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ORIGINAL ARTICLE

Effects of ethylene on shoot initiation, leaf yellowing, and shoot tip necrosis in roses

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Abstract This study was conducted to investigate the in vitro influence of ethylene on shoot branching and leaf yellowing in the rose cultivar Tineke by using different compounds that regulate ethylene inhibition and stimulation. Aminoethoxy vinyl glycine (AVG), silver thiosulfate (STS), and sodium nitroprusside (SNP) caused enhanced apical shoot initiation and reduced leaf yellowing, via inhibition of ethylene production, in the following order: AVG > SNP > STS. In contrast, the addition of 1-aminocyclopropane-1-carboxylic acid (ACC) or 3-indoleacetic acid (IAA) stimulated ethylene production and had greater negative effects on the studied parameters than the control; the negative effects of IAA were further confirmed in combination with AVG, STS, or SNP. The effects of ethylene on apical shoot initiation and leaf yellowing in Tineke were confirmed in another rose cultivar, Innocence. Hence, this study provides strong support for the hypothesis that ethylene-inhibiting agents have beneficial effects on apical shoot initiation and reduction of leaf yellowing in other rose cultivars.

Keywords Ethylene · Ornamental plant · Plant growth regulators · Shoot initiation

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Introduction

For several decades, roses have been considered as commercially important cut flowers that are grown worldwide. The development of new rose cultivars has recently increased substantially on a yearly basis. The main breeding techniques utilized for roses include mutation breeding and genetic transformation; however, successful in vitro shoot regeneration is a prerequisite for both these techniques. Direct or indirect shoot regeneration from various explants has been studied in a variety of roses (Hsia and Korban 1996; Rosu et al. 1995; Schum et al. 2001; Pati et al. 2004; Tian et al. 2008). Direct shoot regeneration from explants, without the callus induction phase, is a desirable breeding approach because it increases rapidity and reduces the costs of regeneration.

Ethylene concentration plays an important role in shoot regeneration during the in vitro regeneration of roses (reviewed by Pati et al. 2006). Kevers et al. (1992) suggested that pulse treatments with low concentrations of ethylene enhanced shoot proliferation in roses, while Horn (1992) reported that high concentrations of ethylene markedly increased senescence and shoot tip necrosis while reducing shoot growth.

Ethylene biosynthesis inhibitors, such as aminoethoxyvinylglycine (AVG) and silver nitrate ($AgNO_3$), have been shown to enhance multiplication rates by inducing the number of axillary shoots (Kevers et al. 1992; Dubois and de Vries 1995; Gaspar et al. 1989); $AgNO_3$ accelerated the regeneration response by at least a week via the inhibition of ethylene biosynthesis. In addition, Mekers et al. (1984) reported that AVG significantly reduced the leaf senescence of roses in vitro. Recently, positive effects of the ethylene inhibitors on shoot regeneration and genetic transformation have been also reported in *Prunus avium* (L.) cv Stella (Sgamma et al.

2015). Reid et al. (1980) reported that immersion of rose flower stems in silver thiosulfate (STS) solution doubled the vase life (from 5 to 10 days) by inhibiting ethylene; however, its effect on in vitro shoot responses is yet to be examined. Xu et al. (2009) demonstrated that sodium nitroprusside (SNP) promoted callus induction and shoot regeneration in micropropagated *Dioscorea opposita*. In addition, Zhu and Zhou (2007) have shown that SNP could extend the storage period of strawberries by inhibiting ethylene production. However, the effects of SNP on in vitro responses via ethylene inhibition have not been well characterized in ornamental plants.

Our preliminary studies have demonstrated leaf senescence in in vitro shoots of the rose cultivar Tineke leading to shoot growth inhibition. Bogaert et al. (2006) reported similar findings in roses and suggested that this is caused by high accumulation of ethylene in in vitro cultures. Thus, we were interested to evaluate whether the compounds AVG, STS, and SNP, which have been shown to inhibit ethylene production and promote in vitro responses, could promote in vitro shoot initiation and reduce the number of yellowing leaves per shoot in the cultivar Tineke. Previous studies have focused mainly on the role of ethylene biosynthesis inhibitors in the vase life of roses as well as leaf senescence and in vitro rose shoot-tip necrosis, without investigating the effect of ethylene concentration on these phenomena in in vitro rose shoots.

In this study, we analyzed the effect of different ethylene-inhibiting compounds on ethylene concentration as well as leaf senescence and apical shoot initiation. In addition, we further examined whether in vitro responses were associated with culture ethylene concentrations by combining ethylene-inhibiting compounds or the ethylene promoter 1-aminocyclopropane-1-carboxylic acid (ACC) with 3-indole acetic acid (IAA).

Materials and methods

Plant material

In this study, 6-week-old in vitro shoots of *Rosa hybrida* 'Tineke', obtained by shoot tip culture of mother plants grown in a greenhouse at the Gumi Flower Research Station (Gumi, South Korea) on Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962), were used as the primary source. The in vitro shoots were subcultured for 6 weeks on hormone-free MS medium to obtain young and uniform shoots for further experiments.

Effects of plant growth regulators on shoot multiplication

Young shoots, approximately 1–2 cm in size, were cultured on MS medium containing 3% (w/v) sucrose (Duchefa Biochemie), 0.7% plant agar (Duchefa Biochemie), and various concentrations of 6-benzylaminopurine (BAP) alone or in combination with various concentrations of α-naphthalene acetic acid (NAA). The culture vessels were maintained under a 16 h photoperiod (50 μmol m^2 s^{-1}) for 30 days prior to evaluating the number of shoots per explant.

Effects of different ethylene-inhibiting compounds on in vitro responses

Based on the above-mentioned experiment, a combination of 2 mg L^{-1} BAP and 0.5 mg L^{-1} NAA was found to be optimal for shoot multiplication. To verify the effects of different ethylene-inhibiting compounds on in vitro shoot responses, same-sized shoots were cultured on selected regeneration medium; different concentrations of AVG (Sigma Aldrich), SNP (Enzo Life Sciences), or STS (Duchefa Biochemie) were added to the medium following autoclaving. Culture vessels (100 mL Erlenmeyer flasks) containing four explants were maintained for 30 days under the same conditions used in the above-mentioned experiment, at which point, the in vitro shoot responses, such as the number of yellowing leaves and the number of shoots per explant, were evaluated. In addition, the inhibition of ethylene by different concentrations of the compounds was determined.

Effect of the ethylene precursor ACC on in vitro shoot responses

To further determine the effect of ethylene precursors on in vitro shoot responses, same-sized shoots grown on regeneration medium with or without 1 mg L^{-1} IAA were treated with different concentrations of ACC (Sigma Aldrich). ACC and IAA were added to the medium after autoclaving. Culture vessels (100 mL Erlenmeyer flasks) were maintained under a 16 h photoperiod (50 μmol m^2 s^{-1}) for 30 days, at which point the number of yellowing leaves, the number of shoots per explant, and shoot tip necrosis were evaluated. In addition, the induction of ethylene in vitro by ACC and IAA was determined.

Effect of genotype on apical shoot initiation and leaf yellowing

To determine cultivar effects, same-sized shoots of the rose cultivar Innocence were cultured on regeneration medium supplemented with 12.5 μM AVG, 1 μM STS, or 20 μM SNP. In addition, Podwyszyńska and Goszczyńska (1998) demonstrated that the presence of IAA promoted ethylene biosynthesis in roses and increased the number of yellowing leaves and shoot tip necrosis. Thus, we simultaneously cultured the explants on medium containing the same concentration of the ethylene-inhibiting compounds and 1 mg L^{-1}

IAA, to determine the effect of IAA on the in vitro shoot responses of this cultivar. Culture vessels were maintained under the above-mentioned culture conditions and the number of yellowing leaves and the number of shoots per explant were evaluated. In addition, the concentration of ethylene in vitro was determined.

Determination of ethylene concentration

Ethylene concentrations were determined as described by Podwyszyńska and Goszczyńska (1998), with some modifications. Briefly, shoots from the experimental media were transferred into 20 mL vials containing 2 mL of distilled water. The vials were left open for 1 h to avoid ethylene release because of post-transplantation stress. Next, the vials were tightly sealed with rubber stoppers for 4 h. The concentration of ethylene in the headspace of the vials was then measured by withdrawing the ethylene with a 1 mL syringe and using a gas chromatograph (GC-2010, Shimadzu). The results were expressed as $\mu L\ g^{-1}$ fresh $wt \cdot h^{-1}$.

Experimental design and statistical analysis

The experiments were conducted using a randomized complete block design (RCB) with the following experimental conditions: pH 5.8, 3.0% sucrose, and 0.7% agar. Each experiment included three Erlenmeyer flasks (100 mL) containing four explants and all experiments were performed in triplicate. Data were collected after 30 days of culture and were analyzed with SAS (9.4). The means were compared using Duncan's multiple test with a significance level of $P < 0.05$.

Results

Explants cultured on medium containing various BAP concentrations responded differently. Of the different concentrations tested, 1 mg L^{-1} BAP was found to be less effective for shoot regeneration. Increasing the BAP concentration to 2 mg L^{-1} promoted the number of shoots per explant; however, concentrations >2 mg L^{-1} resulted in decreased shoot regeneration (Table 1). In addition, significant enhancement of shoot regeneration (2.92) was observed in explants cultured on medium containing a combination of 2 mg L^{-1} BAP and 0.5 mg L^{-1} (but not 1 mg L^{-1}) NAA.

Although the BAP (2 mg L^{-1}) and NAA (0.5 mg L^{-1}) combination was found to be best for shoot regeneration, the number of yellowing leaves per shoot was 2.83. Thus, in order to reduce the number of yellowing leaves per shoot, we added different concentrations of ethylene-inhibiting compounds to the shoot regeneration medium. The results shown in Fig. 1 demonstrate that ethylene plays a role in the

Table 1 Effect of plant growth regulators on in vitro shoot regeneration in *Rosa hybrida* 'Tineke' following 30 days of culture

Plant growth regulator (mg L^{-1})		Mean number of shoots
BAP	NAA	
0	0	1.22 c
1.0	0	1.94 c
2.0	0	2.46 ab
3.0	0	2.23 b
5.0	0	2.02 b
2.0	0.5	2.92 a
2.0	1.0	2.51 ab

Values in the column followed by the same letter do not differ at significance level $P < 0.05$ (Duncan's multiple range test)

reduction of leaf yellowing as well as in shoot initiation in vitro. Explants cultured on medium containing AVG (7.5–12.5 μM) exhibited a linear positive effect on the reduction of leaf yellowing (Table 2). Interestingly, 12.5 μM AVG significantly promoted apical shoot initiation and reduced the number of yellowing leaves per explant (Fig. 1d), whereas AVG concentrations >12.5 μM resulted in a decrease of these positive effects. Thus, 12.5 μM AVG appears to be the optimal concentration (Table 2). Moreover, the addition of AVG to the medium inhibited ethylene production and 12.5 μM AVG was found to be the most effective ethylene inhibition treatment (Fig. 2).

A significant reduction in the number of yellowing leaves was observed when the explants were grown on medium containing STS (Table 2). However, while the presence of 1or 3 μM STS promoted apical shoot initiation (Fig. 1), 5 or 10 μM STS had negative effects on apical shoot initiation and 10 μM STS had a greater negative effect on apical shoot initiation than 5 μM STS. In contrast to AVG, STS did not significantly inhibit ethylene production during the culture period (Fig. 2).

The addition of 10–15 μM SNP lowered the number of yellowing leaves per explant but had no effect on apical shoot initiation (Table 2). When the SNP concentration was increased to 20 μM, a greater reduction in the number of yellowing leaves per explant was observed; in addition, the number of shoots per explant induced by this concentration was also higher than that in the control (Table 2). Increasing the SNP concentration to 25 μM reduced the effect on apical shoot initiation. The presence of SNP induced lower amounts of ethylene compared with the control; the highest level of inhibition was observed at 20 and 25 μM SNP (Fig. 2).

In addition, we investigated the in vitro response of shoots to the ethylene promoter ACC alone or in combination with IAA to determine the correlation between ethylene production and in vitro responses such as apical shoot initiation,

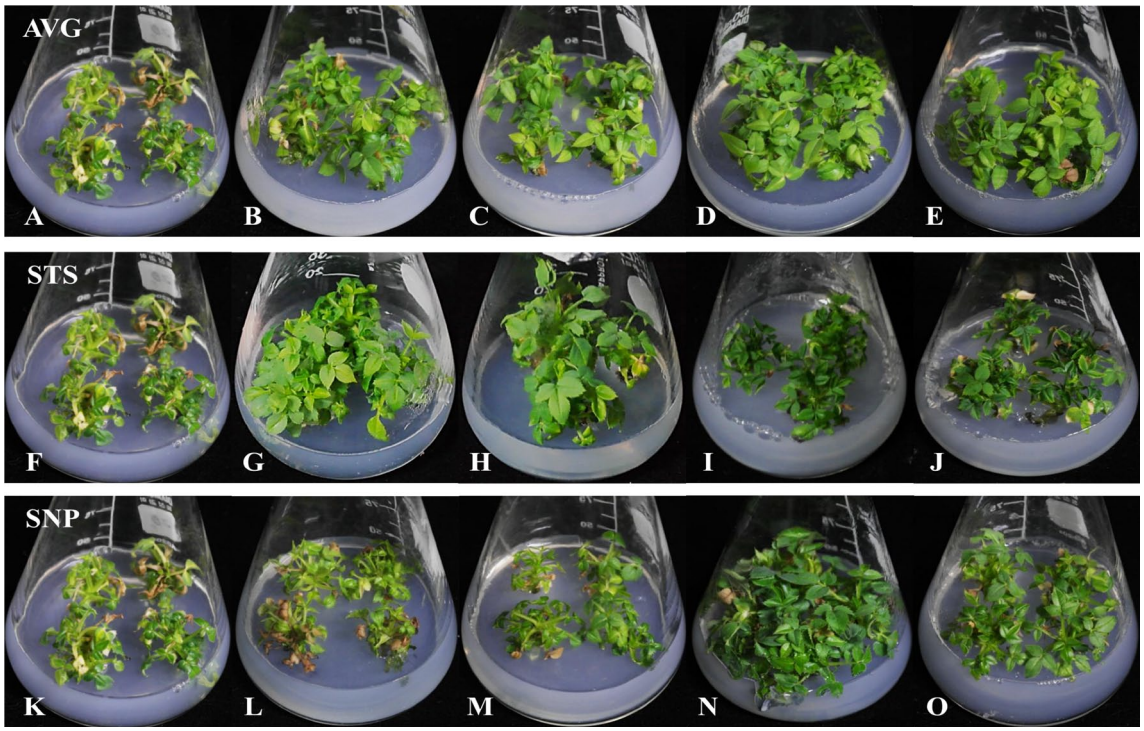

Fig. 1 Effects of AVG, STS, and SNP on shoot proliferation of cultivar Tineke after 30 days. **a** Control; **b** AVG, 7.5 μM; **c** AVG, 10 μM; **d** AVG, 12.5 μM; **e** AVG, 15 μM; **f** control; **g** STS 1 μM; **h** STS, 3 μM; **i** STS, 5 μM; **j** STS, 10 μM; **k** control; **l** SNP, 10 μM; **m** SNP, 15 μM; **n** SNP, 20 μM; **o** SNP, 25 μM

Table 2 Effect of AVG, STS, and SNP on leaf senescence and number of shoots in *Rosa hybrida* 'Tineke' following 30 days of culture

Ethylene inhibitor (μM)	Mean number of yellow leaves per shoot	Mean number of shoots
Control AVG	2.83 d	2.92 c
7.5	2.20 c	3.06 c
10	1.70 b	3.17 c
12.5	0.74 a	5.75 a
15	1.11 a	4.50 b
Control STS	2.83 c	2.92 c
1	1.32 a	3.67 a
3	1.55 a	3.33 b
5	2.07 b	2.42 d
10	2.13 b	1.67 e
Control SNP	2.83 c	2.92 c
10	2.09 b	2.92 c
15	1.96 b	2.97 c
20	1.22 a	4.17 a
25	1.34 a	3.83 b

Values in the column followed by the same letter do not differ at significance level $P < 0.05$ (Duncan's multiple range test)

the number of yellowing leaves, and shoot tip necrosis. Explants grown on medium containing only ACC or IAA exhibited reduced apical shoot initiation and increased numbers of yellowing leaves and shoot tip necrosis. Increasing ACC concentrations exhibited a linear negative effect on these parameters. The negative effects were more dramatic when ACC was combined with IAA (Table 3). The negative effects of IAA and ACC on the in vitro responses were supported by the effect of increasing concentrations of ACC on ethylene amount and production; addition of IAA to the control and medium containing 100 μM ACC resulted in distinct and enhanced ethylene production (Fig. 3).

As shown in Table 2, AVG and SNP exhibited a positive effect on apical shoot initiation and reduced the number of yellowing leaves via inhibition of ethylene production; 12.5 μM AVG and 20 μM SNP were the optimal concentrations for these effects. Similarly, 1 μM STS positively affected the studied parameters, although ethylene inhibition by STS was not significantly different. In contrast, as shown in Table 3, IAA negatively affected apical shoot initiation and increased the number of yellowing leaves as well as the severity of shoot tip necrosis; subsequently, enhancement of ethylene production by IAA was also observed. To examine genotypic effects, the rose cultivar Innocence was cultured on the same medium and treated with the same concentrations of the ethylene-inhibiting compounds with or without IAA. Similar to the results in Tineke, the addition of ethylene-inhibiting compounds significantly enhanced apical shoot initiation and reduced the number of yellowing leaves (Table 4); however, the further addition of IAA negatively affected these parameters.

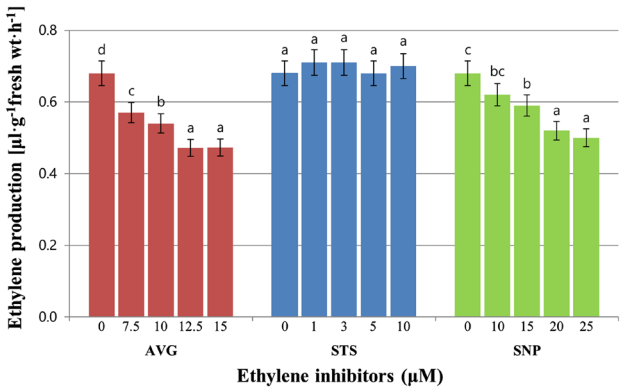

Fig. 2 Inhibition of ethylene production in in vitro rose cultures by different ethylene inhibitors (AVG, STS, and SNP)

Table 3 In vitro response of *Rosa hybrida* 'Tineke' shoots to ACC-containing medium with or without IAA following 30 days of culture

ACC (µM)	Presence (+) or absence (−) of IAA 1 mg L^{-1}					
	Number of shoots		Shoot-tip necrosis		Number of yellow leaves per shoots	
	(−)	(+)	(−)	(+)	(−)	(+)
0	2.67 a	2.08 b	0.00 a	0.38 cd	0.74 a	1.13 ab
10	1.08 c	0.86 cd	0.06 a	0.38 cd	3.63 bc	5.13 c
25	0.67 de	0.62 e	0.13 ab	0.56 de	8.32 d	13.21 e
50	0.33 f	0.25 f	0.31 bc	0.69 ef	18.42 e	38.52 g
100	0.17 f	0.19 f	0.38 cd	0.81 f	51.94 h	64.47 i

Values in the column followed by the same letter do not differ at significance level $P < 0.05$ (Duncan's multiple range test)

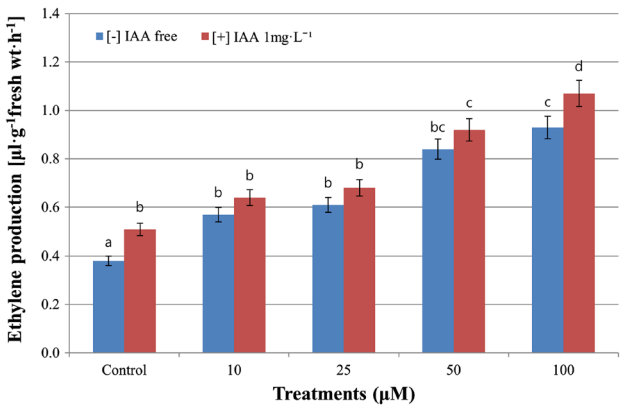

Fig. 3 Amount of ethylene produced by shoots cultured on medium containing ACC alone or in combination with IAA for 30 days. C, ACC free medium

The number of shoots per Tineke explant induced by AVG was higher than that induced by STS, followed by SNP (Table 4). To elucidate the association between ethylene production and ethylene-inhibiting compounds and IAA, the amount of ethylene induced by the compounds alone or in combination with IAA was determined after 30 days of culture. As expected, the compounds induced lower levels of ethylene; inhibition was highest with AVG followed in decreasing order by SNP and STS. In both cultivars, addition of IAA to the control induced greater amounts of ethylene than did the control without IAA. Similarly, ethylene production was higher when the compounds were added without IAA (Fig. 4).

Discussion

Several studies have shown that ethylene plays an important role in the shoot regeneration of several plant species (Burgos and Alburquerque 2003; Kevers et al. 1992; Kumar et al. 2007; Podwyszyńska and Goszczyńska 1998; Martinez et al. 2015; Sgamma et al. 2015; Würschum et al. 2015). Our study confirms the correlation between ethylene production and rose growth parameters, such as apical shoot initiation, leaf yellowing, and shoot tip necrosis, by using ethylene-inhibiting compounds (AVG, STS, and SNP), an ethylene promoter (ACC), and an auxin (IAA). AVG, STS, and SNP inhibited ethylene production and promoted the studied parameters, while the opposite effects were observed in the presence of ACC, IAA, or their combination. Merkers et al. (1984) reported that AVG significantly promoted the reduction of leaf senescence and enhanced rose growth with a high multiplication rate, while Gaspar et al. (1989) did not show any effect of AVG on the multiplication of roses. A similar study performed by Kevers et al. (1992) confirmed the positive effects of AVG on roses. Our results also support the positive effect of AVG on in vitro apical shoot initiation, reduction of leaf yellowing, and ethylene inhibition. STS has been similarly used in a number of plant species (Martinez et al. 2015; Würschum et al. 2015); however, there is limited information regarding its effect on ornamental plants (Chae et al. 2012) and, to the best of our knowledge, there are no available reports for roses. Our results indicate that similar to AVG, STS positively affected the inhibition of ethylene production and apical shoot initiation. However, AVG had a greater positive effect on these studied parameters compared to STS, in contrast to the findings of Chae et al. (2012) and Naing et al. (2015), who examined the effects of STS on apical shoot initiation in the ornamental plants *Sinningia speciosa* Bail and chrysanthemum. In addition, the recent studies also claimed that STS improved embryogenesis in *Quercus alba, Quercus rubra*, and hexaploid triticale (Martinez et al. 2015; Würschum et al. 2015). Thus, the effects of these compounds seem to vary greatly among species. Higher concentrations of both AVG and STS resulted in negative effects in this study, which confirms the findings of Naing

Table 4 Effects of AVG, SNP, and STS alone or in combination with IAA on leaf senescence and shoot regeneration in *Rosa hybrida* 'Innocence' following 30 days of culture

Treatment (μM)	Mean number of yellow leaves per shoot	Mean number of shoots
Control	3.95 g	2.17 f
AVG 12.5	1.13 a	4.00 a
STS 1	1.23 b	3.50 b
SNP 20	1.24 c	3.08 c
Control + IAA (1 mg L^{-1})	4.81 h	1.17 h
AVG 12.5 + IAA (1 mg L^{-1})	1.36 d	2.75 d
STS 1 + IAA (1 mg L^{-1})	1.53 e	2.50 e
SNP 20 + IAA (1 mg L^{-1})	1.59 f	1.83 g

Values in the column followed by the same letter do not differ at significance level $P < 0.05$ (Duncan's multiple range test)

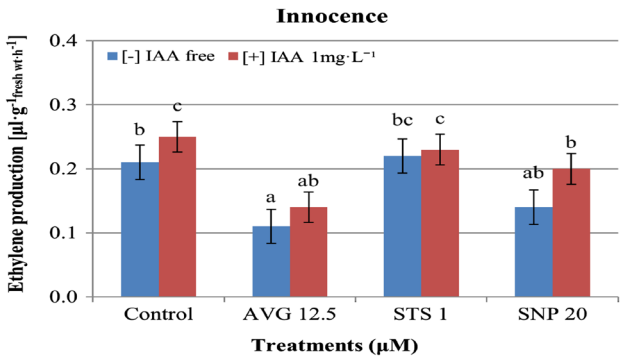

Fig. 4 Amount of ethylene produced by shoots cultured for 15 days on medium containing AVG, STS, or SNP alone or in combination with IAA

et al. (2014, 2015), who hypothesized this was because of the toxic effects of the compounds.

Although SNP has been used to promote shoot regeneration (Xu et al. 2009), extend the storage period of strawberries (Zhu and Zhou 2007), and expand the vase life of cut flowers such as chrysanthemum, to the best of our knowledge, the effects of SNP in in vitro rose experiments and on ethylene inhibition are yet to be examined. Here, we found that SNP affected both ethylene inhibition and apical shoot initiation in roses and that its ability to induce shoots was higher than that of STS; the optimal SNP concentration (20 μM) induced 4.17 shoots per explant, while STS induced 3.67 shoots per explant. In addition, SNP had a greater ethylene inhibiting effect than STS. However, similar to AVG and STS, higher concentrations of SNP inhibited apical shoot initiation, consistent with the findings of Xu et al. (2009), who reported that higher than optimal concentrations of SNP inhibited callus induction in Dioscorea opposite.

Addition of the ethylene promoter ACC to the regeneration medium negatively affected the studied parameters in a concentration dependent manner by enhancing ethylene production. Thus, ethylene produced by cultured explants inhibits shoot initiation and enhances the number of yellowing leaves per explant. Similarly, the positive effects of AVG, STS, and SNP demonstrated in this study can be explained by their ability to inhibit ethylene production.

Our results further confirm the findings of Podwyszyńska and Goszczyńska (1998) that the presence of IAA promoted ethylene biosynthesis in roses and increased the number of yellowing leaves and shoot tip necrosis. The combination of IAA and ACC resulted in higher ethylene levels and a greater negative effect on apical shoot initiation, leaf yellowing, and shoot tip necrosis than ACC alone. This further supports the findings of Horn (1992) and Bogaert et al. (2006) that the occurrence of leaf yellowing and shoot tip necrosis in roses was because of ethylene; however, the amount of ethylene affecting leaf senescence was not examined.

Similar effects of AVG, STS, and SNP on apical shoot initiation and ethylene inhibition were observed in the cultivar Innocence. Moreover, the addition of IAA to medium containing ethylene inhibitors also resulted in negative effects. Comparable effects were observed in maize by using a combination of an ethylene inhibitor and promoter (Songstad et al. 1988).

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