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The influence of ethylene and ethylene modulators on shoot organogenesis in tomato

--Manuscript Draft--

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Abstract:	The influence of ethylene and ethylene modulators on the in vitro organogenesis of tomato was studied using a highly regenerating accession of the wild tomato Solanum pennellii and an F1 plant resulting from a cross between Solanum pennellii and Solanum lycopersicum cv. Anl27, which is known to have a low regeneration frequency. Four ethylene-modulating compounds, each at four levels, were used, namely: cobalt chloride (CoCl2), which inhibits the production of ethylene; AgNO3 (SN), which inhibits ethylene action; and Ethephon and the precursor 1-aminocyclopropane-1-carboxylic acid (ACC), which both promote ethylene synthesis. Leaf explants of each genotype were incubated on shoot induction medium (SIM) supplemented with each of these compounds at 0, 10 or 15 days following bud induction. The results obtained in our assays indicate that ethylene has a significant influence on tomato organogenesis. Concentrations of ethylene lower than the optimum (according to genotype) at the beginning of the culture may decrease the percentage of explants with buds (B), produce a delay in their appearance, or indeed inhibit bud formation. This was observed in S. pennellii and the F1 explants cultured on media with SN (5.8-58.0 μM) as well as in the F1 explants cultured on medium with 21.0 μM CoCl2. The percentage of explants with shoots (R) and the mean number of shoots per explant with shoots (PR) also diminished in media that contained SN. Shoots isolated from these explants were less developed compared to those isolated from control explants. On the other hand, ethylene supplementation may contribute to enhancing shoot development. The number of isolable shoots from S. pennellii explants doubled in media with ACC (9.8-98.0 μM). Shoots isolated from explants treated with ethylene releasing compounds showed a higher number of nodes when ACC and Ethephon were added at 10 days (in F1 explants) or at 15 days (in S. pennellii) after the beginning of culture. Thus, the importance of studying not only the concentration but also the timing of the application of regulators when developing regeneration protocols has been made manifest. An excess of ethylene supplementation may produce an inhibitory effect, as was observed when using Ethephon (17.2-69.0 μM). These results show the involvement of ethylene in tomato organogenesis and lead us to believe that ethylene supplementation may contribute to enhancing regeneration and shoot development in tomato.
Response to Reviewers:	Dear Dr. Schuyler, Attached please find the revised version of our manuscript TICU5251, “The role of

ethylene in tomato shoot regeneration," which we would like to publish in Plant Cell, Tissue and Organ Culture.
The revised version has been prepared taking into account your comments and those of the reviewers. Below, we have provided a detailed list of the changes made in response to the reviewers' comments.
Looking forward to hearing from you.
Best Regards,
Carmina Gisbert

Answers to the Editor's questions:
1.The explants used were leaves. This is specified in the materials and methods, but I have also specified the type of explants that were used in both the abstract and the text.
2.The R and PR parameters are different. R is the frequency of shoot regeneration (percentage of explants with shoots) whereas PR is the "mean number of shoots per explant with shoots". Shoots are shoots developed from buds that can be isolated (cut) and transferred for rooting in standard medium. All isolable shoots are able to root, although differences in root length were observed depending on their origin (explants cultured on SIM or SIM supplemented with ethylene-releasing or -inhibiting compounds) as is shown in Table 2. As suggested by the referee, in this version of the manuscript, I have changed the description of the measured parameters.

List of changes following suggestions of the Editor-in-Chief:
1.The Abstract and the Text now state the compounds used in full.
2.Light intensity has been written properly.
3.Writing style, grammar and syntax of the paper have been reviewed and improved. Please inform of any further changes deemed necessary.
4.We have included three articles published in PCTOC, Dias et al. (2009), Lu et al. (2011) and Ptak et al (2010), in addition to two that were cited in the previous version (Bhatia et al. 2004 and Mohiudding et al. 1997).

Changes according to the suggestions of Reviewer 1
1.The title has been modified as suggested by this referee. Thus, the title of the manuscript in this version is: "The influence of ethylene and ethylene modulators on shoot organogenesis in tomato".
2.We have modified the first sentences in the abstract following the referee's instructions.
3.The explant used is specified in the abstract and throughout the text.
4.I have introduced page numbers.
5.The last paragraph in the introduction has been modified as suggested: "The aim of the present work is to investigate the influence of ethylene-releasing and -inhibiting compounds on shoot organogenesis in tomato".
6.In M&M I have deleted the s from pH.
7.Sentences for defining parameters were changed as suggested: a) frequency of explants with organogenic buds (B); b) frequency of shoot regeneration (R). PR is defined as "mean number of shoots per explant with shoots."
8.At the end of M&M, the sentence "10 or 15 days following initiation of culture, explants with organogenic buds were subcultured to SIM or SIM-supplemented media with SN, $CoCl_2$, ACC or Ethephon at 5.8, 4.2, 9.8 and 6.9 μM, respectively," has been included in the substitution to the original sentence.
9.The sentence "Parallel to previous experiment" has been deleted.
10.In Results I modified the subheadings as follows: "Effects of different doses of ethylene inhibitors or ethylene-releasing compounds on organogenic bud initiation"; "Influence of ethylene inhibitors or ethylene-releasing compounds added to medium following bud induction on shoot regeneration"
11.In all tables, the letter "a" has been assigned to the highest value. This change was also made in Figure 2.
12.We have tried to improve our writing style, following the suggestions of other researchers and English reviewers.

Plant Cell, Tissue and Organ Culture

The influence of ethylene and ethylene modulators on shoot organogenesis in tomato

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Abstract

The influence of ethylene and ethylene modulators on the in vitro organogenesis of tomato was studied using a highly regenerating accession of the wild tomato *Solanum pennellii* and an F1 plant resulting from a cross between *Solanum pennellii* and *Solanum lycopersicum* cv. Anl27, which is known to have a low regeneration frequency. Four ethylene-modulating compounds, each at four levels, were used, namely: cobalt chloride ($CoCl_2$), which inhibits the production of ethylene; $AgNO_3$ (SN), which inhibits ethylene action; and Ethephon and the precursor 1-aminocyclopropane-1-carboxylic acid (ACC), which both promote ethylene synthesis. Leaf explants of each genotype were incubated on shoot induction medium (SIM) supplemented with each of these compounds at 0, 10 or 15 days following bud induction. The results obtained in our assays indicate that ethylene has a significant influence on tomato organogenesis. Concentrations of ethylene lower than the optimum (according to genotype) at the beginning of the culture may decrease the percentage of explants with buds (B), produce a delay in their appearance, or indeed inhibit bud formation. This was observed in *S. pennellii* and the F1 explants cultured on media with SN (5.8-58.0 µM) as well as in the F1 explants cultured on medium with 21.0 µM $CoCl_2$. The percentage of explants with shoots (R) and the mean number of shoots per explant with shoots (PR) also diminished in media that contained SN. Shoots isolated from these explants were less developed compared to those isolated from control explants. On the other hand, ethylene supplementation may contribute to enhancing shoot development. The number of isolable shoots from *S. pennellii* explants doubled in media with ACC (9.8-98.0 µM). Shoots isolated from explants treated with ethylene releasing compounds showed a higher number of nodes when ACC and Ethephon were added at 10 days (in F1

explants) or at 15 days (in *S. pennellii*) after the beginning of culture. Thus, the importance of studying not only the concentration but also the timing of the application of regulators when developing regeneration protocols has been made manifest. An excess of ethylene supplementation may produce an inhibitory effect, as was observed when using Ethephon (17.2-69.0 µM). These results show the involvement of ethylene in tomato organogenesis and lead us to believe that ethylene supplementation may contribute to enhancing regeneration and shoot development in tomato.

Keywords

Organogenesis, ACC, $AgNO_3$, $CoCl_2$, Ethephon

Abbreviation

ACC: 1- aminocyclopropane- 1 -carboxylic acid

BM: Murashige and Skoog basal medium (1962)

$CoCl_2$: Cobalt chloride

Ethephon: 2- chloroethanephosphonic acid

SN: Silver nitrate ($AgNO_3$)

SIM: Shoot Induction Medium

Introduction

The regeneration of in vitro-cultured explants is required for various in vitro culture techniques (transformation, protoplast fusion, etc.) and has been described for a large number of crops (Brown and Thorpe 1995). Even though many studies have been carried out in order to improve our understanding of the metabolic processes related to regeneration, the standardization of the conditions for in vitro plant regeneration is still an empirical process (Devi et al. 2008). Successful regeneration depends on the nutrient medium, the concentration and combination of growth regulators, the light and temperature regimes in the growth chamber and the genotype and explant type (Bhatia et al. 2004, 2005). In addition to auxin and cytokinin, which take part in in vitro morphogenesis, there is considerable evidence that ethylene also plays an important role in this process (Reviewed Biddington 1992; Moshkov et al. 2008).

Ethylene is the only gaseous plant hormone and may be produced endogenously and/or as the consequence of explant isolation (Kumar et al. 1998; Moshkov et al. 2008). Moreover, exogenous auxin and cytokinin, which are used to induce shoots, have been shown to stimulate ethylene production by inducing (reviewed Tsuchisaka and Theologis 2004) or modifying (Vogel et al. 1998) specific biosynthetic enzymes. The manipulation of the ethylene levels in different in vitro systems has revealed that the hormone has the potential to both inhibit (Mohiuddin et al. 1997) or enhance (Dimasi-Therion

and Economou 1995) shoot organogenesis depending on the species, genotype, tissue, culture system and the presence of other plant growth regulators (Biddington 1992; Kumar et al. 1998; Moshkov et al. 2008; Dias et al. 2009; Ptak et al. 2010). In tomato (*Solanum lycopersicum* L.), organogenesis is the most common regeneration pathway and is strongly influenced by genotype, culture media and culture conditions (Bathia et al. 2005). Even though regeneration in tomato has been a frequent subject of research due to the commercial value of the crop, little is known about the influence of ethylene on organogenesis. To the best of our knowledge, only two reports have focused on the effect of this hormone: Lima et al. (2009) reported that ethylene is essential for callus formation in Micro-Tom tomato root explants carrying the high-regeneration *Rg-1* gene, and Osman and Khalafalla (2010) found that silver nitrate (SN) and cobalt chloride ($CoCl_2$), both at a unique concentration of those experimentally tested, enhanced the number of shoots per explant in *S. lycopersicum* cv. Omdurman.

The aim of this study is to investigate the influence of ethylene releasing and inhibiting compounds on shoot organogenesis in tomato. For this purpose we tested four ethylene-modulating compounds, each at four concentrations, namely $CoCl_2$, which inhibits the production of ethylene, SN, which inhibits ethylene action and Ethephon and the precursor 1- aminocyclopropane- 1 -carboxylic acid (ACC), which both promote ethylene synthesis. The effects of adding these compounds prior to or following bud induction (at 10 or 15d) were also studied. The plant materials used in this work are well-characterized tomato genotypes that presented different organogenesis responses The *S. pennellii* accession has a high regeneration potential (Gisbert et al. 1999). The F1 genotype is obtained by crossing this wild tomato accession with a tomato cultivar (cv. Anl27) that has very low regeneration capacity (Trujillo-Moya et al. 2011). These materials have been used for detecting QTLs related with the organogenic response (Trujillo-Moya et al. 2011). The presence of ethylene response factors was detected in 5 of the 6 QTLs identified.

Materials and methods

Plant material and culture conditions

Clones of the genotypes *S. pennellii* (accession PE-47) and an F1 plant (*S. lycopersicum* cv. anl27 x *S. pennellii* PE-47) were cultured in vitro in tubes containing fresh basal medium (BM: Murashige and Skoog salts including vitamins, 1.5%sucrose and 0.7% plant agar). The plants were maintained in in vitro culture by transferring nodes every 3-4 weeks. Leaf explants (0.6-0.8 cm^2) were obtained from plants in order to carry out the experiments.

The pH of all the media was adjusted to 5.8 before sterilization at 121ºC for 20 min, and cultures were incubated in a growth chamber at 26ºC ± 2ºC under a 16h photoperiod with cool white light provided by Sylvania cool white F37T8/CW fluorescent lamps (90 µmol $m^2 s^{-1}$).

Culture of leaf explants in SIM or SIM supplemented with ethylene inhibitors or ethylene-releasing compounds

S. pennellii and F1 leaf explants were obtained and cultured on SIM (Murashige and Skoog's salts (Murashige and Skoog, 1962), 3% sucrose, 0.7% plant agar and 0.57 µM Zeatin riboside), and on SIM supplemented with SN (at 5.8, 14.5, 29.0, 58.0 µM), $CoCl_2$ (at 4.2, 10.5, 21.0, 42.0 µM), ACC (at 9.8, 24.5, 49.0, 98.0 µM) or Ethephon (at 6.9, 17.2, 34.5, 69.0 µM). Explants were cultured on these media for 30 days and then transferred to BM for 20 days.

Zeatin riboside, SN, $CoCl_2$, ACC and Ethephon were filtered (0.22 µm Millipore filters) and then added to sterilized SIM medium. The media were plated in Petri dishes (90 x15 mm) with 30 ml of culture medium (plates used for the first 30 days of culture) or in 90 x 25 mm plates, with 40 ml of medium per plate (used for the following 20 days of culture). For each genotype, five explants per plate and 4 to 5 repetitions per genotype and treatment were evaluated.
The following parameters were determined every 7 days until day 50 of culture: a) frequency of explants with organogenic buds (B), b) frequency of shoot regeneration (R) and c) mean number of shoots per explant with shoots (PR).

Addition of ethylene inhibitors or ethylene-releasing compounds to SIM after bud induction

Ten or 15 days following initiation of culture, explants with organogenic buds were subcultured to SIM or SIM-supplemented media containing SN, $CoCl_2$, ACC or Ethephon at 5.8, 4.2, 9.8 and 6.9 µM, respectively (the lowest concentration of the assay explained above). Thirty days after initiating the experiment, all explants were transferred to BM for 20 days. At the end of this period (50 days), B, R and PR were measured. About 16 shoots per treatment were isolated and cultured individually in tubes (14.8 cm length, 2.5 cm diameter, 15 ml medium) containing 25 ml of BM. Root length (cm), shoot length (cm), number of nodes and node size (mm) were measured 25 days after culture.

Statistical analysis

Data were subjected to a unifactorial analysis of variance (ANOVA) and the means for the different traits were subsequently separated by a Duncan test.

Results and Discussion

Ethylene is a simple hydrocarbon (C_2H_4) gas that affects growth, differentiation and senescence in plants in concentrations as low as 0.01 µM (Reid 1995). We studied the role of ethylene in tomato organogenesis by evaluating the effect of two ethylene-releasing compounds (ACC and Ethephon) and two ethylene inhibitors (SN and $CoCl_2$) using two tomato genotypes which displayed different organogenic responses.

Effect on organogenesis of different doses of ethylene inhibitors or ethylene-releasing compounds

The organogenic response in leaf explants of the wild tomato *S. pennellii* and the F1 (*S. lycopersicum* cv. Anl27 x *S. pennellii*) genotypes did not differ in SIM (control medium). Thus, the

frequency of explants with organogenic buds (B) and the frequency of shoot regeneration (R) was 100% in both cases (Table 1; chemical concentration = 0). The mean number of shoots per explant with shoots (PR) proved to be higher in *S. pennellii* than in F1, giving 5.05 and 3.40 shoots per explant, respectively (Table 1). These results are in accordance with those previously obtained (Trujillo-Moya et al. 2011).

SN has been used in several studies in a range of concentrations from 0 to 100 µM (Pua et al. 1996), and its addition to culture media has resulted in contradictory effects depending on the plant materials and experimental conditions (Biddington 1992; Moshkov et al. 2008). In our study, a reduction in B of about 20% was observed in *S. pennellii* explants cultured on medium with SN concentrations higher than 14.5µM (Table 1). In these media, R and PR traits were also reduced, displaying a range of reduction between 20-64% and 71-81%, respectively (Table 1). Inhibition of bud induction in F1 explants was higher than in *S. pennellii*; no buds were induced in explants cultured on SN-containing media (Table 1). These results indicate that ethylene is necessary for organogenesis, as has been described in a variety of culture systems (Kumar et al. 1998). Our result is not in accordance with that reported by Osman and Khalafalla (2010) in the tomato cultivar Omdurman, which found a higher number of shoots per explant when explants were cultured on medium with SN at 29.0 µM and $CoCl_2$ at 12.6 µM. In tomato, it has also been reported that ethylene is essential for callus formation (Lima et al. 2009). Callus initiation commonly appears before bud formation in certain tomato genotypes. Different results between tomato genotypes may be due to varying endogenous hormone concentrations. This may be a putative explanation for the drastic regeneration inhibition produced by SN in the F1 explants compared to that observed in *S. pennellii*; the wild species may have a higher endogenous ethylene concentration than the F1 genotype.

The addition of $CoCl_2$, an inhibitor of the ACC synthase, had no effect on the organogenic response (B, R and PR) of *S. pennellii* explants. However, a decrease in B and R (on average 11% and 28%, respectively) was observed in media supplemented with $CoCl_2$ (> 21.0 µM) in F1. This result is in accordance with those obtained when SN is added, although the differences are not as pronounced as in this case. This is most likely due to the fact that the range of $CoCl_2$ concentrations used in our study was low for our genotypes. As occurred in medium with SN, the inhibitory effect was higher in F1 compared to *S. pennellii* for similar concentrations of inhibitor.

The addition of ACC at the highest concentration (98.0 µM) diminished B and R in *S. pennellii* and F1 explants. However, PR increased (was doubled) in *S. pennellii* explants cultured on media supplemented with ACC, even at the highest concentration tested (Table 1). This result indicates that ethylene is clearly influencing the development of buds into shoots in *S. pennellii*. In the F1, however, PR did not increase in these media, probably because both the number of induced buds was lower in F1 than in the *S. pennellii* explants and, putatively, in the F1 the endogenous ethylene concentration is lower than in *S. pennellii*. As occurred at the highest ACC concentration in this study, a scarce bud formation but enhanced bud development (higher PR) in media supplemented with ethylene has also been reported by other authors, e.g. Huxter et al. (1981). Ethylene production has also been described as influencing the development of somatic embryos in several works that have reported regeneration through the embryogenic pathway (Ptak et al. 2009; Lu et al. 2011).

The addition of Ethephon, which decomposes to release ethylene at physiological pH (Abeles et al. 1992), inhibited bud induction and/or the development of buds in *S. pennellii* and in the F1 at concentrations ≤6.9 µM (Table 1). A lower PR than that of the control medium was also observed in *S. pennellii* in media with Ethephon (>34.5 µM), whereas no differences for this parameter were observed in the F1 genotype (Table 1). The inhibition observed, mainly in *S. pennellii*, may be the product of toxic effects; the concentrations of Ethephon are quite possibly too high to stimulate the development of buds. It has been reported that there is a critical ethylene concentration at which morphogenesis is affected; concentrations above or below this level are respectively inhibitory or ineffective (Huxter 1981; Biddington 1992). The inhibition of shoot regeneration by adding Ethephon in a range of concentration from 1 to 10 µM has also been reported (Chraibi et al. 1991).

B and R were measured every 7d (from day 0 to day 50). At Figure 1 it is represented both traits for *S. pennellii* and F1 explants cultured on control medium and media supplemented SN (a-d) or Ethephon (e-h). Even though B was 100% for both genotypes in the control medium, the maximum percentage of explants with buds was only achieved in *S. pennellii* at 14 days of culture (Fig. 1a), whereas in F1 this maximum occurred later, at 28 days (Fig. 1c). Differences were also observed in R, which had a value of 50% at 28d in *S. pennellii* (Fig. 1b) and at 42d in the F1 (Fig. 1d). When SN (Fig. 1 a-d) was added, a total inhibition of B was produced in the F1 genotype whereas a decrease and a delay in bud formation (B) and bud development (R) were produced in *S. pennellii* (for instance, R reached 50% at 42 days). In the case of Ethephon, both B and R were reduced and delayed in both *S. pennellii* and the F1 genotype (Fig. 1 f and h). Both effects were increased with increasing the Ethephon concentrations.

Application of ethylene inhibitors or ethylene-releasing compounds following bud induction

The results of applying inhibitors or ethylene-releasing compounds after bud induction are presented in Figure 2. Explants directly cultured on SIM (C0d) and explants cultured on SIM and transferred to SIM 10 days (C10d) or 15 days (C15d) after the initial culture were the controls in this experiment. No differences between controls were observed except for those explants of *S. pennellii* transferred to fresh SIM 15 days after the beginning of culture, which showed a slight increase in PR (Fig. 2 a).

The addition of SN at 10 or 15 days after initial culture reduced R in *S. pennellii*, which was not the case when SN was added at day 0. However, for this genotype, PR was inhibited in explants treated at 0, 10 or 15 days (Fig. 2a). In the case of the F1 explants, the delay in the supplementation of the ethylene inhibitor SN, permitted the production of buds (B) and the development of some of these buds into shoots (R and PR). A higher percentage of plants was observed in the explants whose treatment occurred latest (at 15d). These results demonstrate the importance of ethylene in tomato bud development.

The addition of $CoCl_2$ after bud induction did not modify the results obtained when it was added at the beginning (Fig. 2 b). As was mentioned previously, the $CoCl_2$ concentrations used in our study may be low for the selected genotypes.

As was expected, the addition of ACC (Fig. 2c) or Ethephon (Fig. 2d) when buds were induced (T10d, T15d) produced no effect on B or R. In both cases, almost all the explants produced buds. The increase in PR observed in *S. pennellii* when ACC was applied at the beginning of the experiment (T0d) was also observed when it was applied 10 days later (T10d) but not at 15 days (T15d). This underlines the importance of studying not only the concentration but also the timing of application of regulators when developing regeneration protocols.

Shoots isolated from explants cultured on SIM and SIM supplemented with ethylene modulators were transferred to culture tubes with BM in order to observe their growth. After 25 days of culture, all plants were rooted and root length, shoot length, the number of nodes and node size were measured (Table 2). *S. pennellii* plants isolated from explants cultured on SIM showed a longer shoot length and higher number of nodes than F1 plants isolated in this medium. Shoots of *S. pennellii* isolated from explants cultured on SN-containing medium showed a growth inhibition that was manifested in all of the measured traits, even when ethylene modulators were added after 10 or 15 days of culture. These results indicate that SN not only affects bud development into shoots but also subsequent shoot growth. Vitrification and malformation of shoots were also observed in some of these plants (data not shown). As expected, plants isolated from explants cultured on media with $CoCl_2$ did not differ from those isolated from explants cultured on control medium. With regards to those plants isolated from explants cultured on media with ethylene-releasing compounds, a higher number of nodes were produced in *S. pennellii* when ACC and Ethephon were added 15 days after the beginning of culture. In the case of F1 explants, a similar effect was produced when these compounds were added at 10 days. Shoot and node length was also increased in the F1 plants. These results indicate that the addition of ethylene-releasing compounds could be regarded as a putative strategy for improving the development of buds into shoots, which can prove to be difficult in some genotypes.

Conclusions

These results confirm the importance of ethylene in tomato regeneration. Optimal concentrations of ethylene are important for bud induction, for the development of buds into shoots as well as in the subsequent growth of shoots isolated from treated explants. A decrease in ethylene had a negative effect on tomato regeneration which was manifested in low regeneration as well as in a delay in the organogenic response. On the other hand, ethylene supplementation may contribute to enhance shoot development. Therefore, ethylene supplementation could be tested to improve those protocols of regeneration where shoot isolation proves difficult. The importance of adjusting the concentration and timing of the application of inhibitors or ethylene-releasing compounds is made manifest in this study. Differences between genotypes with regard to the effects on organogenesis of different doses of ethylene inhibitors or ethylene-releasing compounds may be due to different endogenous ethylene concentrations as well as to the influence of different alleles involved in ethylene perception and response.

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Figure

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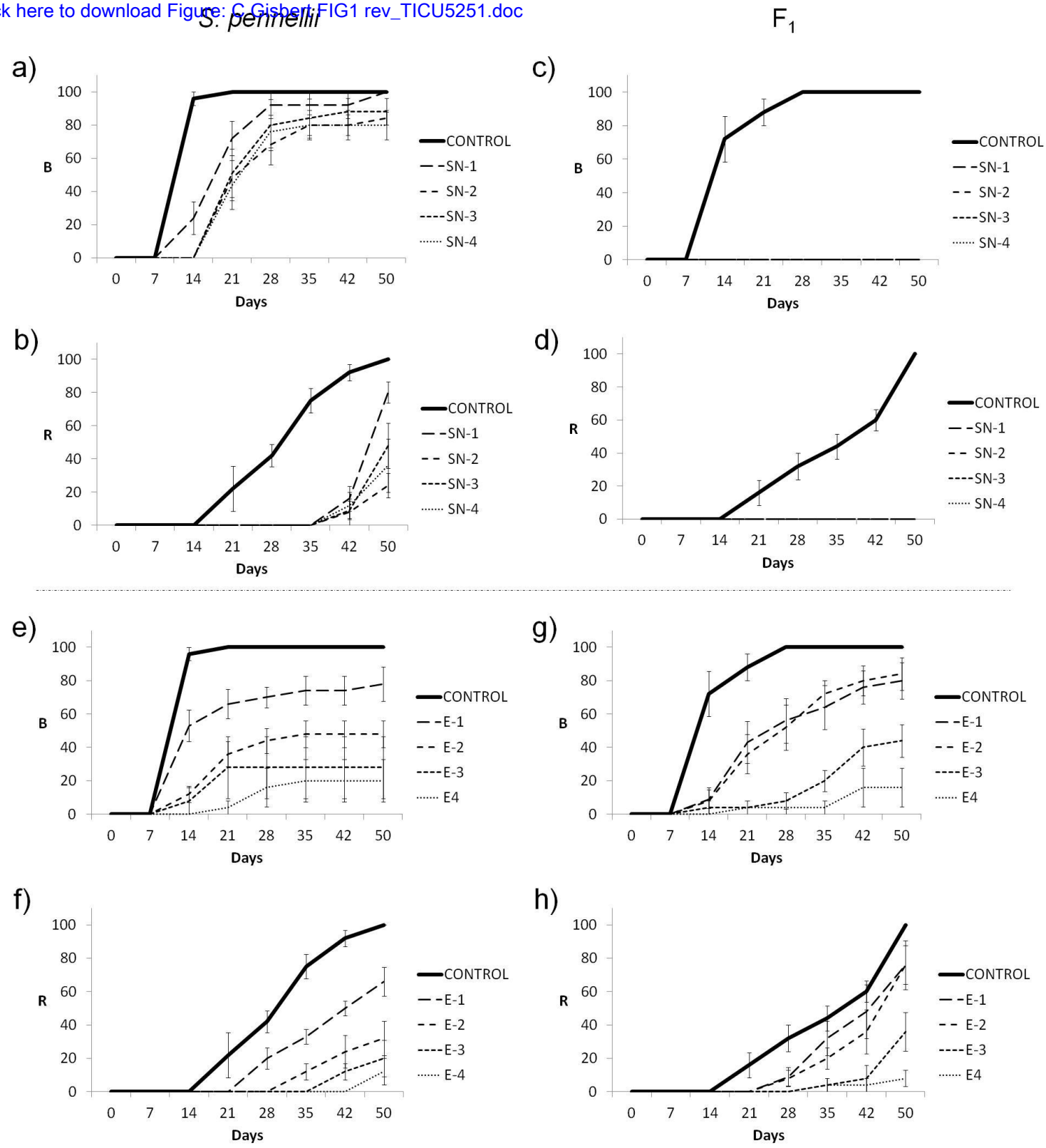

Figure
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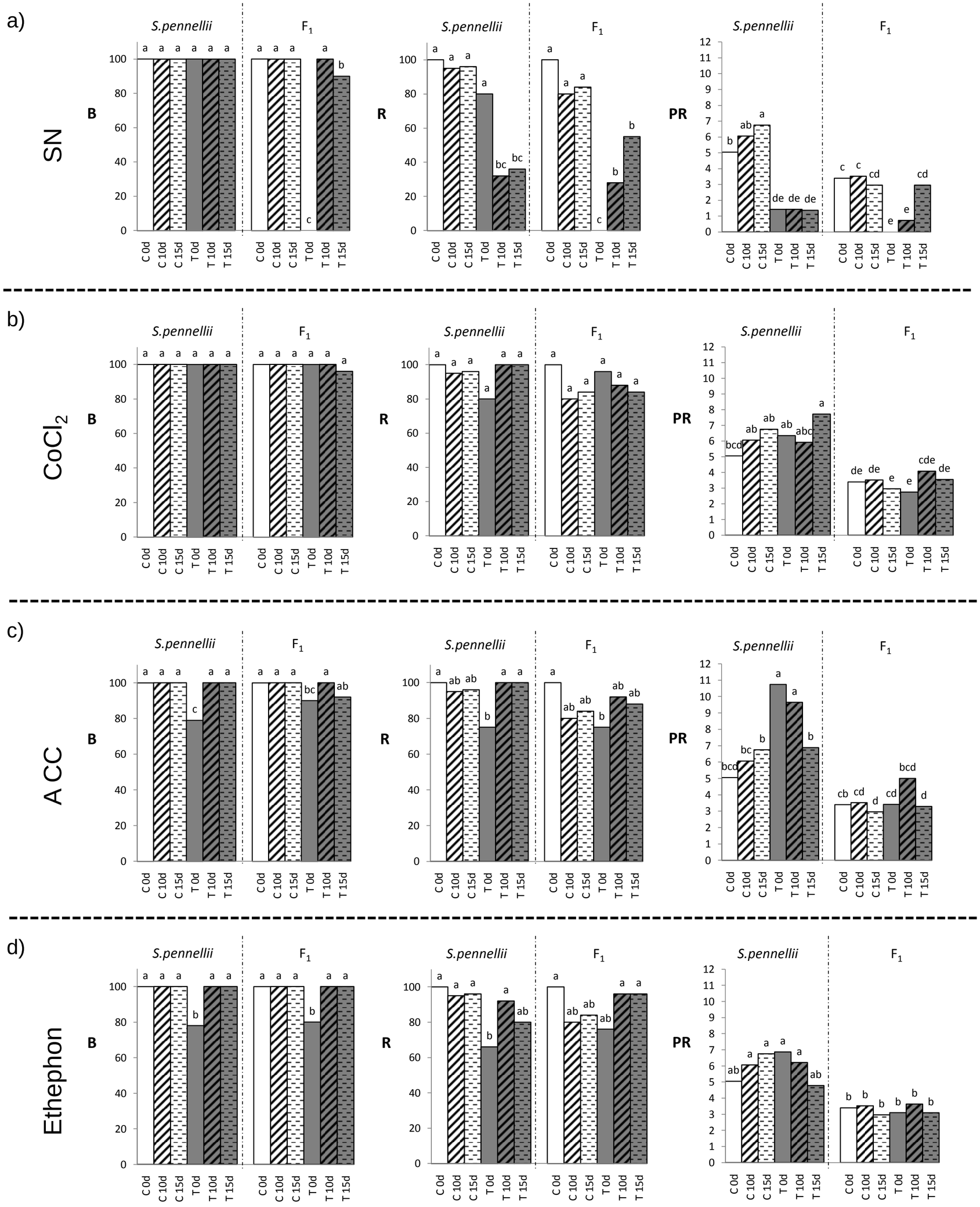

Legends

Figure 1. Frequency of explants with organogenic buds (B) frequency of shoot regeneration (R) of *S. pennellii* (a, b, e, f) and F1 (c, d, g, h) explants after 0, 7, 14, 21, 28, 35, 42 and 50 days of culture. The first 30 days, explants were cultured on SIM (CONTROL) or SIM supplemented with SN (a-d) and ethephon (e-h), and the 20 following days in plates with BM. Concentrations for SN in µM: 5.8 (SN-1), 14.5 (SN-2), 29.0 (SN-3) and 58.0 (SN-4). Concentrations for ethephon in µM: 6.9 (E-1), 17.2 (E-2), 34.5 (E-3) and 69.0 (E-4).

Figure 2. Frequency of explants with organogenic buds (B), frequency of shoot regeneration (R) and mean number of shoots per explant with shoots (PR) of *S. pennellii* and F1 explants after 50 days of culture. The first 30 days, explants were cultured on SIM (Controls) or SIM supplemented with SN (5.8 µM), $CoCl_2$ (4.2 µM), ACC (9.8 µM) or Ethephon (6.9 µM) and the following 20 days in plates with BM. Chemicals were added at 0 days (0d), 10 days (10d) or 15 days (15d) after initial culture. One C and one T before the day of treatment were assigned for controls (C0d, C10d, C15d) and treated explants (T0d, T10d, T15d), respectively.

Table

Table 1. Effect of SN, $CoCl_2$, ACC and Ethephon on in vitro regeneration of *S. pennellii* and an F1 (*S. lycopersicum* cv. Anl 27 x *S. pennellii*). Percentage of explants with buds (B), percentage of explants with shoots (R) and number of shoots per explant with shoots (PR) at 50 days of culture: the first 30 days of culture in plates with SIM and SIM supplemented with SN, $CoCl_2$, ACC or ethephon and the following 20 days in plates with BM.

Genotype	Chemical Concentrations[a]	SN			$CoCl_2$			ACC			Ethephon		
		B	R	PR	B	R	PR	B	R	PR	B	R	PR
S. pennellii	0	100 a	100 a	5.05 a	100 a	100 a	5.05 abc	100 a	100 a	5.05 bc	100 a	100 a	5.05 ab
	1	100 a	80 a	1.43 c	100 a	80 ab	6.35 a	79 ab	75 ab	10.74 a	78 a	66 bc	6.86 a
	2	84 b	24 b	1.20 c	100 a	80 ab	5.80 ab	84 ab	60 b	8.10 ab	48 b	32 d	2.50 bcd
	3	88 b	48 b	1.37 c	100 a	92 a	5.42 ab	75 ab	71 ab	10.04 a	28 b	20 d	1.67 cd
	4	80 b	36 b	0.96 c	96 ab	92 a	5.14 abc	68 b	60 b	10.15 a	20 b	12 d	1.00 d
F1	0	100 a	100 a	3.40 b	100 a	100 a	3.40 cd	100 a	100 a	3.40 c	100 a	100 a	3.40 abc
	1	0 c	0 c	0 d	100 a	96 a	2.75 d	90 ab	75 ab	3.41 c	80 a	76 b	3.09 bcd
	2	0 c	0 c	0 d	100 a	92 a	3.57 cd	78 ab	61 b	2.80 c	84 a	76 b	2.75 bcd
	3	0 c	0 c	0 d	88 c	68 b	3.17 d	80 ab	72 ab	4.28 c	44 b	36 cd	3.00 bcd
	4	0 c	0 c	0 d	90 ab	80 ab	4.05 bcd	70 b	50 b	4.17 c	16 b	8 d	2.40 cd

[a]Chemical concentrations are in µM: 5.8 (1); 14.5 (2), 29.0 (3) and 58.0 (4) for SN; 4.2 (1), 10.5 (2), 21.0 (3) and 42.0 (4) for $CoCl_2$; 9.8 (1), 24.5 (2), 49.0 (3) and 98.0 (4) for ACC and 6.9 (1), 17.2 (2), 34.5 (3), and 69.0 (4) for ethephon. Means followed by different letters within columns are significantly different at $P \leq 0.05$, Duncan test

Table

Table 2. Rooting and development of shoots (*S. pennellii* and F1) cultured for 25 days in tubes with BM medium. Shoots were isolated from explants after 30 days of culture in plates with SIM and SIM supplemented with 5.8 µM SN, 4.2 µM $CoCl_2$, 9.8 µM ACC and 6.9 µM ethephon and 20 days in plates with BM. Chemicals were added at the beginning of the experiment or at 10 or 15 days after culture on SIM medium.

Shoot origin		**Chemicals added at day 0**				**Chemicals added at day 10**				**Chemicals added at day 15**			
	Treatment	**Root Length (cm)**	**Shoot length (cm)**	**Nº of nodes**	**Node length (mm)**	**Root Length (cm)**	**Shoot length (cm)**	**Nº of nodes**	**Node length (mm)**	**Root Length (cm)**	**Shoot length (cm)**	**Nº of nodes**	**Node length (mm)**
S. pennellii	SIM	8.71 a	4.69 a	8.62 a	6.06 abc	9.61 a	4.42 a	9.50 a	6.22 cd	7.53 ab	3.82 abc	8.12 b	5.24 d
	SIM+SN	5.80 b	1.47 d	4.00 e	3.50 d	3.67 d	2.58 c	5.72 bcd	4.25 d	5.00 c	2.94 bcd	5.90 c	2.91 e
	SIM + $CoCl_2$	7.56 ab	4.03 ab	8.12 a	5.54 bc	9.46 a	4.46 a	10.69 a	6.74 bc	8.18 a	3.74 abcd	8.25 b	5.30 d
	SIM +ACC	7.36 ab	3.42 abc	7.62 ab	5.50 bc	9.46 a	4.48 a	10.56 a	6.15 cd	9.74 a	3.90 ab	10.66 a	5.46 cd
	SIM + ethephon	8.00 ab	3.64 abc	8.31 a	4.71 cd	8.48 ab	3.91 ab	9.37 a	5.87 cd	9.00 a	4.80 a	10.50 a	6.38 bcd
F1	SIM	7.30 ab	2.76 bcd	5.38 cde	7.29 ab	6.13 c	2.52 c	4.93 cd	5.68 cd	8.84 a	2.66 bcd	5.31 c	8.61 ab
	SIM+SN	-*	-	-	-	7.92 abc	2.94 bc	4.00 d	7.40 abc	5.52 bc	2.25 d	4.37 c	6.72 bcd
	SIM + $CoCl_2$	7.22 ab	3.65 abc	5.57 cd	7.82 a	7.43 abc	3.35 bc	5.69 bcd	8.42 ab	7.83 a	2.35 cd	5.50 c	7.62 abc
	SIM +ACC	8.00 ab	2.45 cd	4.67 de	6.92 ab	7.72 abc	4.40 a	6.37 b	8.31 ab	7.90 a	2.58 bcd	5.43 c	9.09 a
	SIM + ethephon	6.06 b	4.02 ab	6.62 bc	7.26 ab	6.40 bc	4.50 a	6.81 b	9.09 a	8.74 a	2.55 bcd	5.87 c	7.50 abcd

-*No regeneration obtained. Means followed by different letters within columns are significantly different at $P \leq 0.05$, Duncan test