serum cap glued in the center. Ethylene was serially diluted to 1000 ppm in a separate bottle and 0.23 mL was injected into 0.23 L jars to yield 1.0 ppm ethylene during subsequent dark incubations. During the period of etiolated growth, the plates were kept in the dark at room temperature. After five days the jars were opened, the Petri dishes were placed face-down (without lids) on a scanner. The Adobe Photoshop images were analyzed by NIH image J software to obtain dimensions of epicotyl elongation, width and hook angle.

Treatment of citrus trees and fruit storage evaluation: Sorenson ¹¹ compared BEYOND with a control by one 35 mL (total) application by spraying the leaves and two applications of 35 mL (total) each time to the soil around orange trees during a period several weeks preceding harvest. The subsequent post-harvest procedures that were used add more information to our understanding of the signal transduction process. The post-harvest gassing procedure with 10 ppm ethylene gas for four days at 68°F turns out to be important. This is applied to start the ripening process in harvested fruit; it is widely used for bananas and tomatoes.

Results and Discussion

Many germination experiments demonstrate improved responses in BEYOND treatments. Such observations will be reported using seeds of tomatoes, mung beans, adzuki beans and sweet corn. The first instance of improved germination involves Leading Lady tomato seeds (Sunseeds, Morgan Hill, CA) in sterilized trays and toweling in the laboratory following germination in the presence of 1.25 mL of BEYOND per litre, as shown with data presented in Table 1. After 17 days of growth at room temperature in the dark, digital photographs were taken and the entirety of each treatment of seedlings was dried overnight at 50°C. The dried hypocotyls from the treated seeds weighed 72% more than did the controls. Image analysis of the hypocotyls revealed an 87 percent greater length for BEYOND treated materials compared to the untreated controls.

The second instance of enhanced seed germination is noted using sweet corn. Two independent organizations, STA Labs (Longmont, Colorado) reported 14 percent increased seed germination viability of sweet corn under laboratory conditions. The germination rates

of the control (water) and the BEYOND treated seeds were 65% and 71%, respectively. Also an organization associated with Bayer Crop Science, Celpril of Manteca, CA (www.celpril.com) conducted a sweet corn seed germination trial in the field in the fall of 2003. These data, presented in Fig. 1, show effects of treatments using 1.25 mL and 2.50 mL of BEYOND per litre compared to control treatments that received only water. Emergence rates, 21 days after sowing, were approximately 60, 65 and 15 percent, respectively. The extent of the differences between treatments and controls points out the difficulty growers experience with germinating sweet corn, because of high sugar content (Lynn Loken, Loken Associates, Loveland Colorado; private communication).

The third instance of germination enhancement is with adzuki beans grown in sterilized trays and toweling in the laboratory. Growth rates were again measured at room temperature during the first seven days in darkness following wetting of 10 gram of seeds with either 200 mL of water or the same quantity of 0.75 mL of BEYOND per litre water. The mass of dried hypocotyls originating from the respective treatments was as follows:

Treatment	Dry weight	Percent increase
Control	0.40 g	
BEYOND	0.45 g	13%

In preparation for tests that were conducted aboard the NASA space shuttle ATLANTIS and the MIR space station in late 1997 and early 1998⁶, another parameter was measured in adzuki bean and mung bean seedlings. The enzyme β -1,3-glucanase specific activity was assayed using laminarin (a soluble β -1,3-glucan) as substrate. Crude homogenates of the seedlings yielded the data in Figs 2 and 3. An increase of the β -1,3-glucanase activity was obtained in the BEYOND treatments between seven and twelve days following germination of both bean types (Fig. 2 for adzuki beans and Fig. 3 for mung beans). The data in Fig. 3 also indicate advantageous differences between the treatments of mung bean seeds with BEYOND over those treated with various concentrations of purified colloidal chitin and the derived chitin oligosaccharide containing six glycan moieties, N-acetylchitohexose. The concentration of BEYOND used in this study is greater than the concentrations of chitin and oligosaccharide used. However, any indication of dose response relationship to the oligosaccharide concentration is negative; i.e. lower doses after ten days resulted in greater specific enzyme activities.

Table 1. Dry weight and image analyses of hypocotyls from germinated (0.5 g) tomato cv. Leading Lady seeds treated with 1.0 mL/L solution of BEYOND or water after 17 days of growth in the dark at room temperature. Approximately 180 seeds represent 0.5 g of these seeds. Image analysis of approximately 20 representative seedlings were conducted to obtain the data shown. The experiment was repeated twice.

Treatment	Quantity, ml	Dry wt. g	Average length, mm	Std dev, mm	% Dry wt. increase
BEYOND	6 of solution 6 of water	0.19	16.3	4.5	73%
Control	only	0.11	8.7	3.3	na

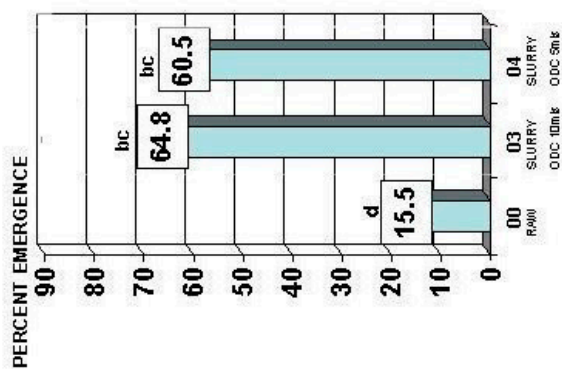
A dose response for BEYOND in induction of elevated β -1,3-glucanase activity in adzuki beans is demonstrated by data in Fig. 4. Induction of this enzymatic activity increases with quantity of BEYOND applied to the seeds and with time. Twelve days after germination there was no differences between treatments and controls in specific enzyme activity in both hypocotyls and epicotyl tissues. However, 21 days after germination the specific enzyme activity in both hypocotyls and epicotyl tissues increase with dosage, becoming significant at 2.0 mg per seed. Both with adzuki and mung beans, a delay between germination and elevation of specific enzyme activity is noted.

In summary, β -1,3-glucanase enzyme activity aids germination of many types of seeds. According to Leubner-Metzger from the University of Freiburg⁷, one form of β -1,3-glucanase (class I), which has been studied extensively in several types of seeds, including tobacco, tomato and other solanaceous seeds, is induced in the endosperm of the seeds just prior to penetration of the radicle, and is believed to help weaken the endosperm wall⁸. A thick β -1,3-glucan layer, which imparts limited permeability to the seed envelope of cucurbitaceous species, is degraded during germination⁹. The difference between stimulated germination in some cases (tomato; Table 1, sweet corn; Fig. 1 and adzuki beans) and improved seedling vigor in other cases may be related to differences in seed anatomy, the composition of the endosperm barrier represented by a β -1,3-glucan layer and other unknown factors.

BEYOND is not considered a systemic agent in plants, because it does not result in hydrogen peroxide production (data not shown). Instead it causes receptors on the cell surface to initiate molecular level processes called signal transduction¹⁰. BEYOND does not stimulate ethylene biosynthesis, as do some elicitors. By conducting a test using *Arabidopsis thaliana* seeds that is widely known as the "triple response"¹⁰, seeds are germinated in the dark, a condition that produces elongated epicotyls (stems). One of the triple responses, epicotyl elongation, is easily measured and is seen in Fig. 5 to be severely reduced by concentrations as low as 1 ppm ethylene. When seeds are germinated on agar medium containing 0.1 mg/mL BEYOND, the results of this very sensitive test indicate ethylene is not produced by the seedlings (Fig. 6). These data can be summarized to say BEYOND did not increase ethylene formation by the seedlings either in the presence or absence of exogenous 1 ppm ethylene.

Ethylene is a plant hormone commonly associated with senescence (aging) and abscission¹⁰. The shedding of leaves, flowers, and fruits from the living plant is known as abscission. These parts abscise in a region called the abscission zone (AZ), which is located near the base of the respective petioles. In most plants, leaf abscission is preceded by the differentiation of a distinct layer of cells, the abscission layer, within the AZ. During senescence, the walls of the cells in the abscission layer are digested by cellulase enzymes, which cause them to become soft and weak. As a result of stress on the weakened cell walls, the leaves, flowers and fruit eventually break off at the abscission layer⁵.

Ethylene stimulates abscission by causing cellulase formation, which breaks down the cellulose in cell walls in the AZ. Recent citrus



PLANTED-10-29-03 ORDER#6074 COUNTED DAY:21 Alpha level: 0.05 CV:20.3%
 BEYOND all natural Plant Amendment - test data provided by www.celpril.com (BAYER Crop Science)

Figure 1. Sweet corn germination study comparing treatment with BEYOND at two application rate (5 and 10 mL per gal = 1.25 and 2.50 mL /litre) with water controls. Data with different letters represent statistical significance at 0.05 level.

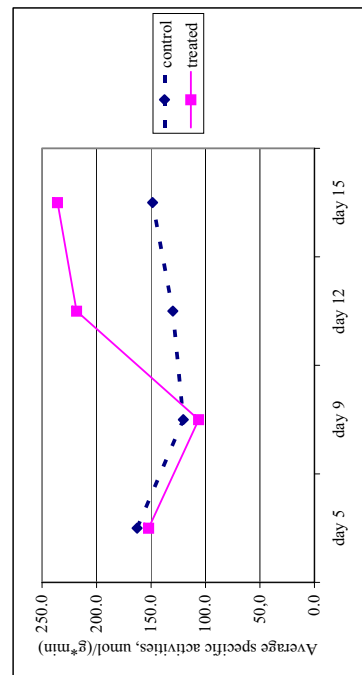


Figure 2. Kinetics of β -1, 3- glucanase formation in homogenates of adzuki bean seedlings between five and fifteen days after germination in test tubes in the presence of 1 mg BEYOND per seed.

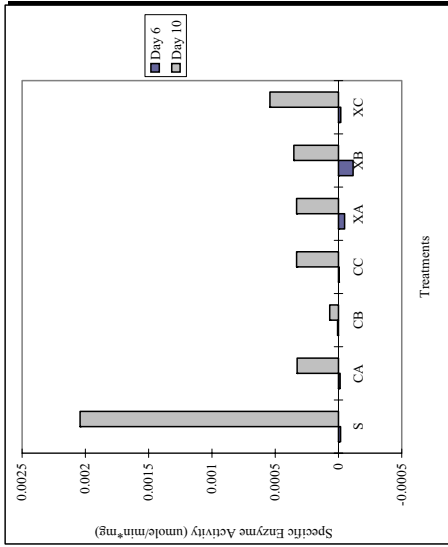


Figure 3. Differences between seed treatments with BEYOND, colloidal chitin and N-acetylchitohexose oligosaccharide on specific activities of β -1,3-glucanase in homogenates of mung bean seedlings six and ten days after germination in test tubes in the presence of S: BEYOND, 1 mg/seed; CA: Chitin, 9.06 mg/seed; CB: Chitin, 0.906 mg/seed; CC: Chitin, 0.0906 mg/seed; XA: N-acetylchitohexose, 0.5 mg/seed; XB: N-acetylchitohexose, 0.05 mg/seed; XC: N-acetylchitohexose, 0.005 mg/seed.

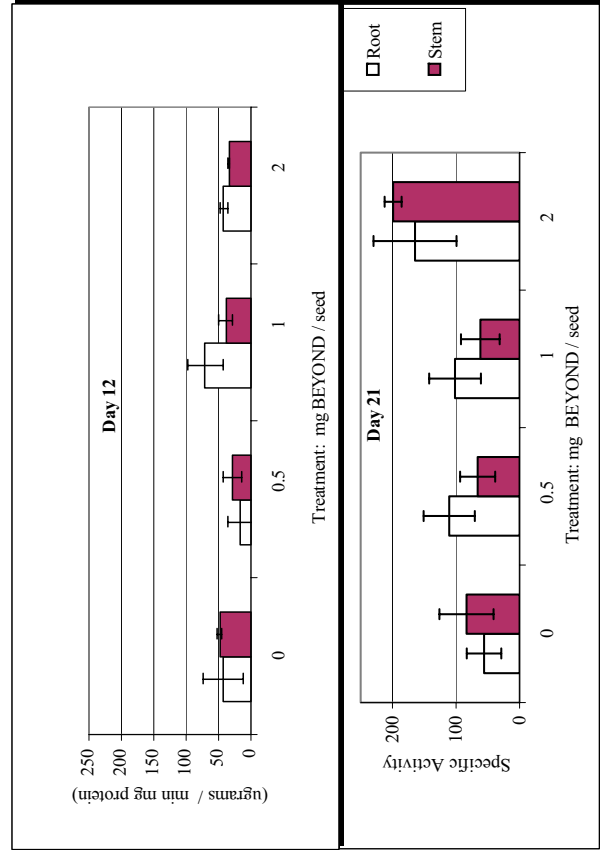


Figure 4. Specific activities of β -1,3-glucanase of treatments and those of water controls in homogenates of adzuki bean epicotyl tissue (stem-red) and hypocotyls (root-blue) twelve and twenty-one days after germination in test tubes in the presence of various concentrations of BEYOND; 0, 0.5, 1.0, and 2.0 mg / seed.

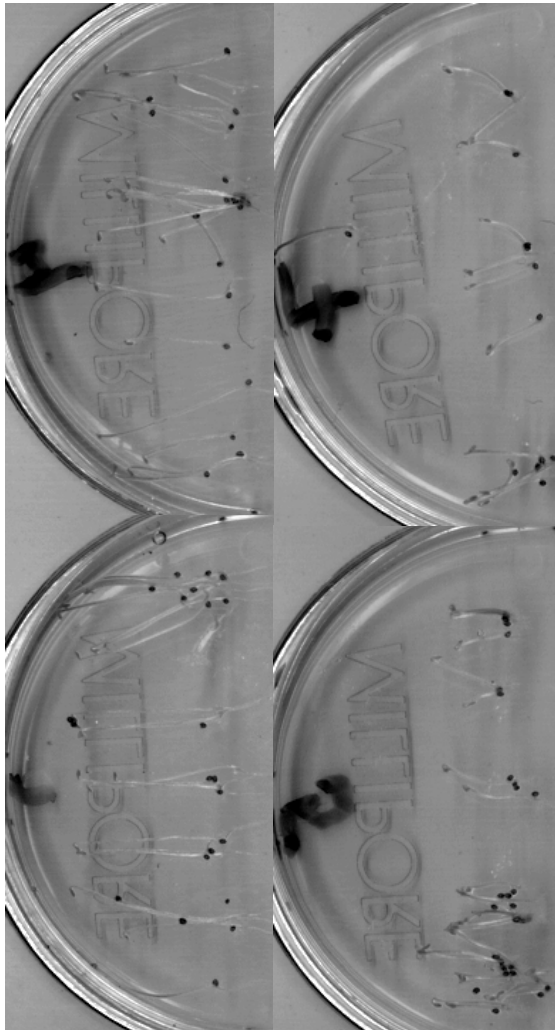


Figure 5. Etiolated seedlings of *Arabidopsis thaliana* Col exposed for five days to ethylene (C_2H_4) as follows: Zero ppm C_2H_4 without BEYOND (Petri dish labeled 1); BEYOND and zero ppm C_2H_4 (Petri dish labeled 4); BEYOND + 1 ppm C_2H_4 (Petri dish labeled 5); One ppm C_2H_4 without BEYOND (Petri dish labeled 7).

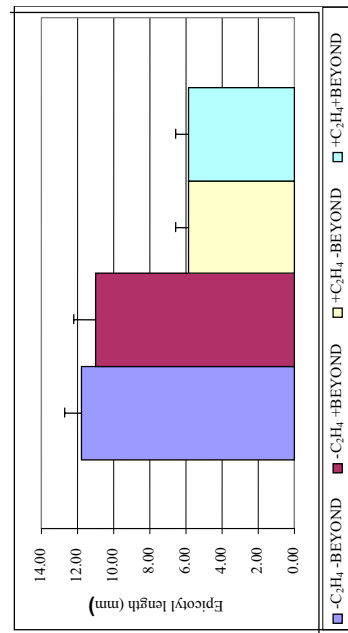


Figure 6. Average epicotyl elongations of etiolated seedlings of *Arabidopsis thaliana* exposed to BEYOND and C_2H_4 in four combinations from image analysis of scanned material represented in Fig. 5. The error bars represent standard deviations from two independent studies of each treatment consisting of 15-20 analyses each.

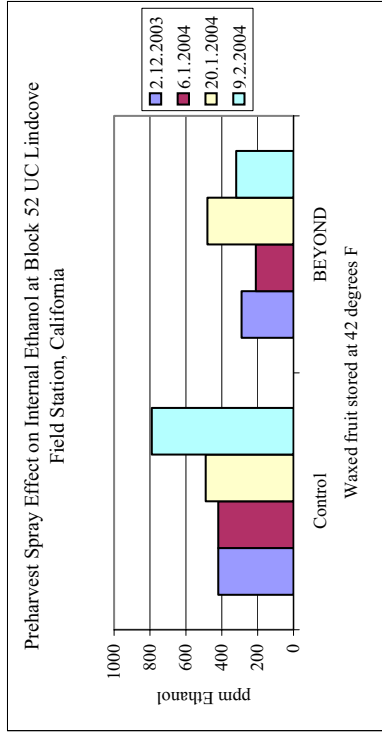


Figure 7. Ethanol production internalized in citrus fruit at four sampling dates during three months of storage following preharvest treatment of trees with BEYOND is lower than in fruit from water-treated control trees and indicates lower rates of fruit senescence and better fruit quality with BEYOND treatments. Data kindly provided by Sorenson¹¹.

field tests conducted by Sorenson¹¹ verify our laboratory results with *A. thaliana*, and reinforce our original hypothesis concerning the induction of signal transduction processes by BEYOND². BEYOND-treated fruit exhibit improved fruit quality, which apparently results from shutdown of the ethylene biosynthesis process in the pre-harvested fruit and consequent delayed senescence. Data in Fig. 7 represent citrus treated with BEYOND in comparison with fruit picked from trees treated in a manner identical, except with water¹¹. Application of BEYOND results in the delay of fruit senescence, and gassed fruit does not exhibit expected signs of aging, as demonstrated by lower quantity of ethanol produced during a three month storage period¹¹. Because the fruit treated with BEYOND requires longer than normal gassing with ethylene to initiate ripening post-harvest, it may be concluded that ethylene biosynthesis is reduced by BEYOND application in citrus. Controlled harvesting is a benefit of the BEYOND application. More uniform fruit retention on the tree until picking translates to higher field yields. The use of BEYOND also allows for a greater degree of control in the ripening process and longer shelf life. Other fruit, called preclimacteric fruit that are similarly affected by ethylene include melons, pears, kiwi fruit, apples, nectarines and avocados¹⁰. Cut flowers also benefit from blockage of ethylene synthesis and the delay of senescence.

Broader experience by Sorenson¹¹ as well as other commercial producers with multiple applications of BEYOND following planting, has demonstrated improved biological responses compared to a single treatment. Hence, the question arises: If BEYOND reduces ethylene formation by signal transduction processes at fruit senescence, are there physiological factors in the young or developing plant that are negatively impacted by ethylene? Review of the literature indicates physiological effects on plant seed germination, root and shoot growth, flower development, senescence and abscission of flowers and leaves, ripening of fruit and modulation of responses to biotic and abiotic stresses¹⁰. The endogenous ethylene biosynthesis pathway is fairly well understood¹⁰, but how BEYOND impacts any one of the enzymatic steps in the pathway needs to be investigated. Similarly, the observed up-regulation of β -1,3-glucanase by treatment with BEYOND requires further investigation.

Conclusions

The ability of the proprietary elicitor to amend plant growth characteristics by means of signal transduction processes makes BEYOND a unique plant amendment that differs from other types of elicitors and treatments, including chitin and chitosan. As a non-systemic agent in plants BEYOND impacts receptors on the cell surface and initiates molecular level signal transduction processes. BEYOND All Natural Plant Amendment naturally activates the signal transduction pathways in a wide and diverse range of plant species and cultivars. During the past 13 years BEYOND has proven to significantly increase seed germination and sprouting under laboratory conditions and field conditions¹². This natural elicitor reduces ethylene formation, thus reducing the impact of physiological factors that negatively control development in plants. Scientists and growers across North America, Mexico, Europe, Asia and India attest that by incorporating BEYOND into seed treatments and field applications by side dressing, drip irrigation systems, flood irrigation and overhead sprays, resulted in better crop yields of higher quality and improved shelf life of produce.

For more information regarding BEYOND All Natural Plant Amendment and its benefits to agriculture contact Loken-Flack at (970) 669-5399 or visit www.agrihouse.com.

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