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The elicitor AsES stimulates ethylene synthesis, induce ripening and enhance protection against disease naturally produced in avocado fruit

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ABSTRACT

Acremonium strictum Elicitor Subtilisin (AsES) is a natural elicitor capable of inducing disease resistance in strawberry and *Arabidopsis thaliana* plants. In this paper, the effect of AsES on ripening and defense response in the climacteric fruit, avocado (*Persea americana*) was studied. With this purpose ethylene production, respiration rate, weight loss, firmness and soluble solids content were studied. The effect of AsES on natural infestation with local pathogens was also evaluated to assess its capacity to activate a defense response. Controls consisted in fruits treated with water, or treated with 1-Methylcyclopropane prior to AsES (1-MCP + AsES).

Results showed that AsES treatment increases significantly ethylene production at early stages of ripening (3 days post treatment); while in fruits treated with water or 1-MCP + AsES the maximum production occurs later (6 and 7 days post treatment, respectively). Enhanced respiration rate, weight loss and soluble solids content, accompanied with the decreased in firmness were observed in fruits treated with AsES. Also, fruits treated with AsES halted the growth the opportunistic pathogens, whereas the protection effect was not observed when avocado fruit were pre-treated with 1-MCP, suggesting that the effect is due to the activation of the ET defense signaling pathway. These results uncover a potential use of AsES on the postharvest management of ripening, and open new research lines to study the relationship between fruit quality and induction of disease resistance in AsES-treated fruit.

1. Introduction

Avocado (*Persea americana* Mill.), is an important commercial fruit in which, ripening is accompanied by autocatalytic increases in ethylene production and fruit respiration rate (Biale, 1941; Bower and Cutting, 1988; Abeles et al., 1992). Ethylene diffuses freely from cell to cell through membranes and integrates the ripening process throughout the fruit. Fruit ripening is a highly coordinated genetic program that involves a series of physiological, biochemical, and organoleptic changes that leads to fruit softening, color change, aroma development, sugar accumulation and reduction in acidity (Saltveit, 1999). Ethylene gas is used as a phytohormone in horticulture due to its beneficial effects of plant growth and quality. These uses include ripening triggering, color enhancement, de-greening of citrus fruit, flowering promotion in pineapple, and inhibition of stem elongation, among others (Prasanna et al., 2007). Exogenous application of ethylene can also induce these irreversible events (Yang, 1987). There are a variety of techniques currently applied to induce or accelerate the ripening of climacteric fruits, thus reducing the costs of handling and storage and achieving a homogeneous ripening. One of the most used practices is the application of exogenous ethylene. Ho et al. (2011) obtained capsules of ethylene powder to replace the use of ethylene gas. However, ethylene postharvest applications are mainly using compressed gas cylinders that are diluted with air and since ethylene is explosive, safety is an important issue in these practices (Blankenship and Sisler, 1991). Other techniques applied are the use of ethylene release chemicals (e.g. ethephon) and the catalytic production of ethylene from ethanol, propylene and acetylene that can cause the same physiological effects of ethylene, but often require very high concentrations (Abeles et al., 1992).

Plants are in contact with a myriad of microorganisms found in the environment; however, in order for the disease to develop the host must be susceptible to a virulent pathogen and the environment must be conducive to the infection (Ferreira et al., 2006). Although disease

Abbreviations: AsES, *Acremonium strictum* Elicitor Subtilisin; 1-MCP, 1-Methylcyclopropane; dpt, dayspost treatment; SSC, soluble solids content

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response processes in plants have been extensively studied, in the case of postharvest fruits many of them are still unknown, and also the results are different for each pathosystem. The role of ethylene in pathogen-infected plants have been reviewed by Hoffman et al. (1999) who studied resistance to various pathogens in ethylene-insensitive mutants of soybean. The novel elicitor AsES (Patent EPC N∘12.720.221.6-1410) is an extracellular protein produced by the avirulent fungal pathogen *Acremonium strictum* and has been characterized as an effective activator of systemic acquired resistance (SAR) in strawberry and *A. thaliana* plants against *Colletotrichum acutatum* and *Botrytis cinerea*, respectively (Chalfoun et al., 2013; Hael-Conrad et al., 2015). Recently, by using *A. thaliana* knockout mutants, Hael Conrad et al. (2015) observed that AsES induces a defense response by activating the three main phytohormones signaling pathways (e.g. salicylic acid, jasmonic acid and ethylene).

The objective of this work was to evaluate the action of the defense elicitor AsES on key parameters of the ripening process, the protection against natural pathogen in avocado fruit, and elucidate the participation of the ethylene in this effect.

2. Materials and methods

2.1. AsES purification and solution

AsES elicitor protein was purified as previously described by Chalfoun et al. (2013). Briefly, the supernatant of a 21 days static liquid culture of *A. strictum* SS71 was centrifuged, filtrated through 0.2 µm Millipore membrane, ultrafiltrated (cut-off 30 kDa), and chromatographed first by anionic exchange (Q-Sepharose, pH 7.5), and then by hydrophobic interaction (phenyl-Sepharose). AsES purity was confirmed by 2D-PAGE. Finally, the purified protein was lyophilized and kept at 4 °C until use, then it was dissolved at a concentration of 60 nM in distilled sterile water and kept at −20 °C.

2.2. Fruit material

Fruits of avocado (*Persea Americana* Mill.) cv. Torres were harvested at the mature green stage, selected by shape, size, lack of physical injuries, and disease evidence. The fruits were packaged in cardboard boxes, transported to the laboratory, gently washed with distilled sterile water and air dried until treatments.

2.3. Sampling and treatments

Selected avocado fruits were randomly divided into three groups of 50 fruits, and subjected to following treatments: group 1 with water, and group 2 with AsES. Fruit were sprayed with 1 cm^3 of water or 60 nM AsES per fruit, respectively. The group 3 was treated with 1-MCP prior to AsES treatment (1-MCP + AsES). Fruits were placed in 18 L plastic containers, treated with 1-MCP (0.5 $\mu L.L^{-1}$) by using a commercial formulation (Ethyblock® Floralife, Burr Ridge, IL) and sealed. After 24 h, fruits were sprayed with 1 cm^3 of 60 nM AsES per fruit. After the three treatments, fruits were stored at 25 °C, 55–60 % RH. The assay was repeated three independent times.

2.4. Respiration rate and ethylene production

Respiration rate and ethylene production were measured daily for the different treatments (water, AsES or 1-MCP + AsES). Fruits were randomly picked and individually placed once a day in 2 L plastic jars, sealed with a rubber stopper, and held at 25 °C for 1 h. Ethylene concentration was measured by taking a 1 cm^3 gas sample from the headspace of jars using a gastight syringe. Then the sample was injected in a AGILENT 6890 N Gas Chromatograph equipped with a 30 m x 0.53 mm alumina column; running conditions: 120/80/240 °C for the injector/column/FID temperatures, respectively, and 0.50 $cm^3 s^{-1}$ carrier gas (N_2) flow rate. CO_2 production was measured by taking a 100 cm^3 gas sample from each bottle and injected in an Oxygen/Carbon Dioxide Headspace Analyzer equipped with an infrared detection cell (Servomex 01514/701 infrared transducer; Servomex PLC, Crowborough, East Sussex, UK). Measurements were repeated every day over 8 days. Ten fruits were evaluated by treatment (n = 10), and the assay was repeated three independent times.

2.5. Fruit quality measurements

Firmness (N) of whole, unpeeled fruits was determined using a penetrometer (IRC Force Gage®, Norfolk, VA, USA.), with a 1 mm diameter plunger tip, at two equidistant points on the equatorial region of each fruit. Soluble solids content (SSC) (%) was determined from avocado juice using a refractometer (Arcano REF103) and three readings were taken for each fruit. Both parameters were determined at 2, 4, 6 and 8 days post treatment (dpt). Fruit weight loss was recorded using a scale with an accuracy of 0.01 g and expressed as the percentage of initial weight. Ten fruits were evaluated by treatment and by time (n = 10), and the assay was repeated three independent times.

2.6. Disease incidence

The influence of AsES on the occurrence of latent natural infection was evaluated. After being treated with water, AsES or 1-MCP + AsES, fruit were placed in trays, covered with plastic film to maintain a high relative humidity (RH: 95%), and incubated at 25 °C. Symptoms were evaluated at 12 dpt by measuring lesion size (cm^2) using the ImageJ Software. Ten fruits were evaluated by treatment (n = 10), and the assay was repeated three independent times.

2.7. Statistical analysis

The statistical analysis was carried out using the software InfoStat 2013 version (http://www.infostat.com.ar). ANOVA analysis was performed to detect significant variances among treatments and was followed by a Fisher test with a 99% confidence level.

3. Results and discussion

The relationship between fruit ripening and ethylene/respiration pattern allows the classification of fruit as climacteric or non-climacteric. In climacteric fruit, ethylene biosynthesis increases and shows a peak that coincides with respiration pattern, while in non-climacteric fruit the ethylene production declines with fruit ripening and senescence (Abeles et al., 1992). In this paper, various physiological parameters were measured to obtain a representative data set describing fruit development, climacteric ripening, and postharvest storage. Immediately after treatment of fruit, ethylene production and respiration rates were measured together with SSC and firmness, two important fruit quality parameters. Typical climacteric changes in respiration rate and ethylene production were observed in both AsES- and water-treated (control) fruit, while a minor change was observed in fruit pre-treated with 1-MCP. The increase in ethylene production occurred three days earlier, and was 49.6% higher, in fruit treated with AsES compared to water-treated, reaching a peak of 726 $pmol.kg^{-1}.s^{-1}$ at 3 dpt (Fig. 1A). Furthermore, AsES treatment enhanced the respiration rate of fruit during the storage being 50.8% higher than the water-treated fruits, reaching a peak of 5.9 $mmol.kg^{-1}.s^{-1}$ at 3 dpt (Fig. 1B). Bower and Cutting (1998) observed an increase of ethylene production and an acceleration of ripening after the application of isopentenyl adenosine. When fruit were pre-treated with 1-MCP and then with AsES, ethylene production and respiration peaks were delayed and less pronounced as compared to fruit treated with AsES alone, with peaks of 120 $pmol.kg^{-1}.s^{-1}$ and 3.6 $mmol.kg^{-1}.s^{-1}$ at 7 dpt respectively (Fig. 1A and 1B). Similar effects have been observed in avocado fruit treated

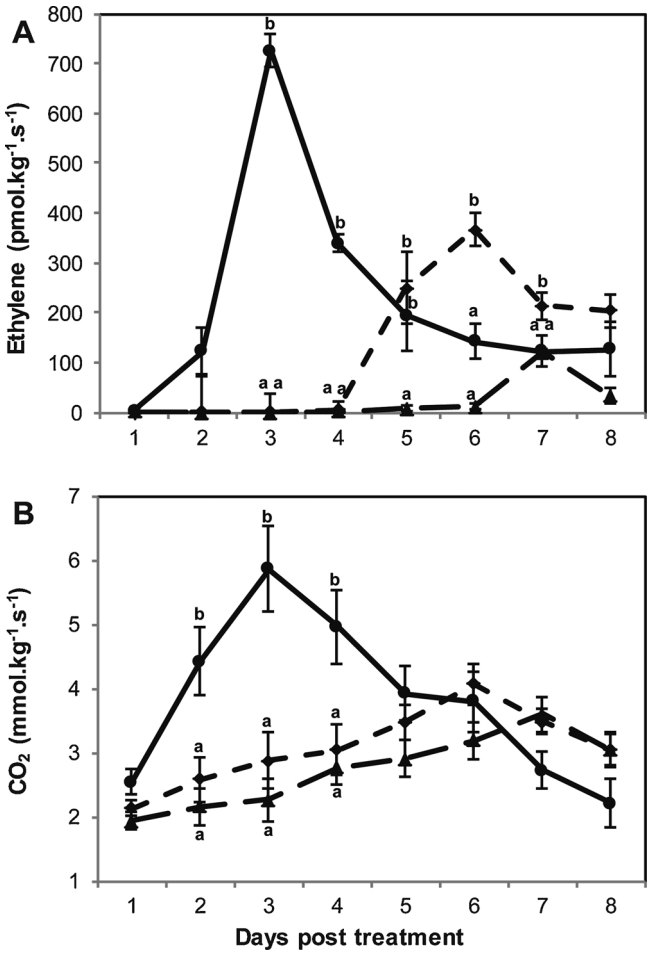

Fig. 1. Effect of AsES on ethylene and CO_2 production of avocado fruit during ripening. Fruits were treated with AsES (●), 1-MCP + AsES (▲), and water (♦). A) in vivo ethylene production (pmol.kg^{-1}.s^{-1}), B) in vivo CO_2 production (mmol.kg^{-1}.s^{-1}). Mean values were obtained from ten independent samples. Vertical bars represent standard error (± SE). Analysis of variance (ANOVA) followed by a Fisher test was performed using InfoStat/L software. Different letters correspond to statistically significant differences among the treatments ($p < 0.05$).

only with 1-MCP; the timing of the peak of ethylene production and the respiratory climacteric were delayed (Feng et al., 2000; Jeong et al., 2003; Hershkovitz et al., 2005). Therefore AsES was able to accelerate and increase the key parameters of ripening.

Weight loss occurs during fruit ripening and is due to the respiration process and the loss of water through fruit peel (Ayranci and Tunc, 2003; Maqbool et al., 2011). Throughout the evaluation, avocado fruit showed a continuous weight loss along the postharvest period (Fig. 2). Fruits treated with AsES showed a significantly greater weight loss being the maximum difference of 10.6% more weight loss at 8 dpt compared to control of fruits treated with water (Fig. 2). The fruit that received the combined treatment (1-MCP + AsES) showed 21.5% less weight loss with respect to the AsES treated fruit at 8 dpt (Fig. 2). The latter agrees with Jeong et al. (2002) who reported that 1-MCP treated fruit have a lower weight loss than untreated fruit.

Firmness also was significantly diminished in response to AsES treatment (Fig. 3A) consistent with the fact that softening is one of the most ethylene-sensitive processes of ripening (Lelievre et al., 1997). AsES treatment accelerated the decline of fruit firmness; an AsES-treated fruit was 15% less firm compared to the water control at 8 dpt. In the case of double treatment (1-MCP + AsES), firmness is much greater at 6 and 8 d with respect to the fruit treated with AsES (Fig. 3A).

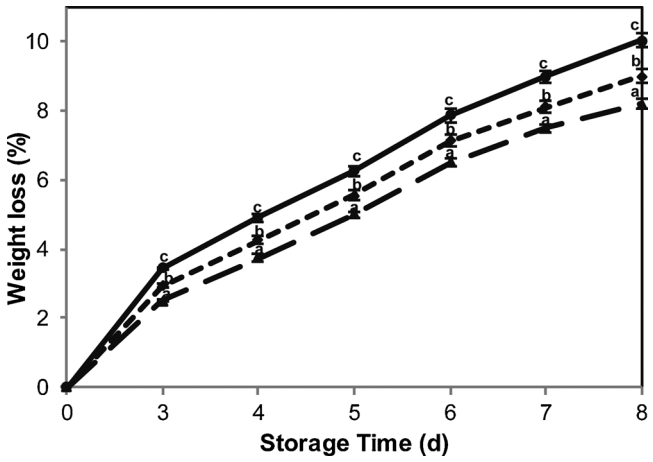

Fig. 2. Effect of AsES on weight loss expressed as a percentage (%) relative to the initial weight of avocado fruit stored at 25 °C. Fruits were treated with AsES (●), 1-MCP + AsES (▲), and water (♦). Mean values were obtained from ten independent samples. Vertical bars represent standard error (± SE). Analysis of variance (ANOVA) followed by a Fisher test was performed using InfoStat/L software. Different letters correspond to statistically significant differences among the treatments ($p < 0.05$).

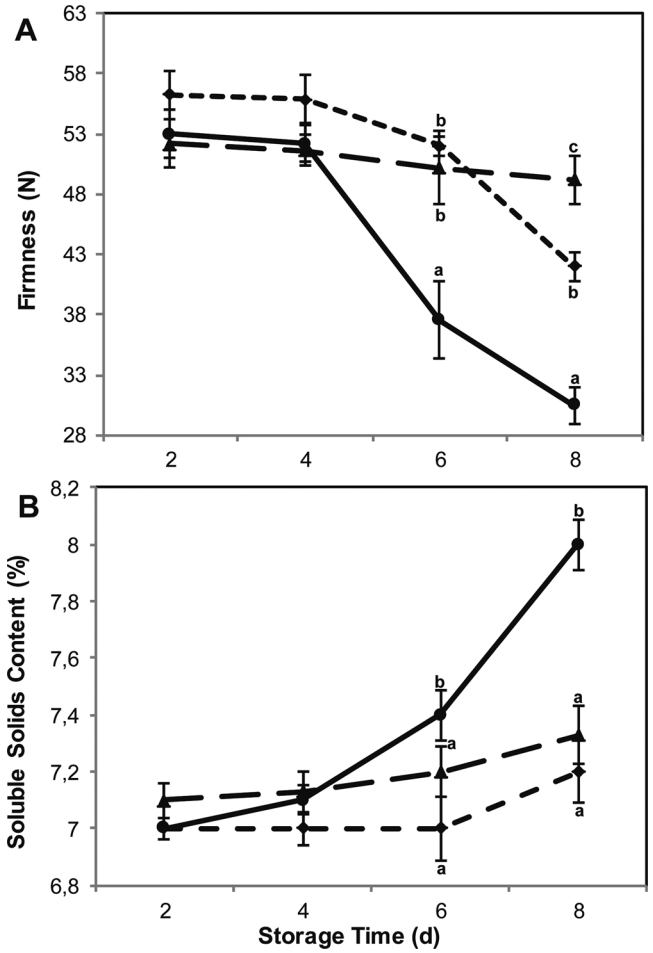

Fig. 3. Effect of AsES on the firmness and soluble solids content during fruit ripening at 2, 4, 6 and 8 dpt. Fruits were treated with AsES (●), 1-MCP + AsES (▲), and water (♦). **A)** firmness (N); **B)** soluble solids content (%). Mean values were obtained from ten independent samples. Vertical bars represent standard error (± SE). Analysis of variance (ANOVA) followed by a Fisher test was performed using InfoStat/L software. Different letters correspond to statistically significant differences among the treatments ($p < 0.05$).

AsES-treated fruit showed higher SSC compared to water controls at 6 and 8 dpt being 5.4 and 10% higher, respectively, while difference observed in the SSC was 8.4% lower when fruit were subjected to double treatment with respect to AsES treated fruit (Fig. 3B). Similar effects of 1-MCP in attenuating fruit softening have been observed for 'Hass' avocado, apricot, and apple fruit (Feng et al., 2000; Fan and Mattheis, 1999; Rupasinghe et al., 2000). The softening delay observed when using 1-MCP confirms that ethylene is involved in the softening-related metabolism.

The effect of ethylene on ripening can be explained by the fact that ethylene affects the expression of 1-aminocyclopropane carboxylicacid oxidase (ACO) and 1-aminocyclopropane carboxylicacid synthase (ACS) genes, and a fruit-specific polygalacturonase, involved in the depolymerization of cell wall pectin during ripening (Awad and Young, 1979).

AsES protein has been reported to provide strawberry plants protection against the hemi-biotrophic pathogen *C. acutatum* and in *Arabidopsis thaliana* plants against *B. cinerea* (Chalfoun et al., 2013; Hael-Conrad et al., 2015). In this work, we evaluated the influence of AsES on the spontaneous-natural infection occurring in avocado fruits.

Results showed that after 6 days under 25 °C and 95% RH fruits treated with water exhibited an incipientrot of dark brown color at the insertion of the peduncle that gradually progressed (not shown). After 12 days the dark area increased around the scar and a white mycelial growth covering scar was observed, while the avocado pulp became softer and browner with clear symptoms of fruit decay as shown in Fig. 4A. These processes of fruit decay are characteristic symptoms produced by the pathogenic fungus *Lasiodiplodia theobromae* causal agent of peduncular rot disease (Nishijima, 1994). But it can also indicate the participation of opportunistic fungal pathogens such as *Colletotrichum gloeosporioides* that is the causal agent of anthracnose (Binyamini and Schiffmann-Nadel, 1972). Anyhow, both pathogens correspond to postharvest disease agents. It is well known that latent infections cause postharvest diseases, and is due to an inhibitory effect exerted on the pathogen growth by the host, due to the particular physiological conditions imposed until ripening. At that stage changes in the surface of the fruit take place, providing adequate conditions for the pathogen growth and infestation (Abd-AllA and Wafaa, 2010). However, when the fruits were treated with AsES no symptoms were observed after 12 days (Fig. 4B), whereas fruits previously treated with the ethylene inhibitor 1-MCP, this protective effect was not observed (Fig. 4C). 12 days after the harvest, AsES-treated fruits showed a 94 and 82% of reduction of the lesion size compared to water and 1-MCP + AsES treated fruit, respectively (Fig. 4D). These results indicated that AsES exerts a protective effect against native spontaneous microbiota in the non-model avocado fruit. The latter agrees with the results reported by Hael-Conrad et al., (2015) in strawberry fruit challenged with *B. cinerea*. Hael Conrad et al. (2015) by using knockout mutants of *A. thaliana,* observed that AsES triggers defense responses through the three main signaling pathways (e.g. salicylic acid, jasmonic acid and ethylene). Consistently, ethylene production was enhanced when fruits were treated with AsES at early stages of avocado ripening (Fig. 1A), and this effect was suppressed when fruits were pretreated with 1-MCP, clearly indicating that AsES accelerates the fruit ripening by activating the ethylene synthesis.

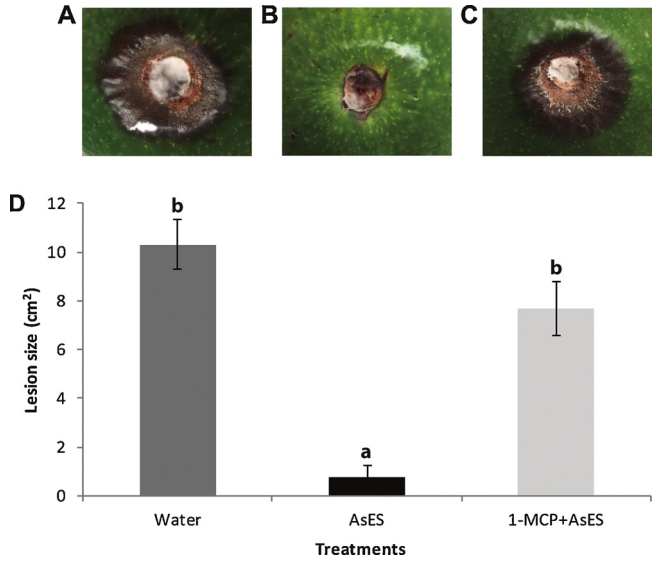

Fig. 4. Effect of AsES in avocado fruit latent natural infection. Fruits were treated with Water or 1-MCP + AsES. Lesion size was measured 12 days postharvest. **A)** Sick peduncle scar of fruits treated with water; **B)** peduncle scar of fruits treated with AsES (60 nM); **C)** peduncle scar of fruits pre-treated with 1-MCP and then with AsES. **D)** quantification of the injury. Mean values were obtained from ten independent samples. Vertical bars represent standard error (± SE). Analysis of variance (ANOVA) followed by a Fisher test was performed using InfoStat/L software. Different letters correspond to statistically significant differences among the treatments ($p < 0.01$).

Adkins et al. (2005) reported that avocados fruits treated with 1-MCP extended the marketing period suggesting that either the fruits were exposed to a low charge of inoculum or the fruit variety used was more resistant to the pathogens.

Results obtained suggest that the physiological and biochemical changes as well as the increased resistance to natural infection of fruit treated with AsES, may be attributed to the increase in biosynthesis of ethylene. Ravi et al. (2007) observed a similar effect in banana fruit. They reported that ethylene-induced ripening is accompanied by the upregulation of genes associated to stress, defense, detoxification, ethylene biosynthesis, and cell wall hydrolysis. On the other hand, the induction of ethylene production and the expression of genes involved with their synthesisin interactions with citrus fruits and *Penicillium digitatum* were reported (Marcos et al., 2005).

Similarly, increased ethylene levels in oranges heat-treatedand inoculated with *P. digitatum* were observed (Ballester et al., 2011; González-Candelas et al., 2010). However, the role of ethylene in defense responses is complex, and strongly depends on the type of interaction (Berrocal-Lobo et al., 2002). However, more biochemical and functional genetic studies are needed to better elucidate the resistance observed.

4. Conclusions

This is the first report of the influence of the defense elicitor AsES on the physiological and biochemical changes during ripening in avocado fruit.

Our results show that AsES activates the synthesis of ethylene, which in turns induces ripening and defense process in avocado fruit. The use of AsES in postharvest fruit may provide some potential benefits. AsES is a natural product that can be used in aqueous solutions, and is easy and safe to handle as compared to the gaseous form of ethylene. Furthermore, the use of this protein represents a relatively simple way of managing not only the ripening of fruit, but also other plant processes regulated by ethylene, such as seed germination. Also, this could be useful in postharvest practices due the growing consumer concerns regarding food security and demand for fruit produced organically. In this way we provide an important resource for future studies on the subject

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