Rhizogenesis in *in vitro* shoot cultures of passion fruit (*Passiflora edulis* f. *flavicarpa* Deg.) is affected by ethylene precursor and by inhibitors

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Abbreviations: $AgNO_3$ = silver nitrate, ACC= 1-aminocyclopropane-1-carboxylic acid, AVG= aminoethoxyvinylglycine, $CoCl_2$= cobalt chloride, IAA= indole-3-acetic acid, IBA= indole-3-butyric acid, S.E.= standard error of the mean, STS= silver thiosulphate

Summary: The effects of the ethylene precursor ACC and two inhibitors, $AgNO_3$ and AVG, on root formation were tested in *in vitro* shoots of passion fruit (*Passiflora edulis* f. *flavicarpa* Deg.). The organogenic response was assessed on the basis of percentage of shoot-forming roots, root number and length. The time course of ethylene production was also monitored. ACC inhibited root formation by delaying root emergence and increasing callus formation at the basis of the shoots. In addition, ACC caused a marked increase in ethylene production, coupled to leaf chlorosis and senescence with lower rooting frequencies, number and length of roots. IAA supplementation increased ethylene production. Both ethylene inhibitors, $AgNO_3$ and AVG, at appropriate concentrations reduced callus formation at the basis of shoots. AVG increased the number of roots per shoot, but drastically reduced length of differentiated roots. Regarding to leaf pigments, ACC promoted a marked reduction on carotenoids and total chlorophyll, whereas AVG and $AgNO_3$ delayed explant senescence and pigments degradation, not differing from IAA supplemented and non-supplemented control treatments. The results confirm previous reports on the beneficial effects of ethylene inhibitors on *in vitro* rooting and suggest its reliability to be used as an alternative approach to evaluate sensitivity of *Passiflora* species to ethylene.

Introduction

The plant hormone ethylene affects several aspects of growth and development notably on the ripening of climacteric fruits, inhibition of root, senescence of leaves, and stem elongation. Ethylene plays a role in several developmental processes such as meristemoid formation, bud ontogenesis, xylogenesis, callus proliferation, somatic embryogenesis, rhizogenesis, flowering *in vitro*, and shoot or plantlet morphology (*Biondi* et al. 1998, *Matthys* et al. 1995). As stated by *Kumar* et al (1998) it is clear that a precise definitive statement concerning the role of ethylene in organized growth and development *in vitro* cannot be made, as for all the processes examined, ethylene has been shown either to enhance or inhibit or to be without effect, sometimes even in the same species. For instance, positive effects of ethylene supplementation were reported in *Lilium speciosum* (*Van Aatrijk* et al. 1985), *Triticum aestivum* and *Nicotiana plumbaginifolia* (*Purnhauser* et al. 1987), *Picea abies* (*Bollmark* & *Eliasson* 1990), *Prunus persica* x *P. amygdalus* (*Dimasi-Theriou* et al. 1995), and *Malus* x *domestica* (*Harbage* and *Stimart* 1996). Conversely, negative effects were observed with *Lycopersicon esculentum* (*Coleman* et al. 1980), *Prunus avium* (*Biondi* et al. 1990), *Vitis vinifera* (*Soulie* et al. 1993), *Albizzia julibrissin* (*Sankhla* et al. 1995), *Carica papaya* (*Magdalita* et al. 1997), *Glycine max* (*Santos* et al. 1997), *Nicotiana tabacum* (*Biondi* et al. 1998), *Malus* x *domestica* (*Ma* et al. 1998), among others.

Passiflora is a large genus of perennial climbing species and some of them of edible fruit. *Passiflora edulis* f. *flavicarpa*, the yellow passion fruit, is thought to have originated from tropical areas of Brazil and is one of the

most economically important species of the Passifloraceae family. The fruit has become a popular source of juice and a good source of vitamins A and C (*Arjona & Matta* 1991, *Taylor* 1993). However, the availability of fresh fruit is limited by irregular supply and short postharvest shelf-life (*Pocasangre-Enamorado* et al. 1995). As a climacteric fruit, *Passiflora* spp. shows high rates of ethylene production (*Ludford* 1995; *Pocasangre-Enamorado* et al. 1995; *Shiomi* et al. 1996), which may limit the *in vitro* morphogenic potential of the explanted cultures. The accumulation of ethylene in excessive amounts in tightly enclosed tissue culture vessels is often difficult to control. Therefore, the role of ethylene in plant tissue culture has often been studied by adding the ethylene precursor ACC, and various ethylene inhibitors, such as $AgNO_3$, AVG, STS and $CoCl_2$, to the tissue culture media (*Matthys* et al. 1995). To date, as far as we know, the only report on the effects of ethylene affecting *in vitro* morphogenesis of passion fruit was the one by *Faria & Segura* (1997). The addition of silver thiosulphate (STS) to the culture medium significantly increased the differentiation and development of adventitious shoots from hypocotyl and leaf explants of *Passiflora edulis* f. *flavicarpa;* no ethylene quantification along the culture period was made however.

In order to evaluate the effect of this plant growth regulator upon the senescence and rhizogenesis of passion fruit shoot cultures *in vitro*, inhibitors of ethylene biosynthesis (AVG) or ethylene action ($AgNO_3$) and its precursor (ACC) were added to the culture medium.

Material and methods

Plant material and tissue culture

Greenhouse grown seedlings (14–18 d after germination) were used as the source of explants. Surface-sterilization was performed by immersion of the shoots in 70% (v/v) ethanol for 1 min, followed by 15 min in a 2.5% (v/v) sodium hypochlorite solution containing 1% (v/v) Tween 80, followed by four rinses in sterile distilled water. Shoots (10 mm long) were transferred to a MS-based medium (*Murashige & Skoog* 1962), with 2% (w/v) sucrose, solidified with 0.3% (w/v) Phytagel (Sigma), lacking growth regulators. The pH was adjusted to 5.8 prior to adding Phytagel. Established shoot cultures (30–40 d-old) were used as source of shoot tips to perform the experiments. Defoliated shoot tips (1–1.5 cm) were placed individually in test tubes (25 x 150 mm) containing 10 ml of rooting medium supplemented with 0.88 mg l^{-1} IAA, as described by *Drew* (1991). Cultures were maintained under 16/8 h light/dark regime, 24 mol m^{-2} s^{-1} light radiation, and temperature of the culture room at 26 2 C. *In vitro* rhizogenic ability of the passion fruit shoot cultures was assessed onto MS medium supplemented with several levels (0, 1, 3, 10 and 30 µM) of AVG, ACC or $AgNO_3$ The following characteristics were evaluated: rooting frequency (%), number and length of roots, leaf chlorophyll and carotenoid pigments, at the end of 30 days in culture. Ten replicates were used per treatments. To investigate further the time course of ethylene production, as influenced by ACC, AVG or $AgNO_3$ (0, 3, and 30 µM), a second experiment was performed. AVG, ACC and $AgNO_3$, all experiments, were dissolved in milli-Q water, filter-sterilized with Millipore filters (0.22 µm; 2.5 cm diameter; Millex), and added to the medium following autoclaving (1.2 kg cm^{-2} at 121 °C; 15 min) and cooling. Eighteen replicates were used for each treatment, and three samples were analyzed every six days, over 30 days of culture.

Gas evolution

Ethylene evolution was assayed by a gas chromatograph Shimadzu – CG 14B equipped with a flame ionization detector and an 1.0 cm Poropak-Q (80–100 mesh) column. Test tubes (150 x 25 mm) were sealed with air-tight rubber caps for 30 min. One-milliliter gas samples were withdrawn with a hypodermic syringe with a needle (29G 1/2 inches, B-D, Becten Dickinson, EUA) from the headspace and injected onto the gas chromatograph injector. The carrier gas was nitrogen with a flow rate 30 ml s^{-1}. The temperature was set at 60 °C for the column, at 75 °C for the injector and at 135 °C for the flame ionization detector. At different times in culture (6, 12, 18, 24, and 30 days) ethylene was measured. Ethylene concentration was expressed as nl C_2H_4 g^{-1} FW h^{-1} based on shell vial volume (0.024 l), plantlet fresh weight (g), and incubation time (0.5 h). Every 6 days three replicates per treatment were taken for analysis of ethylene evolution.

Determination of leaf pigment contents

Leaf pigments were extracted by homogenizing 2g of fresh tissue with 80% acetone, the extract filtered through filter paper Whatman 1 and total chlorophyll and carotenoid contents estimated as described by *Lichtenthaler* (1987). Absorbances were determined at 451 and 503 nm for carotenoids, and 647 and 664.5 nm for chlorophyll. Pigments were expressed as milligrams per gram of fresh tissue (mg g^{-1} FW).

Statistical analysis

Analyses were performed using a completely randomized design. Data were subjected to analysis of variance using the Statistical and Genetics Analysis System (SAEG) and the appropriate means were compared by Tukey's test (P=0.05).

Results and discussion

In vitro rooting of shoot cultures as affected by AVG, $AgNO_3$ and ACC

At all concentrations tested, ACC inhibited rooting clearly and affected shoot morphology; leaves curled

downwards and early signs of senescence were visible as shown by pigment contents (*Figure 1*, *Figures 2K-N*, *Figure 3*). The addition of ACC to the rooting medium led to prolific callusing (*Figures 2K-N*) at the base of the shoots, as also observed in the IAA-supplemented control (*Figure 2B*). Rooting frequency was negatively influenced by ACC (*Figure 1A*) and the mean root length was diminished (*Figure1C*) as compared to IAA-free and IAA-supplemented control treatments. Thus, the negative effects of ACC on rooting should be attributed to a strong chlorosis and ethylene-induced senescence after 10 days in culture. Similarly, ACC induced callusing from root explants in *Albizzia julibrissin* (*Sankhla* et al. 1995) and at the base of shoot cultures in *Malus* x *domestica* (*Ma* et al. 1998). As for AVG and $AgNO_3$ treatments, shoots appeared healthy (*Figures 2C-F*, *Figures 2G-J*) and the frequency of rooted cultures was less affected by AVG as compared to $AgNO_3$. Nonetheless, at the end of the experiment, mean rooting percentage in AVG and $AgNO_3$-treated cultures, in general, remained below that of the control (*Figure 1A*). Interestingly, the mean number of roots tended to increase with increasing AVG concentrations (*Figure 1B*, *Figures 2C-F*), a phenomenon accompanied by a drastic decrease of root length. Indeed, AVG 30 M induced the differentiation of several short root primordia, most of them barely emerged (*Figure 2F*). $AgNO_3$ (1, 3, and 10 µM) caused reduction of rooting frequency. Conversely, $AgNO_3$ (30 M) promoted mean rooting frequency about 80% (*Figure 1A*), and a mean number of roots comparable to control treatments (*Figure 1B*). A marked delay on root emergence was observed that occurred by the 15-18th day, accompanied by the appearance of browning of the medium probably as a consequence of silver degradation by light (*Figure 2J*). Similarly to AVG effects, $AgNO_3$-treated cultures also caused a shortening in root length, especially when compared to the auxin-free control (*Figure 1C*). Several studies revealed that sometimes morphogenesis *in vitro* is stimulated by the inhibition of ethylene biosynthesis, and in other cases, by its synthesis (*Biondi* et al. 1998). Major role in rooting of cuttings has been assigned to ethylene repeatedly and the finding that auxins can induce ethylene biosynthesis in most plant tissues has led to the suggestion that auxin-stimulated rooting may be mediated by ethylene; but experimental results, however, have been contradictory (*Kevers* et al. 1997). It promoted root formation in poplar shoots produced *in vitro* (*Hausman* 1993) and in hypocotyl cultures of *Digitalis obscura* in the absence of auxin and cytokinin (*Pérez-Bermúdez* et al. 1985). However, the presence of auxin was essential for the promotive effect of ethylene on root induction in hazelnut cotyledons (*Gonzalez* et al. 1991). Interestingly, *Harbage* & *Stimart* (1996) and *Ma* et al. (1998) presented conflicting results on the role of ethylene in relation to *in vitro* rooting of apple microcuttings. The former suggested that ethylene was not associated with rooting of apple microcuttings. However, the latter argued in favor that ACC inhibited root formation at the base of the shoots, whereas both AVG and $AgNO_3$ stimulated root emergence and enhanced root growth. As pointed out by *Ma* et al. (1998), apart of increasing root number per shoot, a negative effect of AVG was to reduce growth rate and delay root emergency at concentrations between 3 and 30 M. *De Klerk* et al. (1999) pointed out that contradictory results in literature about the role of ethylene may be partly explained by different culture conditions resulting in different levels of trapped ethylene, and due to differences in ethylene sensitivity among rooting

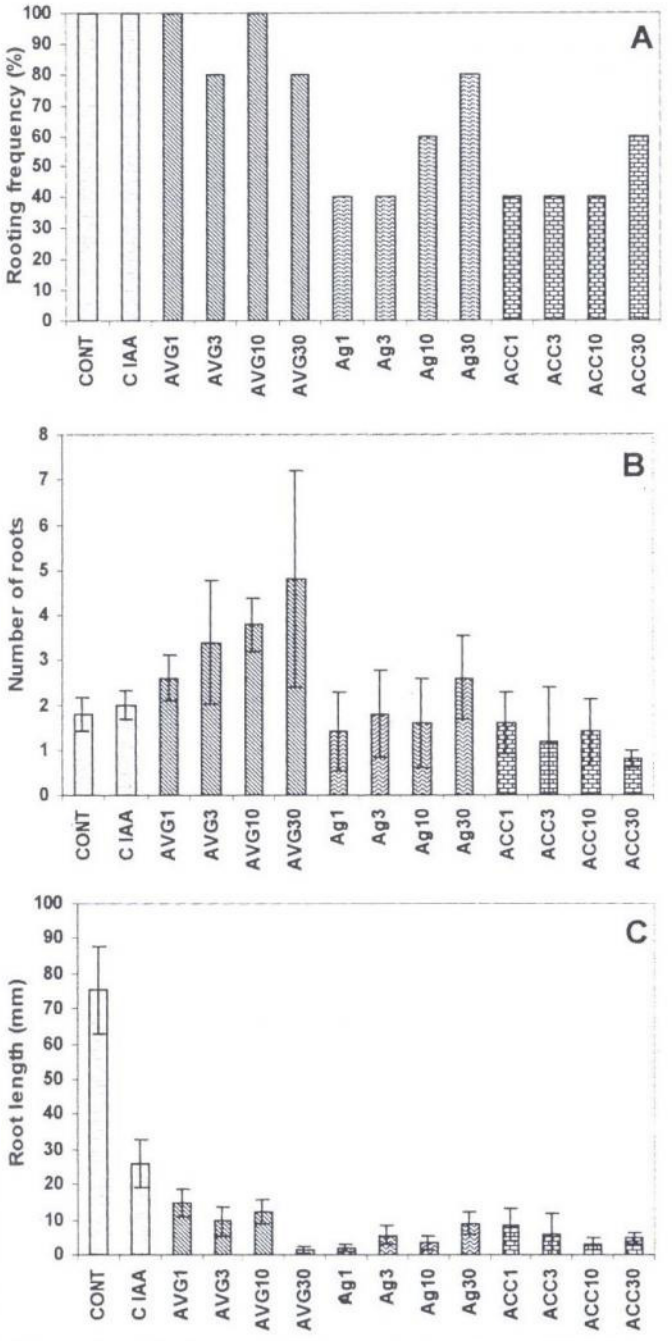

Figure 1 Rooting frequency (**A**) mean number of roots (**B**) and root length (**C**) in *in vitro* grown yellow passion fruit plants, after 30 days in culture onto MS medium supplemented with 1, 3, 10, and 30 µM of AVG, $AgNO_3$ or ACC (Cont = IAA-free control; C IAA = control plus 0.88 mg l^{-1} IAA). Vertical bars correspond to the mean ± S.E.

Figure 2 Detail of *in vitro* grown passion fruit plants cultured onto MS medium supplemented with 1, 3, 10, and 30 μM AVG, $AgNO_3$ or ACC. **A.** IAA-free control; **B.** Control with IAA; **C–F:** 1, 3, 10, 30 μM AVG; **G–J:** 1, 3, 10, and 30 μM $AgNO_3$; **K-N:** 1, 3, 10, and 30 μM ACC (Bar = 10 mm)

phases. Ethylene was promotive during the initial days of the rooting treatment at a low auxin concentration. At high concentration of auxin, apple microcuttings produced high levels of ethylene which resulted in leaf senescence and poor growth of shoot and roots. When STS was given together with auxin, the microcuttings had an excellent appearance: the leaves do not show signs of senescence and the shoots grew well during rooting treatment (*De Klerk* et al. 1999).

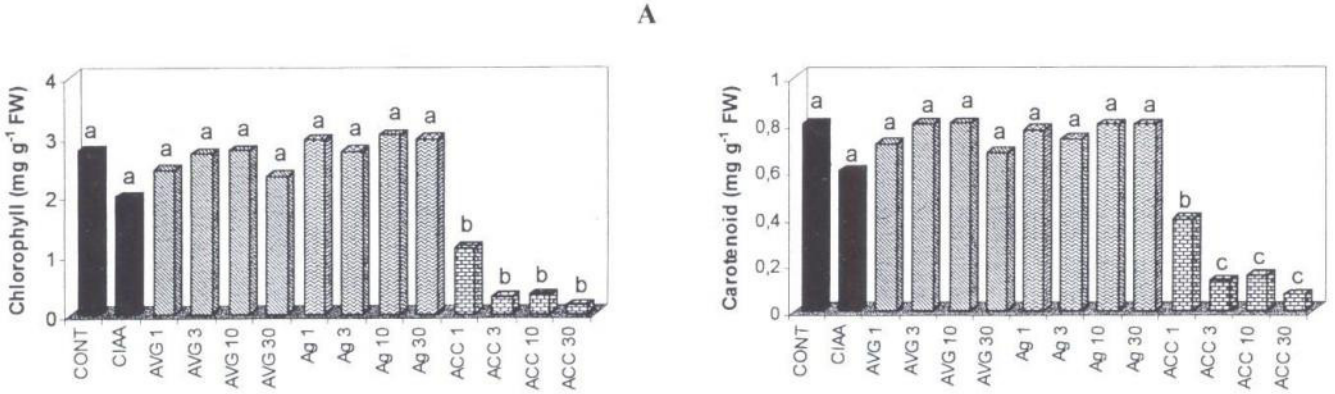

Figure 3 Total chlorophyll (**A**) and carotenoid (**B**) contents (mg g^{-1} FW) in leaves of *in vitro* grown passion fruit plants cultured onto MS medium with 1, 3, 10, and 30 μM AVG, $AgNO_3$ or ACC. The treatments CONT and CIAA correspond to MS medium supplemented and non-supplemented with IAA, respectively. Means followed by the same letter are not statistically significant by Tukey test ($P < 0.05$)

Ethylene synthesis measurements

Ethylene evolution (nl g^{-1} h^{-1}) time course is shown in *Figure 4*. Evolution of ethylene was characterized by a rise at the 18th day, except for the auxin-free control, in which ethylene levels remained basal, with lower values compared with the IAA-supplemented control (*Figures 4A, B, C*). These data are in accordance with several reports showing auxin as an inducer of ethylene synthesis (*Coleman* et al. 1980; *Gonzalez* et al. 1991). Interestingly for the IAA-supplemented control a discrete ethylene peak was observed comparable to that induced by AVG at 30µM. It could be noted that ethylene production showed slight variations as a consequence of ACC addition at 3µM to the culture medium (*Figure 4C*), but always showing higher values compared to the controls supplemented and non-supplemented with IAA. It is worth noting that ACC at 30 M, induced an increase with time and compared with controls in ethylene evolution rate up to the 18th day, thereafter a decrease due to plant senescence occurred, leading to higher rates of ethylene production. It is suggested that stimulation of the biosynthesis of ACC synthase as an effect of auxin treatment, which usually is present at low levels in the plant tissue, increases the ethylene production in plant tissues. Our data confirm that, most likely, ACC supplementation promoted higher substrate availability for ACC oxidase, resulting in elevation of ethylene production by passion fruit shoot cultures. Recently, *Mita* et al. (1998) reported that increases in the enzyme activity of ACC synthase and ACC oxidase induced the ethylene evolution during ripening of the purple passion fruit. The pattern of expression of two ACC synthase genes (PE-ACS1 and PE-ACS2), and one ACC oxidase gene (PE-ACO1), and two ethylene receptor genes (PE-RTR1 and PE-ERS1) revealed gene expression of these genes is differentially regulated.

Regarding to AVG and $AgNO_3$ it a decrease in ethylene production was observed up to the 12th day followed by a marked increase by the 18th day (*Figures 4A-B*). Shoot cultures treated with 3 M AVG or $AgNO_3$ presented higher ethylene production as compared to 30 M, with slight differences up to the 24th day. After this the ethylene production increased in cultures on both levels of $AgNO_3$. This can be explained by the photodegradation of silver which likely reduced its inhibitory effect upon ethylene action. In addition, this rise may be associated with a higher root differentiation in these cultures leading to an increment of plant metabolism. Ethylene inhibitors, AVG (*Figures 5C–D*) and $AgNO_3$ (*Figures 5E-F*), and its precursor, ACC (*Figures 5G-H*) differentially affected rhizogenic responses in shoot explants of passion fruit. The course of differentiation and expansion in adventitious roots is marked by high mitotic activity and enhanced cellular metabolism during this morphogenic process. Indeed, as pointed out by Huxter et al. (1981), much of the ethylene produced in culture is likely a consequence of wound metabolism at culture initiation and as a byproduct of growth-associated metabolism, and as being a gas, ethylene readily diffuses out from its site of production.

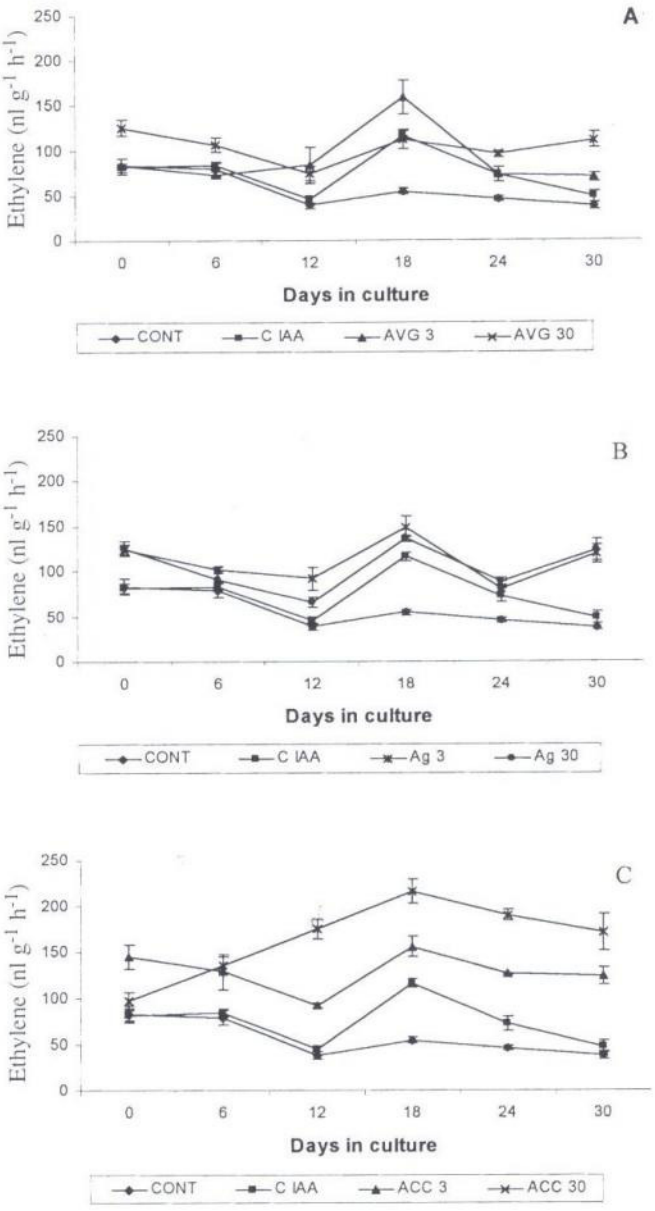

Figure 4 Course of ethylene evolution (nl g^{-1} h^{-1}) by *in vitro* grown passion fruit plants cultured onto MS medium supplemented with by 3 and 30 µM AVG **(A)**, $AgNO_3$ **(B)** and ACC **(C)**, respectively. The treatments CONT and CIAA correspond to MS medium supplemented and non-supplemented with IAA, respectively. (Vertical bars correspond to the mean ± S.E.)

Leaf pigments

In the first experiment ACC (all concentrations) applied to the medium led to significant reduction on total chlorophyll (*Figure 2A*) and carotenoid (*Figure 2B*) contents compared to AVG, $AgNO_3$, and both IAA-supplemented and non-supplemented media. This difference could be attributed to the chlorosis and senescence observed in all ACC-supplemented media (*Figures 3K–N, Figures 5G–H*). After 12–15 days in culture it was possible to detect chlorosis under 10 and 30 M ACC. On the other hand, there

Figure 5 Shoots of yellow passion fruit cultured onto a MS-medium supplemented with AVG, $AgNO_3$ or ACC. **A**: IAA-free control; **B**: control plus 0.88 mg l^{-1} IAA); **C**: AVG (3 µM); **D**: AVG (30 µM) ; **E**: $AgNO_3$ (3 M); **F**: $AgNO_3$ (30 M); **G**: ACC (3 µM); **H**. ACC (30 µM) (Bar = 10 mm)

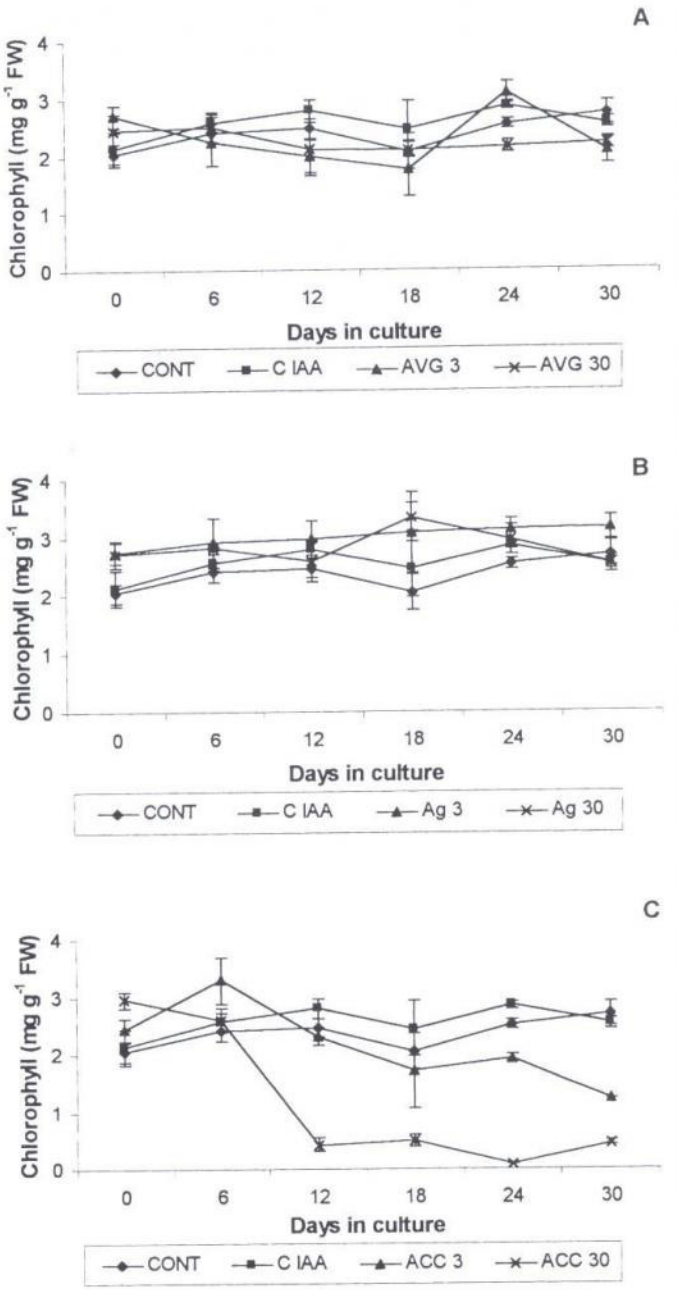

Figure 6 Total chlorophyll contents (mg g^{-1} FW) from *in vitro* grown passion fruit derived plants cultured onto MS medium, as affected by 3 and 30 µM AVG (**A**), $AgNO_3$ (**B**) and ACC (**C**), over a period of 30 days in culture. The treatments CONT and CIAA correspond to MS medium supplemented and non-supplemented with IAA, respectively. (Vertical bars correspond to the mean ± S.E.)

was no significant difference in respect to pigment degradation among AVG, $AgNO_3$, and both IAA-supplemented and non-supplemented media (*Figures 2A–B*). Likewise, the experiments involving the time course of ethylene evolution showed differential pigment degradation as affected by AVG, ACC and $AgNO_3$ (*Figure 6, Figure. 7*). ACC-supplemented treatments led to higher pigment degradation as compared to the other treatments. ACC 30 µM drastically reduced chlorophyll and carotenoid contents, coupled chlorosis symptoms and precocious leaf senescence after the 6th day. It can be observed that leaf total chlorophyll and carotenoid contents remained almost constant when the ethylene inhibitors AVG and $AgNO_3$ were added to the medium (*Figure 6, Figure 7*). This tendency reflects similar behavior in comparison to auxin-free and auxin-supplemented treatments. Therefore, some variations, i.e. AVG at 3 µM (*Figure 6A, Figure 7A*) and $AgNO_3$ at 30 µM (*Figure 6B, Figure 7B*), observed on the curves led an ethylene drop at the 18th day. This might reflect the low number of samples analyzed (n=3), or the variation of the natural senescence process. The supplementation of the culture medium with ACC is a procedure adopted by various authors to evaluate possible effects on *in vitro* morphogenesis for several species (*Biondi* et al. 1990, *Biondi* et al. 1998, *Bollmark & Eliasson* 1990, *Harbage & Stimart* 1996, *Huxter* et al. 1981, *Lakshmanan* et al. 1997, *Ma* et al. 1998, *Magdalita* et al. 1997, *Sankhla* et al. 1995). However there are no available informations on the effects of ethylene in respect to pigments quantification

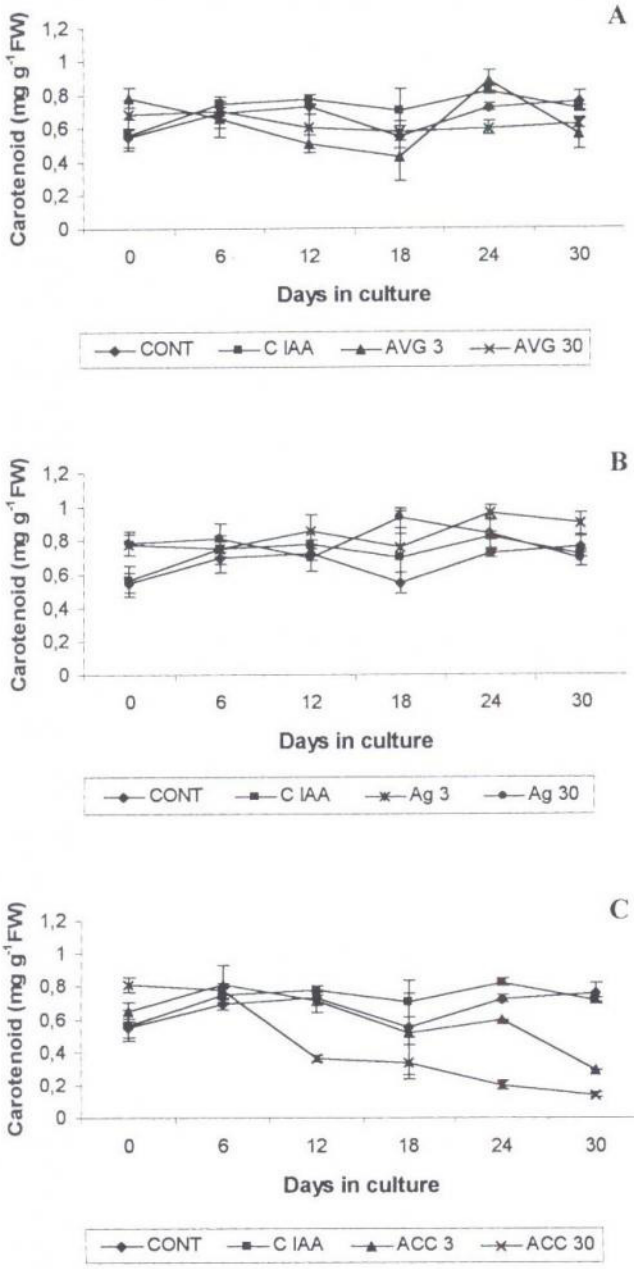

Figure 7 Carotenoid contents (mg g^{-1} FW) from leaves derived from *in vitro* grown passion fruit plants cultured onto MS medium, as affected by 3 and 30 µM AVG (**A**), $AgNO_3$ (**B**) and ACC (**C**), over a period of 30 days. The treatments CONT and CIAA correspond to MS medium supplemented and non-supplemented with IAA, respectively. (Vertical bars correspond to the mean ± S.E.)

throughout the *in vitro* culture, making difficult any confrontation of the present data. In the process of pigment degradation thylacoid membrane degeneration follows a fairly ordered process, although what initiates the degradation is unknown. Ethylene has been implicated in senescence and may be the initial trigger of membrane degradation (*Heaton* & *Marangoni* 1996, *Matile* et al. 1996). The only report on the effect of ethylene on passion fruit *in vitro* morphogenesis was made by *Faria* & *Segura* (1997). STS did not affect the percentage of hypocotyls producing buds; in contrast the compound significantly increased the frequency of buds from leaf explants when added to a medium supplemented with 8.8 M BA and 2.7 M IAA. Furthermore, STS also delayed senescence of the primary explants, especially of the leaf-derived ones. However, the authors did not quantify ethylene evolution or pigment degradation throughout culture (*Faria* & *Segura* 1997).

Our results taken together reinforce the high sensitivity of passion fruit tissues to ethylene and that its accumulation may limit *in vitro* morphogenesis of this species by inducing the senescence of the cultures. Further works need to be undertaken in order to extend this system for evaluating morphogenesis in other explants and *Passiflora* species.

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