

Review

Modulation of organogenesis and somatic embryogenesis by ethylene: an overview

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Abstract: Ethylene is a plant hormone controlling physiological and developmental processes such fruit maturation, hairy root formation and leaf abscission. Its effect on regeneration systems, such as organogenesis and somatic embryogenesis (SE), has been studied and progresses in molecular biology techniques have contributed to unveil mechanisms behind its effects. This compound affects regeneration differently, depending on the species, genotype and explant. In some species, ethylene seems to revert recalcitrance in genotypes with low regeneration capacity. However, its effect is not additive, since in genotypes with high regeneration capacity this ability decreases in the presence of ethylene precursors, suggesting that regeneration is modulated by ethylene. Several lines of evidence have shown that the role of ethylene on regeneration is markedly connected to biotic and abiotic stresses as well as to hormonal-crosstalk, in particular with key regeneration hormones and growth regulators of the auxin and cytokinin families. Transcriptional factors of the ethylene response factor (ERF) family are regulated by ethylene and strongly connected to SE induction. Thus, an evident connection between ethylene, stress responses and regeneration capacity is markedly established. In this review the effect of ethylene and the way it interacts with other players during organogenesis and somatic embryogenesis is discussed.

Keywords: ethylene biosynthesis; ethylene inhibitors; *in vitro* culture; plant hormones; S-adenosylmethionine; stress responses

1. Introduction

Micropropagation has been studied and developed through the years, currently being one of the best alternatives for large-scale propagation and conservation of a wide range of plant species [1]. In practical terms, micropropagation is the culture of plant cells, tissues or organs in a well-defined culture medium, under controlled aseptic conditions [2]. Several *in vitro* culture methods have been developed and, in combination with molecular techniques, have allowed for the multiplication of selected genotypes with better resistance to diseases and stress tolerance capacity [1,3]. Advances in plant tissue culture have also provided a deep analysis of several developmental plant processes and have helped to understand some of their molecular mechanisms [4].

Apart from the genotype and the type of explant, nutritional and environmental conditions are critical factors to achieve an optimal response from plant tissues. Mineral composition, carbon source,

plant growth regulators (PGRs), gelling agents, pH, light, temperature and also the gaseous environment are the factors to take into consideration for the success of the different *in vitro* plant propagation and regeneration processes [1].

Plant regeneration systems, such as organogenesis and somatic embryogenesis (SE) are well known micropropagation processes based on plant cells totipotency. Both systems have the ability of regenerate a whole plant genetically identical to initial explant, allowing the propagation of selected genotypes [5]. In organogenesis, plant organs, such as shoots, roots and even flowers, can be formed from cultured explants. However, for micropropagation purposes, the most interesting is *de novo* shoot meristem formation followed by shoot growth and rooting. SE is a more complex developmental pathway by which bipolar structures identical to zygotic embryos are developed from somatic cells through a complex dedifferentiation process followed by totipotency acquisition and formation of somatic embryos [4,5]. The presence of PGRs in the culture media, in particular, auxins and cytokinins, is required for the success of plant regeneration, since a balance between both hormones contributes to enhance the *in vitro* regenerative capacity of a myriad of plant species [4]. While these PGRs are deliberately placed in the culture medium in a well-defined added concentration, and a dose-response assay can be easily evaluated, the role of gaseous compounds, such as the hormone ethylene, is more difficult to analyse. This gas is usually liberated during the *in vitro* culture of plant tissues accumulating in the surrounding atmosphere when closed vessels are used. Therefore, its presence and consequent accumulation in culture vessels is a consequence of the method and not of a deliberate addition [6]. Thus, understanding whether ethylene influences *in vitro* culture is relevant for the improvement of *in vitro* regeneration processes and somehow may explain why some species or tissues seem to be more recalcitrant than others.

Taking this into consideration, this review presents a general overview of the ethylene effect on the main *in vitro* regeneration processes, revising and discussing the studies that have been published over the years. A brief overview of the ethylene biosynthesis and signaling pathways and its different modulations are also presented here in.

2. Ethylene: Biosynthesis and Signaling Pathway

The plant hormone ethylene, C_2H_4 , is a simple, small and gaseous molecule synthesized and released by plant tissues. Despite its simplicity, this hormone has a crucial role in controlling several physiological and developmental processes, ranging from plant growth to fruit ripening [7]. Its biosynthesis was intensively studied in the second half of the 20th century, period in which methionine was found as an ethylene precursor by Lieberman *et al.* [8]. Later on, Adams and Yang [9,10] identified and demonstrated that S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) were the intermediates in the conversion of methionine to ethylene. The same authors also proposed that the methionine involved on ethylene production was recycled by a set of reactions termed methionine cycle, nowadays known as Yang cycle. The identification of the ethylene precursors was the major breakthrough for the establishment of the ethylene biosynthetic pathway as it is known today. In a general overview, ethylene biosynthesis begins with methionine, which is converted to ethylene through three successive enzymatic reactions (Fig. 1). In the first reaction methionine is converted to SAM by SAM synthetase (SAMS), then SAM is converted to ACC by ACC synthase (ACS) and finally ACC oxidase (ACO) degrades ACC, releasing ethylene [11].

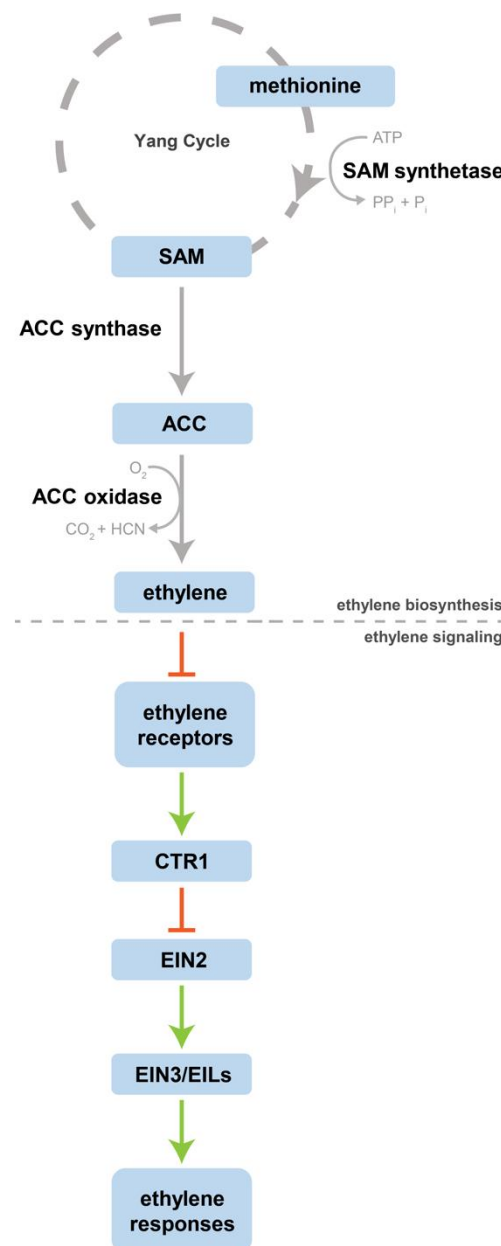


Figure 1. Schematic overview of ethylene biosynthesis and signaling. The different pathways are separated by a dashed line. Adapted from [11,12].

The effect of this hormone on plant tissues is the result of a complex subsequent signaling pathway of which some molecular mechanisms are not yet fully understood. Recently, Binder [12] reviewed the canonical ethylene signaling pathway in *Arabidopsis* and suggested that ethylene signal transduction may be even more complex. Summarizing, canonically, ethylene once biosynthesized, acts as an inverse agonist, binding and inhibiting its receptors in the endoplasmic reticulum (ER) membrane. Downstream, ethylene receptors interact with the serine/threonine-protein kinase CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), inhibiting its activity. Therefore, CTR1 can no longer phosphorylate ETHYLENE INSENSITIVE2 (EIN2), resulting in an increase of EIN2 levels. EIN2 functions as a positive regulator, increasing the levels and the activity of the transcription factors EIN3, ETHYLENE INSENSITIVE3-LIKE1 (EIL1) and EIL2 in the nucleus, which leads to transcriptional changes and consequently promotes ethylene responses [12,13]. As the ethylene signaling pathway is not the main focus of this review it will not be discussed in detail, but for readers who desire detailed information recent reviews by Ju and Binder [13] and Binder [12] are recommended. A brief outline on ethylene biosynthesis and consequent signaling is present herein (Fig. 1).

3. Culture Vessels and Ethylene Accumulation

The use of closed culture vessels to prevent contaminations is one of the requirements of *in vitro* culture. Test tubes, glass vials, Petri dishes and boxes made of polycarbonate or polypropylene are some examples of vessels used in micropropagation. These vessels can then be closed with different types of closures, such as screw lids, polycarbonate or polypropylene lids, plastic film, Parafilm, cotton plug, among others. The different types of vessels and, in particular, the different possible closures allow a greater or lesser gas exchange rate with the external environment [14,15] without compromising aseptic conditions.

To detect and measure ethylene, several methods can be used, such as gas-chromatography (GC), electrochemical sensing and optical detection. All of these detection methods have their advantages and limitations. Therefore, the selection of the most suitable method will depend on the purpose of each study. For instance, when sensitivity and real-time detection is needed, optical detection is recommended. Whether, in addition to ethylene, other gases need to be quantified, GC will be the most suitable method [16]. GC is the oldest and the most used method of ethylene detection accordingly to the literature, with its first application reported by Huelin and Kennett [17]. In *in vitro* studies using GC to measure ethylene (e.g., ethylene accumulation in culture vessels or the rate of ethylene production by plant tissues), sealed vessels with a rubber septum are required. This type of closures allows the withdrawal of gas samples from the headspace which can then be detected and analyzed [6].

Most vessels currently used in micropropagation seem to allow gas exchanges. This aspect prevents the excessive accumulation of ethylene and other gases in culture atmosphere. However, when exogenous treatments of ethylene are performed, its loss should be considered, especially in studies entailing longer culture periods. Jackson et al. [18] studied the ventilation capacity of different vessels and sealing methods, monitoring the rate loss of ethylene previously injected. For instance, in the same type of vessel (polycarbonate box with a polypropylene lid), vessels loosely closed (*i.e.*, using only the respective lid of the vessel) lost half of injected ethylene in 1-2 h, while tightly closed vessels (*i.e.*, using two layers of Nescofilm between the join of the vessel and the lid) increased the half-time of ethylene depletion to 16h. Glass vessels with glass lids were also tested. Whereas the loose sealing presented the same results as the other vessels (≈ 2 h), the tight sealing increased the half-time of ethylene depletion to 100h. The authors also monitored the accumulation of ethylene produced from plant tissues during 28 days. Ethylene accumulated in all vessels, with a greater accumulation in tightly closed vessels, as expected, and seems to decrease during the last days of the culture.

The amount of ethylene accumulated in the atmosphere of culture will depend on both the gas exchange rate between the vessel and the exterior and the rate of ethylene production by plant tissues themselves [6]. Note that even though these gas exchanges occur and thus some ethylene is lost, its accumulation in culture vessels persists, to a greater or lesser extent, and may be a limiting factor for the success of *in vitro* culture, in particular, for the most ethylene-sensitive species. In this regard, understanding the effect of this hormone in plant tissues can be accessed by a chemical or a genetic approach, subject that will be reviewed in the next two sections.

4. Chemical Modulation of Ethylene Responses

All the knowledge acquired regarding ethylene biosynthetic and signaling pathways have allowed both modulation of its biosynthesis and perception in plant tissues, therefore, enhancing, decreasing or inhibiting ethylene responses. Manipulating these responses is a crucial step to understand the influence of this hormone in a myriad of physiological processes. In this context, several chemical inhibitors and enhancers of ethylene action have been studied and widely used.

Regarding chemical inhibitors, they can be divided into two main categories, depending on whether they inhibit its biosynthetic pathway or its signaling [19].

ACS and ACO enzymes are the key targets in the inhibition of ethylene biosynthesis. Compounds such as aminoethoxyvinylglycine (AVG) and aminoxyacetic acid (AOA) are the main ACS inhibitors

used through the diverse ethylene studies. These two compounds revealed an effective inhibition of ACS activity with significant reductions in the production of ACC and, consequently, ethylene [20-22]. ACS is a pyridoxal phosphate-dependent enzyme for the conversion of SAM to ACC [21] and both AVG and AOA are inhibitors of pyridoxal phosphate-mediated reactions [21,23], reason for the great effectiveness of this inhibition. However, in a recent review about ethylene inhibitors [19], it was stressed the non-specificity of both compounds and their undesirable off-target effects. These compounds inhibit other pyridoxal phosphate-dependent enzymes such as tryptophan aminotransferase involved in indole-3-acetic acid (IAA, endogenous auxin) biosynthesis [24].

Regarding the ACO enzyme, cobalt ions, Co(II), and aminoisobutyric acid (AIB) are known for inhibiting its activity [22,25-26]. ACO is a member of the 2-oxoglutarate-dependent dioxygenase superfamily, which depends on Fe(II) as a cofactor to convert ACC into ethylene [27]. Co(II) is reported as an effective inhibitor of ACO activity due to its competitive inhibition with Fe(II) for the active site [28]. AIB, on the other hand, acts as an analogue of ACC, competitively inhibiting the conversion of ACC into ethylene [25]. Salicylic acid also inhibits ACO activity [29] and for this reason has been also used as an ethylene modulator, albeit to a lesser extent.

Due to the non-specific effect of some compounds the demand for specific ethylene biosynthesis inhibitors has increased. Therefore, novel inhibitors of ACS and ACO are being studied using a phenotype-based screening approach in *Arabidopsis* [30,31], identifying small chemical compounds that specifically inhibit these enzymes.

The main target of ethylene signaling inhibitors are ethylene receptors [19]. Before mentioning the principal compounds used in the inhibition of receptors, a brief review on the biochemical characteristics of these receptors is required to understand these inhibitions. Diverse studies with *Arabidopsis* allowed the identification and characterization of the signaling ethylene components, such as the ethylene receptors. There are five ethylene receptors isoforms identified, denominated ETHYLENE RECEPTOR1 (ETR1), ETR2, EIN4, ETHYLENE RESPONSE SENSOR1 (ERS1), and ERS2. Despite some structural differences, all the isoforms are homodimers, constituted by two monomers, stabilized by two disulfide bonds. The ethylene-binding domain is highly conserved at the N-terminus. This ethylene-binding site is formed by three transmembrane α -helices of each monomer, that together coordinate at least a copper ion, Cu(I) (see [32] for a detailed characterization). ETR1 in *Arabidopsis* not only was the first ethylene receptor identified but also the first plant hormone receptor identified and cloned [33,34]. Further studies with ETR1 have allowed the identification of key components responsible for the high affinity of this hormone to its receptors. Therefore, it is known that Cu(I) has a fundamental role since it acts as a cofactor, mediating the binding of ethylene to its receptors [35]. All the receptor isoforms coordinate Cu(I) in their transmembrane portion (ethylene-binding site). In the absence of this cofactor, ethylene binding is compromised and, consequently, this hormone is not perceived by plant tissues [32].

One of the strategies to inhibit ethylene action in plant tissues is the use of silver ions, Ag(I), usually applied as silver nitrate (AgNO₃) or silver thiosulfate (STS). Ag(I) is a widely used potent inhibitor of ethylene perception [36], due to the replacement of the Cu(I) cofactor required for ethylene binding, thus blocking ethylene signaling [37]. Despite the highly conserved ethylene-binding domain among all ethylene receptors isoforms [32], Ag(I) seems to replace Cu(I) mainly at ETR1, however, this inhibition is sufficient to inhibit ethylene responses [38]. In a similar way to AVG and AOA, silver also has off-target effects [19], increasing auxin efflux [39].

Gaseous compounds such as 1-methylcyclopropene (1-MCP), trans-cyclooctene (TCO) and 2,5-norbornadiene (NBD) also can be used as inhibitors of ethylene receptors [19]. These olefin compounds are competitive inhibitors, competing with ethylene for its receptors. From the three compounds, 1-MCP is the best alternative to control ethylene responses due to both its effective inhibition at extremely low concentrations and prolonged effect [40]. These characteristics make 1-MCP one of the best compounds to apply in ethylene studies due to the reduced off-target effects [19]. Due to the gaseous nature of these receptors inhibitors the use of gas-tight chambers is required for their maintenance in the culture atmosphere (see [19] for chambers examples).

It should be noted that the use of inhibitors of ethylene biosynthesis compared with inhibitors of ethylene signaling do not protect against exogenous ethylene. Considering the main subject of this review, *in vitro* culture, this issue is less relevant due to a closed and controlled atmosphere. On the other hand, this fact can also offer an advantage by allowing the reversion of their effect in plant tissues. For instance, the application of exogenous ethylene to the culture atmosphere, and if only ACS was inhibited, exogenous treatments with ethylene's intermediate ACC can also be used [19].

Apart from the use of both classes of inhibitors mentioned above, changing the ethylene atmospheric concentration is another relevant strategy, particularly, in *in vitro* tissue cultures due to the use of closed vessels. In this regard, pure ethylene or its precursors can be applied in order to enhance ethylene action in plant tissues [6]. The ethylene precursor ACC has been widely used *in vitro*, since it promotes an increase of ethylene biosynthesis by ACO, enhancing ethylene production [41]. Another ethylene precursor, 2-chloroethylphosphonic acid, commonly known as ethephon (ETH), proved to cause responses similar to those of exogenous ethylene treatments effectively [42] and, for this reason, has also been widely used throughout several studies. On the other hand, it is also possible to remove ethylene from culture atmosphere using compounds such as mercuric perchlorate (HgClO_4) and potassium permanganate (KMnO_4 ; [6]). Mercuric perchlorate forms complexes with ethylene [43], whereas potassium permanganate oxidizes ethylene [44]. Both act as ethylene absorbents, therefore decreasing the ethylene concentration on culture atmosphere and, consequently, the action of ethylene on plant tissues. A schematic diagram of some of the principal ethylene modulators and their points of action is present herein (Fig. 2).

5. Genetic and Epigenetic Modulations of Ethylene Responses

Aside from the chemical modulation previously reviewed, the development of molecular techniques has helped to understand and modulate the effect of this hormone on plant tissues while contributing to detailed insights on the role of each component of the ethylene biosynthesis and signaling pathways. With genetic engineering it has been possible to alter both ethylene levels in plant tissues via either both ACS and ACO enzymes or its perception through the components of its signaling [45]. Studies using plant mutants with altered ethylene sensitivity or production [46,47] as well as transgenic plants with altered ethylene biosynthesis or perception [45] have been important contributions for the characterization of ethylene signaling and its responses as they are currently known.

Different mutants in *Arabidopsis*, with different mutations regarding the components of ethylene biosynthesis or signaling pathways, are well characterized. Depending on the type of mutation, the plant mutant can exhibit distinct phenotypes, such as ethylene insensitivity [34], ethylene constitutive responses (*i.e.*, exhibit the phenotype observed in plants treated with ethylene; [48]) and ethylene overproduction [49]. For instance, the *etr1-1* mutant, characterized by a missense mutation at *ETR1* gene, exhibits insensitivity to ethylene. This mutation alters a single amino acid at the N-terminus of ETR1 receptor in the ethylene-binding domain [33,34] and for this reason eliminates the capability to bind ethylene [50], comparable to what happens in Ag(I) treatments. Similar phenotypes are exhibited in both *etr2-1* and *ein4-1* mutants, with similar mutations to that of *etr1-1* in *ETR2* and *EIN4* genes, respectively [51,52]. These similarities seem to be in agreement with the conservation of the ethylene-binding domain among the receptor's isoforms. On the other hand, *ctr1-1* mutant exhibits an ethylene constitutive response. This mutant is characterized by a single amino acid substitution in the serine/threonine-protein kinase CTR1 at the highly conserved kinase residues, resulting in a disruption of its catalytic domain [48] and consequently inactivating its function. Regarding the ethylene biosynthesis, the recessive mutant *eto1* and the dominant mutants *eto2* and *eto3* are characterized by increasing the stability of ACS, exhibiting an ethylene overproduction phenotype [46,49,53]. These ethylene-overproducing mutants are characterized by mutations affecting the posttranscriptional regulation of ACS5 and ACS9 isoforms, resulting in an increase of ethylene biosynthesis [49].

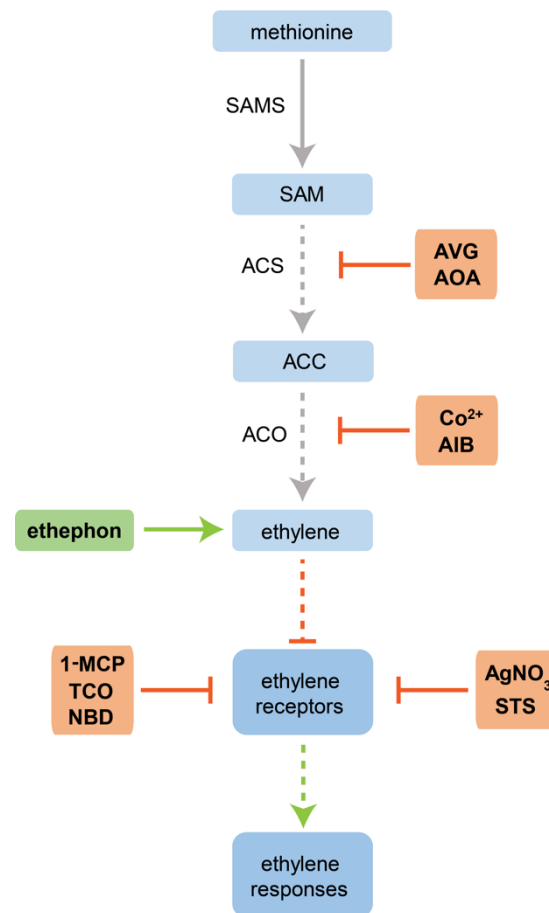


Figure 2. Schematic diagram of some of the principal ethylene modulators and their points of action. Adapted from [19].

Beyond the mutants described, modulating the amount of ethylene produced or its perception, through the creation of transgenic lines in several species, has been a goal of genetic engineering, specially, for commercial purposes [45] due to the effect of excessive amounts of ethylene in fruit ripening and senescence. Therefore, transgenic approaches have been applied using diverse molecular techniques, which have been allowed the silencing or the overproducing of the enzymes involved in ethylene biosynthesis, leading to different accumulations of this hormone in plant tissues. Most of these strategies entail the downregulation of ACS or ACO expression, leading to a reduction of its enzymatic activity and consequent reduction of ethylene production [27]. This modulation can be achieved using sense- or antisense-mediated gene silencing, co-suppression or using an RNA interference (RNAi) approach, with several studies carried out in a myriad of plant species [27,45,54-55]. Regarding an ethylene overproduction strategy, the overexpression of the ACO gene was achieved in poplar [56] and in safflower [57], by transforming these plants with an overexpression vector containing the open reading frame of the specific ACO. Note that ACO enzyme is the most studied and suitable target regarding genetic alterations of ethylene biosynthesis due to its reduced risk of interfering with other metabolic pathways, such as methionine cycle and ACC metabolism [27].

A promising strategy to alter ethylene responses can be the use of genome editing system CRISPR/Cas9, a potent system capable of generate efficiently mutations in specific targeted genes in plants [58]. In fact, very recently, Xu *et al.* [59] reported successfully the generation of ACO-edited mutants in petunia, which exhibited lower ethylene production, through the edition of the specific ACO gene using the CRISPR/Cas9 system.

Currently, using plants with these genetic alterations can give us a more specific approach of the effect of this hormone regarding both *in vivo* and *in vitro* processes, with a decrease of possible off-

targets. Therefore, these plants, especially the *Arabidopsis* mutants, are currently important models to understand the impact of ethylene in the success of several *in vitro* processes. However, the use of transgenic or mutant plants involves more extensive and expensive protocols, compared to the use of chemical inhibitors. A chemical approach is easier to manipulate and its effect can be also easily reverted (e.g., changing plant tissues to a non-treatment medium). Furthermore, these chemicals can be easily applied only at specific points of each *in vitro* process.

Studies related with the involvement of ethylene on epigenetic processes in plants are scarce. However, this line of research needs to be exploited since the precursor of ethylene biosynthesis S-adenosylmethionine is the donor of methyl groups for DNA methylation [60]. In animal cells [61], the levels of metabolic substrates such as SAM and S-adenosylhomocysteine (SAH), the derived compound resulting from the transfer of the methyl group of SAM to a donor, change the expression of genes related to the growth and health. In plants, ethylene is an extra player in this balance since the pool of SAM controls ethylene production [62] and may affect the activity of methyltransferases which catalyse methylation of a large spectra of substrates such as DNA and RNA, thus modifying transcription and translation processes and the subsequent control of mechanisms such as plant growth and response to biotic and abiotic stresses [3].

6. Ethylene Integration in Hormone and Stress-Induced *in Vitro* Plant Regeneration

Plant regeneration depends on cellular plasticity, *i.e.*, the ability of cells have to assume different differentiation pathways ending in different cellular fates [63]. The regeneration capacity differs from species to species and, within the same species, different explants can show different response to regeneration [64]. Juvenile explants, such as young leaves, or immature zygotic embryos, generally have high generation capacity [63]. *In vitro* plant regeneration can occur directly from explant or indirectly through a pre-induced pluripotent mass of cells, denominated callus [5]. A balance between auxins and cytokinins has a fundamental role in promoting organogenesis, inducing callus formation and subsequent shoot development [65-67]. SE is mainly triggered by auxins and by stress factors, such as osmotic and/or oxidative stress, leading to acquisition of embryogenic competence [68-69]. The crosstalk between ethylene with auxins and cytokinins has been progressively unveiled. In a brief overview, Swarup *et al.* [70] have reported an increase in the rate of the endogenous auxin synthesis, IAA, in *Arabidopsis* seedlings in response to 10 and 100 μM ACC. A decrease in IAA content in presence of 10 μM AVG was also observed, suggesting that ethylene upregulates auxin biosynthesis. Furthermore, it was also reported an increase of IAA concentration in presence of 100 μM ACC and a decrease of the IAA synthesis rate at 10 μM AVG in *Arabidopsis* roots [71]. Contrariwise, a reduction in free IAA content in the roots of the ethylene-overproducer mutant *eto1-1* and, albeit to lesser extent, when submitted wild-type *Arabidopsis* to 1 μM ACC was reported [72]. Furthermore, a recent work [73] showed also lower levels of free IAA in the same mutant, due to a downregulation of key auxins biosynthetic genes. Regarding auxin's effect in ethylene biosynthesis, exogenous IAA treatment (20 μM) applied to *Arabidopsis* seedling enhanced the constitutive expression of diverse ACS gene family members in roots [74]. ACS1 and ACS2 genes were also upregulated in presence of 50 and 100 μM IAA in watermelon leaves [75]. In what concerns to cytokinins, *Arabidopsis* seedlings treated with 5 μM of the synthetic cytokinin, benzyladenine (BA), has showed a higher ethylene production due to the stabilization of ACS5 isoform [49]. Similarly, it was found that BA treatments increase the stability of ACS5 and ACS9 isoforms in *Arabidopsis* [76], suggesting that cytokinin acts post-transcriptionally, leading to ACS stabilization and subsequent increase in ethylene production. Ethylene seems also to increase the levels of cytokinins modulated by an upregulation of key cytokinin biosynthetic genes as reported in the *Arabidopsis* mutant *eto1-1* [73].

Apart from the role of plant hormones in regeneration, wounding, initially applied to explants, seems to be essential to initiate both regeneration processes [63]. Wounding triggers stress responses, inducing transcriptional changes with marked changes in metabolism and protein synthesis. In turn, inherent regulators of the cell cycle are activated, leading to cell proliferation and consequent callus formation [63]. Wounding is also crucial to increase the levels of somatic embryogenesis responsiveness

in leaves of *Solanum betaceum* [77] and *Arbutus unedo* [78]. Interestingly, ethylene biosynthesis seems to be stimulated by wounding in several plant tissues [79-82]. Wounding seems to enhance ACS activity over time after cutting, leading to a greater ACC synthesis and consequently increases ethylene production [79]. This increase seems to be in agreement with the role of ethylene in several stress responses, since its role in both biotic [83-85] and abiotic [86,87] stresses has been recognised. Furthermore, wounding also promotes an accumulation of cytokinins at cutting sites leading to callus formation [63], which can also be related to the increase in ethylene biosynthesis [49,76].

As the success of regeneration processes highly depends on hormonal and stress induction, the influence of ethylene in key regeneration hormones, such auxins and cytokinins, and in stress responses, should be markedly considered.

7. Influence of Ethylene Modulation in Regeneration Processes

The influence of ethylene in *in vitro* processes started to be studied more deeply in the last years of the last century, with relevant reviews published by Biddington [78] and Kumar et al. [116]. More recently, numerous studies have been carried out regarding regenerating processes, such as axillary meristem culture, organogenesis and SE. In the next sections an overview of those studies will be presented and discussed in order to elucidate the effect of this hormone in *in vitro* regeneration. A detailed summary of several studies using chemical modulators or using mutants and transgenic lines is presented in Table 1 and Table 2, respectively.

7.1. Organogenesis

Ethylene modulation seems to have different impacts in the organogenesis process, depending on the species. In gloxinia, inhibiting ethylene perception, using silver thiosulfate, increased the number of shoots per leaf explants [91]. Furthermore, inhibition of ethylene biosynthetic enzymes, with AVG and cobalt chloride at 1 mg.L⁻¹, also increased the number of shoots per explant, albeit to a lesser degree when compared to silver thiosulfate treatments. However, at 10 and 20 mg.L⁻¹, both chemicals showed a negative impact on regeneration. Inhibition of ethylene perception seems also to positively affect shoot regeneration from cotyledons in melon [97]. Regeneration capacity has increased by about two-fold using 60 or 120 µM of silver nitrate. In turn, shoot regeneration capacity decreased in presence of 10 and 20 mg.L⁻¹ ETH treatments, where ethylene production was significantly higher compared to both control and silver nitrate treatments. In agreement, using leaves and cotyledons of a melon transgenic line (Table 2), expressing antisense ACO, greatly increased shoot regeneration [110]. Leaves and cotyledons show a 3.5 and 2.8 fold increase in regeneration capacity when compared to wild-type, respectively. Despite a great decrease of ACO activity in both transgenic explants, cotyledons showed a higher ethylene production compared with leaves, which can justify the differences observed in the regeneration response of both transgenic explants. Explants of wild-type or transgenic lines treated with ETH showed a highly decrease in regeneration capacity and, at the higher ETH concentration (100 µM) a complete inhibition of regeneration was reported. A considerable increase of shoot regeneration capacity from hypocotyls of mustard in presence of 3 mg.L⁻¹ silver nitrate was also detected [98]. Also in mustard, both ethylene inhibitors AVG and silver nitrate enhanced the shoot regeneration in leaf discs and petioles [99], whereas combined treatments with ethylene inhibitors and ETH decreased the regeneration capacity in leaf explants. Pre-treated leaf explants with silver nitrate also showed a decrease in shoot regeneration capacity in further organogenesis induction with silver nitrate. However, shoot regeneration capacity of the same explants was not affected in control or AVG treatments [99].

Table 1. Effect of ethylene modulators on different regeneration systems of diverse plant species. Ethylene modulators in the fifth column showed an increase (↑) or a decrease (↓) in the respective parameter when compared to control.

Plant species	Process	Explant	Modulation	Effect	Ref.
<i>Arabidopsis thaliana</i>	SAM-SE system ^a	Seedlings ^a	25 µM ACC; 10 µM AVG or AgNO ₃ ; 100 µM CoCl ₂	<u>Seedlings with embryos (%)</u> : ↑ in ACC treatment (≈ 40%); ↓ in AVG, AgNO ₃ and CoCl ₂ treatments (< 10%); control: ≈ 25%	[88]
Black spruce (<i>Picea mariana</i>)	Somatic embryo maturation	Two embryogenic cell lines, with low (a) or high (b) embryogenic capacity	5, 10 and 100 µM ACC; 0.5, 1 and 2 mM AgNO ₃ ; 5, 10 and 100 µM AOA; 5, 10 and 30 µL.L ⁻¹ C ₂ H ₄	<u>Total embryos formed</u> : ↓ in 100 µM ACC for (a) and ↓ in 10 and 100 µM ACC for (b); ↓ in 30 µL.L ⁻¹ C ₂ H ₄ for (a), but C ₂ H ₄ treatments not affect (b) significantly; ↑ in 10 µM AOA and ↓ 100 µM AOA for (a); ↓ in 10 and 100 µM AOA for (b); ↑ in 1 mM AgNO ₃ for (a) and ↓ in 1 and 2 mM AgNO ₃ for (b) <u>Ethylene production in both lines</u> : C ₂ H ₄ production increased during somatic embryo maturation for (a) and C ₂ H ₄ production maintained constant and low for (b)	[89]
Carrot (<i>Daucus carota</i>)	SE	Embryogenic cell suspension induced from hypocotyls	10, 20, 50 and 100 µM CoCl ₂ ; 10 and 100 mg.L ⁻¹ ETH	<u>No. somatic embryos formed (No./mL of cell suspension)</u> : ↑ in 10, 20 and 50 µM CoCl ₂ , but ↓ in 100 µM (best treatment, 79 No./mL at 50 µM; control: 23 No./mL); ↓ in both ETH treatments; ↓ in 50 µM CoCl ₂ + 10 or 100 mg.L ⁻¹ ETH	[90]
Gloxinia (<i>Sinningia speciosa</i>)	Org ^b	Leaf explants	1, 5, 10 and 20 mg.L ⁻¹ AVG, CoCl ₂ or STS	<u>Regeneration (%) and shoots per explant</u> : ↑ in 5 and 10 mg/L STS (best treatment, 5 mg/L STS, 40% more shoots/explant); ↑ in 1 mg/L AVG or CoCl ₂ and ↓ in 10 and 20 mg/L AVG or CoCl ₂	[91]
Lemon (<i>Citrus limon</i>)	Org	Adult nodal segments from two cultivars a) Verna 51 and b) Fino 49	10, 20 and 30 µM ACC, CoCl ₂ or STS; 5, 10 and 20 µM ETH	<u>Regeneration (%)</u> : ↓ in 10, 20 and 30 µM ACC for both; ↓ in 10 and 20 µM ETH for b); ↑ in 10 and 20 µM STS for both; ↓ in 10 and 20 µM CoCl ₂ for b) and ↓ 30 µM CoCl ₂ for both <u>Buds per explant</u> : ↓ in 10, 20 and 30 µM ACC for both and ↓ in 30 µM CoCl ₂ for a)	[92]
Yellow passionfruit (<i>Passiflora edulis</i>)	Axillary bud culture	Nodal segments	3 and 10 µM ACC, STS or AVG	<u>Buds and leaf area per explant</u> : ↓ in 3 and 10 µM ACC and ↑ in 3 and 10 µM STS or AVG treatments <u>Shoot length per explant</u> : ↓ in 3 and 10 µM ACC treatments	[93]
Alfalfa (<i>Medicago sativa</i>)	SE	Petioles and petiole-derived EC	Between 0 and 4.1x10 ⁻⁴ M NBD	<u>Callus induction/explant and embryo maturation</u> : ↓ as NBD concentration rises, but callus pre induced with NBD forms SE	[94]
			5 and 50 µM AVG	<u>Somatic embryo differentiation and EC proliferation</u> : ↓ in 50 µM AVG; Somatic embryos at cotyledonary stage are reduced in all treatments, but EC induction is not significantly affected by AVG	[95]

		EC induced from petioles (suspension culture in liquid medium)	1, 10, 100 or 500 μM AVG or SA; 4.5×10^{-8} , 9×10^{-8} or 1.12×10^{-7} M 1-MCP	<u>EC proliferation</u>: $\downarrow$ in 10, 100 and 500 μM SA (-50, -70 and -90%, respectively); $\downarrow$ in all AVG treatments (-25, -40, -55 and -90%); $\downarrow$ in all 1-MCP treatments (-30, -40 and -60%) <u>Embryogenic potential of treated suspension cultures (no. of somatic embryos formed)</u>: $\downarrow$ in all SA treatments (-25, -70, -80 and -90%); $\downarrow$ in 10, 100 and 500 μM AVG (-30, -50 and -90%, respectively) and also $\downarrow$ in all 1-MCP treatments <u>Somatic embryo development</u>: $\downarrow$ somatic embryos at cotyledonary stage in 1 and 10 μM SA (-25 and -70%, respectively) but $\uparrow$ somatic embryos at globular stage in 100 μM SA; $\uparrow$ globular embryos in all AVG treatments; cotyledonary embryos $\downarrow$ in 1, 10 and 100 μM AVG (-25, -35 and -40%, respectively); in all 1-MCP treatments $\uparrow$ no. of globular embryos but further development was blocked	[96]
Melon (<i>Cucumis melo</i>)	Org	Cotyledons ^b	60 or 120 μM AgNO ₃ 10 or 20 mg.L ⁻¹ ETH	<u>Shoot regeneration (%)</u>: $\uparrow$ in all AgNO₃ treatment for all genotypes; line with best shoot regeneration, 75% at 60 μM and 68% at 120 μM (control 35%) <u>Shoot regeneration (%) for the best line</u>: $\downarrow$ in both ETH treatments (19% and 10% at 10 mg.L⁻¹ and 20 mg.L⁻¹ ETH, respectively)	[97]
Mustard (<i>Brassica juncea</i> L.)	Org	Hypocotyls	3 mg.L ⁻¹ AgNO ₃	<u>Shoot regeneration (%)</u> : $\uparrow$ in AgNO ₃ treatment with 95.89% shoot regeneration (control: 14.6%)	[98]
		Leaf disc and petioles	20 μM AgNO ₃ and 5 μM AVG; AgNO ₃ or AVG with 10, 25 or 50 μM ETH (combined)	<u>Shoot regeneration (%) from both explants</u>: $\uparrow$ in both AgNO₃ and AVG treatments, with 80-90% (control: 20-30%) <u>Shoot regeneration (%) in combined treatments (leaf explants)</u>: $\downarrow$ in 25 or 50 μM ETH + AVG (50 μM ETH + AVG almost inhibited regeneration and slight $\downarrow$ in ETH + AgNO₃)	[99]
	Plant growth	Shoots	20 μM AgNO ₃ , 5 μM AVG or 50 μM ETH	<u>Plant growth parameters, such as plant height, number of leaves, number of roots and root length</u> : $\downarrow$ in both AgNO ₃ and ETH treatments (AVG does not have a very significant effect on the same parameters)	
Poplar (<i>Populus tremula</i>)	Org	Nodal segments	5, 10 and 15 μM AVG; 0.5, 1 and 5 μM ACC; 5 and 10 μM ETH	<u>Shoot elongation and no. of buds and roots/explant</u> : $\downarrow$ in 10-15 μM AVG, $\uparrow$ in 5 μM ACC and $\uparrow$ in 10 μM ETH	[100]

Robusta coffee (<i>Coffea canephora</i>)	SE	Leaf squares (two genotypes)	30, 60, 150 or 300 μ M AgNO ₃	<u>Number of embryos per explant</u> : $\uparrow$ in 30 and 60 μ M treatment and $\downarrow$ in 150 and 300 μ M treatment; One genotype shows the great yield at 30 μ M (+57%) and the other at 60 μ M (+60%)	[101]
		EC developed from hypocotyl and leaf explants	20 and 40 μ M AgNO ₃ , CoCl ₂ or SA	<u>Callus responded for embryogenesis (%)</u> : $\uparrow$ in all AgNO ₃ treatments (best treatment, 40 μ M, 48%); $\uparrow$ in all CoCl ₂ treatments (best treatment, 40 μ M, 28%); $\uparrow$ in all SA treatments (best treatment, 40 μ M, 32%), control 5% <u>No. of somatic embryos per callus</u> : $\uparrow$ in both all AgNO ₃ and AVG treatments (best treatment, at 40 μ M, 153 and 45 embryos, respectively); in all SA treatments only pro-embryogenic nodular masses appeared (control, did not produce somatic embryos)	[102]
Soybean (<i>Glycine max</i>)	SE	Cotyledons from cultivars with different embryogenic capacity	10 μ M ACC or AVG	Somatic embryo production: $\uparrow$ in ACC treatment for two recalcitrant cultivars (slight increase, but not significantly, for cultivar with high embryogenic capacity); $\downarrow$ in AVG treatment (almost inhibited) for both two recalcitrant cultivars and cultivar with high embryogenic capacity	[88]
Spinach (<i>Spinacia oleracea</i>)	SE	Roots	1, 10, and 100 μ M ETH or AgNO ₃ 1 and 10 μ M AVG	<u>Embryogenic callus (%)</u> : $\downarrow$ in all AgNO ₃ and AVG treatments and $\uparrow$ in 10 and 100 μ M ETH (only in combination w/ 0.1 μ M GA ₃) <u>Calli forming embryos (%)</u> : $\downarrow$ in all ETH treatments (somatic embryos formation inhibited at 100 μ M) and $\uparrow$ in all AgNO ₃ treatments <u>No. embryos/calli</u> : $\downarrow$ in all ETH treatments and $\uparrow$ in all AgNO ₃ treatments (best treatment, 10 μ M AgNO ₃ , 3x more embryos)	[103]
Summer snowflake (<i>Leucojum aestivum</i>)	SE	EC	10 μ M ACC, AgNO ₃ or STS KMnO ₄ (4.5 g solid)	<u>EC increment (%)</u> : $\uparrow$ in both AgNO ₃ and in KMnO ₄ ; $\downarrow$ in both ACC and STS treatments <u>Somatic embryo induction and maturation</u> : $\uparrow$ in ACC treatment <u>Length of plantlets development</u> : $\uparrow$ in KMnO ₄ treatment	[104]
Tomato (<i>Solanum pennellii</i> and <i>Solanum lycopersium</i>)	Org	Leaves from two genotypes (<i>Solanum pennellii</i> and F1: <i>Solanum pennellii</i> vs <i>Solanum lycopersicum</i> cv. Anl27)	5.8, 14.5, 29 and 58 μ M AgNO ₃ ; 4.2, 10.5, 21 and 42 μ M CoCl ₂ ; 9.8, 24.5, 49 and 98 μ M ACC; 6.9, 17.2, 34.5 and 69 μ M ETH	<u>Explants with buds (%)</u> : $\downarrow$ in 14.5, 29 and 58 μ M AgNO ₃ for <i>S. pennellii</i> ; $\downarrow$ in 21 μ M CoCl ₂ for F1; $\downarrow$ in 98 μ M ACC for both genotypes; $\downarrow$ in 17.2, 34.5 and 69 μ M ETH for <i>S. pennellii</i> and $\downarrow$ in 34.5 and 69 μ M ETH for F1; (lowest percentage at 69 μ M ETH for both <i>S. pennellii</i> and F1, with 20% and 16%, respectively) <u>Explants with shoots (%)</u> : $\downarrow$ in 14.5, 29 and 58 μ M AgNO ₃ for <i>S. pennellii</i> ; $\downarrow$ in 21 μ M CoCl ₂ for F1; $\downarrow$ in 24.5 and 98 μ M ACC for both	[105]

				<i>S. pennellii</i> and F1; ↓ in all ETH treatments for both genotypes (lowest percentage at 69 μM ETH for both <i>S. pennellii</i> and F1, with 12% and 8%, respectively) <u>No. of shoots per explant with shoots</u>: ↓ in all AgNO₃ treatments for <i>S. pennellii</i>; ↑ in 9.8, 49 and 98 μM ACC for <i>S. pennellii</i> (↑ x2 more compared to control (≈5 shoots), with ≈10 shoots); ↓ in 17.2, 34.5 and 69 μM ETH for <i>S. pennellii</i>; (lowest no. at 58 μM AgNO₃ and 69 μM ETH with 0.96 and 1, respectively) All AgNO₃ treatments inhibited regeneration for F1	
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^a Shoot apical meristem-somatic embryo (SAM-SE) system, characterized by somatic embryos developed at the shoot apical meristem from seeds germinated in presence of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) as described by Mordhorst et al. [106].

^b Org – Organogenesis.

Furthermore, pre-treated plants with AVG maintained the same regeneration capacity in posterior induction with both inhibitors. This suggests that mustard plants can be more sensible to Ag (I) and to its accumulation than to AVG. Actually, in the same study, further plant growth parameters were negatively affected by silver nitrate, but not by AVG treatment. Shoot regeneration from leaf discs or hypocotyl segments of transgenic lines of mustard (Table 2), expressing antisense ACO, was greatly enhanced compared to wild-type as reported by Pua and Lee [99]. Shoot regeneration (%) in these transgenic lines was similar (about 90%, Table 1) to that reported when a chemical approach using silver nitrate and AVG was used [98,99]. In lemon, ACC and ETH treatments seems to decrease shoot regeneration in adult nodal segments, while silver thiosulfate treatments enhanced it [92]. Induced organogenesis in leaves of two genotypes of tomato (*Solanum pennellii* and F1: *Solanum pennellii* vs *Solanum lycopersicum* cv. Anl27; Table 1) revealed that an excessive amount of ETH or ACC negatively affected the percentage of explants showing buds in both genotypes, whereas lower concentrations of both ethylene precursors had not relevant effect [105]. Silver nitrate also negatively affected the number of explants with buds in *Solanum penelli* and, interestingly, completely inhibited regeneration from F1 explants. Cobalt chloride decrease this percentage from F1 explants at only 21 μM , but not affected *Solanum pennellii*. ETH treatments showed a decrease regarding explants with shoots (%) for both genotypes similarly to silver nitrate treatments in *Solanum penelli*. Although the negative effect of both ETH and silver nitrate in the number of shoots per explant with shoots (%) in *Solanum penelli*, ACC treatments enhanced this parameter. These results show that different genotypes have different responses and sensitivity to ethylene and/or its modulation, affecting its regeneration capacity.

In poplar (Table 1), ethylene precursors, ACC (5 μM) and ETH (10 μM), positively affect regeneration and subsequent plant development and growth, in parameters such shoot elongation, induction and development of buds and also root formation per explant [100]. On the other hand, AVG treatments in a range of 10-15 μM negatively affected these parameters. Experiments carried out during shoot regeneration in cotyledons of different *Arabidopsis* mutants (Table 2) showed that shoot regeneration decreased in ethylene insensitive mutants, *etr1-1* and *ein2-1*, whereas it increased in ethylene constitutive responses mutants, *ctr1-1* and *ctr1-12*, and also in ethylene overproducer mutant, *eto1-1*, suggesting a positive role for ethylene on *Arabidopsis* organogenesis [107].

7.2. Somatic Embryogenesis

First studies in SE process in carrot (Table 1) showed that inhibition of ACO, using cobalt chloride, increased the number of somatic embryos formed from embryogenic cell suspensions [90]. Furthermore, ethylene measurements confirmed that the number of somatic embryos increased with the decrease of ethylene production. The negative effect of ethylene in carrot SE was further confirmed with ETH treatments, in each the number of somatic embryos formed decreased [90]. In robusta coffee [101], the development of somatic embryos from leaf explants of two genotypes greatly increased in presence of silver nitrate treatments (30 and 60 μM). In this species, high silver nitrate concentrations (150 and 300 μM) reduced somatic embryo formation, perhaps, due to its toxicity to plant tissues, rather than due to an inhibitory effect on ethylene perception. Interestingly, the two different genotypes showed a similar great yield in somatic embryo formation ($\approx +60\%$) but at different silver nitrate concentrations, one at 30 and the other at 60 μM , suggesting different sensibilities to ethylene according to the genotype. The effect of ethylene modulators on somatic embryo development from embryogenic callus in robusta coffee was also analysed [102]. The data showed that somatic embryo development was poor in the control (no modulators used), whereas in the presence of silver nitrate, cobalt chloride or salicylic acid, this recalcitrance seemed to be reversed and an increase in somatic embryo formation was

observed. However, results showing a positive effect of ethylene on direct SE from leaf explants of the same species were also reported [117]. SE was directly induced in leaf explants by cytokinin in both Fuentes *et al.* [101] and Hatanaka *et al.* [117] studies. However, a pre-culture of 5 days of leaf explants in induction medium without silver nitrate was carried out in the work of Fuentes *et al.* [101]. This can somehow explain the differences observed, suggesting that ethylene can be necessary in an initial phase to promote embryogenic competence in leaf explants, but later disrupt somatic embryo development. This could justify why an initial ethylene inhibition disrupted direct SE from leaf explants [117], but its later inhibition not affected somatic embryo development [101].

Preliminary studies carried out in our lab with a solanaceous tree species (*Solanum betaceum*; commonly known as tamarillo), showed that at the induction stage, the leaf explants exposed to AgNO₃ and AVG only produced non-embryogenic callus, whereas ETH significantly increased the induction of embryogenic tissue [118]. Furthermore, ETH treatments accelerated the induction of embryogenic callus. During somatic embryo development, following embryogenic callus transfer to an auxin-free medium, the treatment with AgNO₃ and AVG enhanced the number of somatic embryos developed from embryogenic callus, whereas the presence of ETH blocked development beyond the globular stage. Moreover, as found in other species, ethylene may be involved at different steps of somatic embryogenesis induction of tamarillo, from induction to embryo development. These results indicate that ethylene certainly has a role during somatic embryo formation and development in *S. betaceum*, but further studies are necessary to clarify how this hormone interact with other players also crucial for somatic embryo formation in this species, such as auxins and high sucrose levels [77].

In black spruce (Table 1), a gymnosperm, the effect of inhibitors of ethylene biosynthesis and perception, AOA and silver nitrate, and also of ACC and pure ethylene on somatic embryo maturation from two cell lines with different embryogenic capacity was analysed [89]. Inhibiting ACS activity with 10 μ M AOA increased the total number of somatic embryos developed from a cell line with low embryogenic capacity. However, with the same AOA treatment, a decrease in the number of somatic embryos developed from a high embryogenic cell line was observed. Similar results were reported both lines when treated with silver nitrate. Adding pure exogenous ethylene (30 μ L.L⁻¹) along with 10 μ M AOA treatments reverted the previous effect of AOA observed in both cell lines, confirming that somatic embryo formation in this specie is somehow regulated by ethylene. Nevertheless, higher AOA concentration (100 μ M) has a negative impact in somatic embryo formation on both lines. When using the ethylene precursor ACC, the formation of somatic embryos decreased at 100 μ M for both cell lines and 10 μ M also negatively affected this parameter, although, only for the high embryogenic line. Exogenous treatments with pure ethylene at 30 μ L.L⁻¹ also decreased the number of somatic embryos for the low embryogenic line, without affecting significantly the line with high embryogenic capacity. The differences in embryogenic capacity seems to be related to differences in ethylene production in both cell lines. While in high embryogenic cell lines, ethylene production remains constant and low, at a supposing optimal concentration, low embryogenic cell lines have a higher ethylene production, showing a suppose supraoptimal ethylene concentration. Therefore, in this species ethylene below or above an optimal concentration seems to be directly correlated with low embryogenic regeneration capacity.

Table 2. Effect of ethylene modulators, mutants or transgenic lines on different regeneration systems of diverse plant species. Ethylene modulators/mutants or transgenic lines in the fifth column showed an increase (↑) or a decrease (↓) in the respective parameter when compared to control.

Plant species	Process	Type of explant	Modulation	Effect	Ref.
<i>Arabidopsis thaliana</i>	Org ^a	Cotyledons	Ethylene mutants	<u>Shoot regeneration (%)</u> : ↓ in ethylene insensitive mutants (<i>etr1-1</i> and <i>ein2-1</i>), ↑ in ethylene constitutive responses mutants (<i>ctr1-1</i> and <i>ctr1-12</i>) and ↑ in ethylene overproduction mutant (<i>eto1-1</i>)	[107]
	SE	Embryonic calli (induced from primary somatic embryos pre induced from immature zygotic embryos)	10, 20, 50, 100, 150 and 200 μM ACC; ethylene mutants	<u>Somatic embryo regeneration/embryonic calli</u> : ↓ as ACC treatment concentration rises, 100 and 150 μM greatly decreases somatic embryo production and 200 μM almost inhibited its regeneration; ↓ in both ethylene overproduction mutant (<i>eto1-1</i>) and ethylene constitutive responses mutant (<i>ctr1-1</i>); <i>ctr1-1</i> almost inhibited somatic embryo formation; (ethylene insensitive mutants (<i>etr1-3</i> and <i>ein2-1</i>) and double ACS mutant (<i>acs2-1 acs6-1</i>) do not affect somatic embryos production)	[108]
		Immature zygotic embryos	1, 5, 10 μM ACC; 1, 10 μM CoCl ₂ ; 1, 10, 15 μM AVG; 1, 10, 100 μM AgNO ₃ or 250 mM KMnO ₄ ; ethylene mutants	<u>Explants that formed somatic embryos (%)</u> : ↓ in 1, 5 and 10 μM ACC; in 10 μM CoCl ₂ ; in 10 and 15 μM AVG; in 10 and 100 μM AgNO ₃ and also in 250 mM KMnO ₄ treatments (lower % at both 10 μM ACC and 10 μM CoCl ₂ treatments ≈ 20%, control ≈ 90%) <u>Average no. of somatic embryos produced/explant</u> : ↓ in 1, 5 and 10 μM ACC; in 10 μM CoCl ₂ ; in 10 and 15 μM AVG and also in 100 μM AgNO ₃ treatments Both parameters ↓ in ethylene insensitive mutants, in ethylene constitutive responses mutant and in ethylene over- and under-producer mutants	[109]
Melon (<i>Cucumis melo</i>)	Org	Leaves and cotyledons	Transgenic plant line expressing antisense ACO; 50 or 100 μM ETH	<u>Shoot regeneration (%) from leaf explants</u> : ↑ in transgenic line with 53% (control 15%) <u>Shot regeneration (%) from cotyledon explants</u> : ↑ in transgenic line with 37% (control 13%) <u>Shoot regeneration (%) from both explants in response to ETH</u> : ↓ in all ETH treatments (for both explants); <u>transgenic leaf explants</u> + 50 μM ETH treatment shows 5% shoot regeneration and + 100 μM	[110]

				ETH treatment regeneration was inhibited; <u>transgenic cotyledon explants</u> + 50 μ M ETH treatment shows 8% shoot regeneration and + 100 μ M ETH treatment shows 1%	
<i>Medicago truncatula</i>	SE	Two different genotype leaf-derived EC lines, with different embryo production capability	0.1, 1, 10 and 100 μ M ACC, MGBG ^b , AgNO ₃ or AVG; ACS and ACO expression; MtSERF1 ^c knockdown (using RNAi)	<u>No. of somatic embryos developed/explant</u> : $\uparrow$ in 1 and 10 μ M ACC and in 10 and 100 μ M MGBG, best treatments 10 μ M ACC and 100 μ M MGBG, $\approx$ 35 embryos/explant (control $\approx$ 12); $\downarrow$ in 1 and 10 μ M AVG or AgNO ₃ and completely inhibited at both 100 μ M AVG and AgNO ₃ treatments; <u>ACS and ACO expression</u> : $\uparrow$ in line with highly embryo production capacity; <u>MtSERF1 knockdown</u> : Disrupt somatic embryo production	[111]
Mustard (<i>Brassica juncea</i>)	Org	Leaf discs and Hypocotyl segments	10 transgenic plant lines expressing antisense ACO; 5 μ M AVG, 10 μ M AgNO ₃ or 50 μ M ETH (alone or combined)	<u>Shoot regeneration (%) from leaf explants</u> : $\uparrow$ in 9 transgenic lines, between 58% and 92% (control 12-16%), 4 best lines % (83, 79, 80, 92); <u>Shoot regeneration (%) from leaf explants in response to inhibitors and/or ETH</u> : $\uparrow$ in WT plants + AVG or + AgNO ₃ treatments (similar % compared with the 4 transgenic lines w/o treatment), (WT plants + AgNO ₃ + ETH $\approx$ 60%); Both WT plants + AVG + ETH (%) and the 4 transgenic lines + ETH (%) are similar to control; <u>Shoot regeneration (%) from hypocotyl explants</u> : $\uparrow$ in the 4 best transgenic lines, 85-95% (control $\approx$ 5%)	[112]
Potato (<i>Solanum tuberosum</i>)	Plant growth; Callus formation	Nodal segments with unfolded leaf	Different ventilations: a) sealed with silicone rubber bungs; b) capped with polypropylene discs and c) forced ventilation; 3 μ M AgNO ₃ or 2 μ M ACC, applied to cultures under different ventilations	<u>Leaf area/explant</u> : $\uparrow$ in both a) + AgNO ₃ and b) + AgNO ₃ ; $\uparrow$ in a) + ACC and $\downarrow$ in b) + ACC <u>Steam length</u> : $\uparrow$ a) + ACC and $\downarrow$ in both b) + ACC and c) + ACC <u>Roots/explant</u> : $\uparrow$ a) + ACC and $\downarrow$ in both a) + AgNO ₃ and b) + AgNO ₃ <u>Root length/explant</u> : $\downarrow$ in both a) + ACC and b) + ACC and $\uparrow$ in both a) + AgNO ₃ and b) + AgNO ₃ <u>Ethylene concentration in vessels</u> : low ethylene concentration in both b) and c) vessels (similar) and high concentration in a) vessels	[113]

Scots pine (<i>Pinus sylvestris</i>)	SE	Embryogenic cell line cultures with distinct embryogenetic capacity	ACS1 and ACS2 expression and ethylene production during different SE stages	<u>ACS1 transcript</u> is accumulated throughout the lines with different embryogenic capacity and also in somatic embryos, similarly; <u>ACS2 transcript</u> is accumulated only in somatic embryos (the ethylene production is only greatly detected in somatic embryos); ↑ ACS2 gene expression levels in the cell line with higher embryogenic capacity; embryos at cotyledonary stage showed highly ethylene production and during germination into plantlets ethylene production is greatly reduced	[114]
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^a Organogenesis.

^b Methylglyoxal bis(guanylhydrazone), inhibits polyamine biosynthesis, polyamines synthesis use SAM as precursors and its inhibition stimulates ethylene biosynthesis [115].

^c SOMATIC EMBRYO RELATED FACTOR1 (*SERF1*), which its expression depends on ethylene biosynthesis and perception [111].

In scots pine [114] the expression of *ACS1* and *ACS2* genes during different SE stages in cell lines with different embryogenic capacity was studied (Table 2). Although *ACS1* gene was similarly expressed throughout the different lines and in different SE developmental stages, *ACS2* gene was markedly expressed only in somatic embryos, along with high ethylene production. The line with the higher embryogenic capacity showed a positive correlation between the number of somatic embryos developed, ethylene production and *ACS2* expression. Ethylene production increased throughout somatic embryo development, greatly detected at cotyledonary stage, and decreased during subsequent somatic embryo conversion and germination [114].

In spinach (Table 1), SE was induced from root explants using inhibitors of ethylene biosynthesis and signaling, silver nitrate and AVG, and also ETH. A decreased on the induction of embryogenic callus from spinach roots was observed in silver nitrate and AVG treatments, while using ETH, at 10 and 100 μM , significantly enhanced the percentage of embryogenic callus induced [103]. However, during somatic embryo formation, ETH negatively affected the number of somatic embryos produced per explant, whereas inhibiting ethylene perception with silver nitrate greatly increased the number of somatic embryo developed per calli, the highest being a 3-fold increase (at 10 μM) when compared with the control. Once again, the results show that the effect of ethylene depends on the stage of somatic embryo differentiation.

Inhibition of ethylene perception in summer snowflake (Table 1) by silver nitrate or using potassium permanganate, as an ethylene absorbent, increased the proliferation of embryogenic callus [104]. However, this proliferation was negatively affected by silver thiosulfate. Despite the negative impact of ACC on the embryogenic callus proliferation, a great increase on the number of somatic embryos formed and their posterior maturation was reported in ACC treatment. A great number of somatic embryos at torpedo stage in presence of ACC was observed, when compared with both the control and other treatments, suggesting that ethylene is essential for somatic embryo development in *Leucojum aestivum*.

Assays in *Arabidopsis* (Table 2) to evaluate the effect of ACC and ethylene mutants in somatic embryo formation from embryogenic calli [108]. The data showed that as ACC concentration rised, the number of somatic embryos formed per embryogenic callus decreased. A similar effect was observed in both ethylene overproducer mutant, *eto1-1*, and ethylene constitutive response mutant, *ctr1-1*. Along these results, embryogenic calli treated with ACC treatments or from the mutant *ctr1-1* showed a downregulation in *YUCCA* genes expression. *YUCCA* genes are known to encode for the key enzymes of auxin biosynthesis [119] and their requirement to induce SE in *Arabidopsis* from embryogenic calli is known [108]. Taken together, these results provided evidence of the hormonal regulation required to induce SE in *Arabidopsis*' embryogenic tissue, suggesting that ethylene negatively affects SE in this species through inhibition of auxin biosynthesis. Using immature zygotic embryos as explants [108], at first glance, the ethylene effect is not so clear when only ethylene modulators are used, since both ACC and silver nitrate negatively affected the SE process. In other words, SE efficiency (*i.e.*, the percentage of explants that formed embryos) and SE productivity (*i.e.*, the average of somatic embryos produced per explant) decreased in the presence of ETH, but also decreased in presence of inhibitors of ethylene biosynthesis and perception (see Table 2 for more details). However, AVG and cobalt chloride at low concentration (1 μM) do not affect these parameters. Using ethylene mutants, with different and opposite phenotypes, a decrease in the SE efficiency and productivity was also observed, likely to what was observed with the chemical modulation. Taking these results into account, and knowing that in control conditions SE efficiency reaches 90%, it can be concluded that immature zygotic embryos have *per se* an optimal ethylene production, at the induction conditions, which includes 5 μM 2,4-D and 20 $\text{g}\cdot\text{L}^{-1}$ sucrose [108], promoting an efficient SE process. Furthermore, in this work, the authors also studied the effect of the *ETHYLENE RESPONSE FACTOR022* (*ERF022*) gene on *Arabidopsis* SE. *ERF022* is a member of the ethylene response factor (ERF) family genes, which are associated with plant responses to stress [120]. It was also found that a strong inhibition of *ERF022* gene is associated with SE induction in *Arabidopsis* [109]. However, data have also shown that the knock-out *erf022* mutant, exhibited a reduced capacity for SE [121]. Confronting these

results, it was suggested that *ERF022* expression is required to induce SE in *Arabidopsis*. In this regard, the authors [121] also studied a putative molecular function behind this phenomenon. They reported a downregulation of *ACS7*, *ERF1* and *ETR1* gene expression, suggesting that *ERF022* negatively controls ethylene biosynthesis and perception. Furthermore, and even more interesting, they found that *erf022* mutant exhibited a great inhibition of *LEAFY COTYLEDON2 (LEC2)* gene expression, along with the impaired capacity of SE induction. *LEC2* was reported as a promoter of SE in *Arabidopsis* via *YUCCA*-mediated auxin biosynthesis [122]. In line with this, *erf022* mutation was correlated with a downregulation of *YUC1* and *YUC4* gene expression, along with reduced levels of the endogenous auxin, IAA. This study provides evidence that SE induction from embryogenic calli in *Arabidopsis* is based on an ethylene-auxin crosstalk, mediated by *ERF022-LEC2* interaction [122].

In the case of alfalfa, a nice model to analyse somatic embryogenesis, the embryogenic competence, in the presence of NBD, an inhibitor of ethylene perception, was considerably reduced [94]. Further embryo maturation also decreased in the presence of this ethylene receptor's competitive inhibitor. In another study with this species [95], both embryogenic callus proliferation and somatic embryo maturation decreased in presence of 50 μ M AVG. The same group [96] also studied the effect of inhibitors of ethylene biosynthesis and perception on SE induction in alfalfa (see in detail in Table 1). In agreement with the two last studies, these modulators negatively affected some important SE stages, such as somatic embryo formation and further development and maturation. AVG and salicylic acid showed a great disruption on somatic embryo development, with a great decrease on the number of somatic embryos in later developmental stages, such as at the cotyledonary stage; in some cases with only globular somatic embryos being observed. Interestingly, in *Medicago truncatula* (Table 2), a specie from the same genus as alfalfa (*Medicago sativa*), treatments with the ethylene precursor ACC and MGBG increased the number of somatic embryos developed per embryogenic callus, while silver nitrate and AVG greatly decreased the somatic embryo formation [111]. Furthermore, when high silver nitrate and AVG concentrations (100 μ M) were tested, somatic embryo formation was completely inhibited. Moreover, a positive correlation between *ACS* and *ACO* gene expression and the genotype with higher embryo production capacity was found, suggesting that ethylene is required for somatic embryo formation and development in this species [111]. Interestingly, *ACS* and *ACO* expression in somatic embryos was found to be similar to the patterns of expression detected in zygotic embryos. The authors also reported an upregulation of ethylene responsive genes when a transcriptional profile analysis of embryogenic calli was carried out. It was found that a member of ERF family, denominated *MtSERF1* was highly expressed in embryogenic calli and strongly expressed in globular somatic embryos. The same gene was poorly in a line with low embryogenic capacity. Furthermore, ethylene-dependent *MtSERF1* expression was proved by its inhibition with AVG and silver nitrate. These data, together with the fact that silencing *MtSERF1* using RNAi completely inhibited the somatic embryo formation [111], prove that ethylene is a key factor during somatic embryogenesis induction, probably mediating the action of other hormones, such as auxins and cytokinins. *MtSERF1* orthologs genes [88] were found in *Arabidopsis thaliana* (*At5g61590*) and soybean (*GmSERF1* and *GmSERF2*), which are involved in the regulating of *AGAMOUS-Like15 (AGL15)*. This gene promotes SE in the SAM of *Arabidopsis* seedlings [123] and in soybean [124], being upregulated by auxin, in particular, 2,4-D [124].

Ethylene modulation has been tested in both soybean and *Arabidopsis* somatic embryogenesis (see Table 1). The *Arabidopsis* SAM-SE system [106], is highly promoted by the ethylene precursor ACC, with an increase in number of seedlings with somatic embryos. Furthermore, a reduction in the number of seedling with embryos was observed in presence of the inhibitors of ethylene biosynthesis and perception, such AVG, cobalt chloride and silver nitrate, suggesting a positive correlation between SE and ethylene. Similar results were observed in different cultivars of soybean, with different embryogenic capacity [88]. The number of somatic embryos increased with ACC treatments and decreasing with AVG, with the positive impact of ACC treatments highly marked in the recalcitrant cultivars. The relationship between ethylene/*At5g61590/AGL15* and ethylene/*GmSERF1/GmAGL15* in the induction of SE of *Arabidopsis*

and soybean, respectively, where confirmed by the transcript accumulation of *At5g61590* and *GmSERF1* in response to ethylene modulators. Both *At5g61590* and *GmSERF1* transcript levels were enhanced in ACC treatment, and, in turn, a decreased in transcript levels of both genes in presence of inhibitors of ethylene biosynthesis and perception was reported. Transcription levels of *AGL15* were also upregulated or downregulated in the presence of ACC or AVG, respectively. Thus, it can be concluded that ethylene regulates SE induced by 2,4-D in *Arabidopsis* seedlings and in soybean cotyledons, by regulating the ortholog genes of *MtSERF1* and subsequent regulation of *AGL15*, based on an ethylene-auxin crosstalk. Note that *ERF022*, *MtSERF1*, *At5g61590* and *GmSERF1* are all members of the ERF family. Taking into consideration the role of ERF family in response to stress we may conclude that ethylene affects SE differently based on stress response signalling induced by the hormonal stress caused by auxin and/or cytokinin.

8. Conclusion and Future Prospects

The compiled literature presented here in can give us a great insight on how ethylene affects regeneration processes. It is markedly noted that ethylene affects regeneration depending on the species, the explant and the stress conditions. The ethylene capacity in reverting recalcitrance in some species highlights the importance of ethylene modulation studies regarding *in vitro* regeneration. Further studies focused on the ERF members genes regulated by ethylene in response to stress-induced regeneration can contribute to unveil the mechanisms behind the highly regeneration capacity observed in some genotypes. And may also explain why some genotypes and species are recalcitrant to regeneration. Ethylene seems to affect *in vitro* regeneration by a stress response signaling, with evident hormonal crosstalk, at least in some in some species. Considering all the literature, this hormone seems to be an important link between stress, regeneration and development.

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