

REVIEW ARTICLE

Ethylene in seed formation and germination**Angel J. Matilla***

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Abstract

In seed formation the role of ethylene has received little attention. The data available on zygotic embryogenesis suggest an association of the ethylene biosynthetic pathway and seed maturation. Over the course of dicot embryogenesis, ACC-oxidase mRNA can be expressed in the cotyledons and embryonic axis. However, as maturation proceeds, cotyledonary ACC-oxidase expression disappears. In some seeds that develop primary dormancy, ethylene synthesis can be among the prerequisites for breaking dormancy. Moreover, the persistence of dormancy may be related to the difficulty of the embryonic axis to produce the necessary ethylene levels or to low tissue sensitivity. The use of inhibitors of ethylene biosynthesis or its action has provided data implicating an ethylene requirement for seed dormancy or germination in some species. However, the role of ethylene in germination remains controversial. Some authors hold that gas production is a consequence of the germination process, while others contend that ethylene production is a requirement for germination. Furthermore, among seeds that require ethylene, some are extremely sensitive to the gas, while others require relatively high levels to trigger germination. Recent studies with *Xanthium pennsylvanicum* seeds suggest that β -cyanoalanine-synthase is involved in ethylene-dependent germination. In addition, regulation of the partitioning of S-adenosyl-L-methionine (AdoMet) between the ethylene vs polyamine biosynthetic pathways may be a way of controlling germination in some seeds. Such regulation may also apply to the reversal of seed thermoinhibition, which can occur when polyamine synthesis is inhibited, thereby strongly channelling AdoMet towards ethylene. The biological models and approaches that may shed additional light on the role of ethylene during seed germination are presented.

Keywords: abscisic acid, 1-aminocyclopropane-1-carboxylic acid, ACC-oxidase, ACC-synthase, cell expansion, β -cyanoalanine-synthase, dormancy, ethylene, germination, 1-(malonylamino)cyclopropane-1-carboxylic acid, methyl jasmonate, mitochondrial respiration, osmopriming, pollen grains, polyamines, seed development, short-chain saturated fatty acids, thermoinhibition

Introduction

The growth and development of higher plants, from the earliest to the most advanced stages of the life cycle, are strictly regulated by phytohormones, such as ethylene (Fluhr and Mattoo, 1996). Germination, flowering, maturation, senescence and response to pathogens are some of the processes that involve this two-carbon alkene (Esashi, 1991; Mattoo and Suttle, 1991; Kepczynsky and Kepczynska, 1997; Kieber, 1997).

From the time of the discovery of methionine as the precursor of ethylene (Lieberman *et al.*, 1965; Yang *et al.*, 1966) to the first studies of ethylene signal transduction using *Arabidopsis thaliana* mutants (Ecker, 1995; Fluhr, 1998), major breakthroughs have been made in the knowledge and understanding of ethylene physiology. Thus, it has been demonstrated that: (a) methionine is transformed into AdoMet and methyl-thyoadenosine (MTA) which, via the "Yang cycle", regenerates methionine (for review, see Miyazaki and Yang, 1987; Kushad, 1990); (b) AdoMet is transformed into ethylene with 1-aminocyclopropane-1-carboxylic acid (ACC) as the only intermediate (Adams and Yang, 1979); (c) ACC can be alternatively conjugated to 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) (Amrhein *et al.*, 1981) or 1-(γ -L-glutamylamino)cyclopropane-1-carboxylic acid (GACC) (Martin *et al.*, 1995), although MACC is the principal conjugate (Peiser and Yang, 1998); (d) MACC, under stress conditions, can be

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transformed into ACC (Jiao *et al.*, 1986; Hanley *et al.*, 1989); (e) AdoMet can be alternatively channelled towards ethylene or towards the polyamine (PA) pathway, constituting a control mechanism for certain physiological processes in higher plants (Miyazaki and Yang, 1987; Evans and Malmberg, 1989; Mattoo and White, 1991; Matilla, 1996).

Ethylene production in higher-plant tissues is usually low but is greatly increased at certain stages of growth and development, such as the germination of some seeds, the ripening of climacteric fruits or the abscission of leaves. In higher plants, the ethylene synthesis involves ACC-synthase, ACC-oxidase, and in some cases MACC-transferase and GACC-transferase (John, 1991; Kende, 1993; Fluhr and Mattoo, 1996). Since ethylene regulates diverse processes, its production must be closely regulated. Multiple genes encode key enzymes of ethylene biosynthesis (ACC-synthase and ACC-oxidase), and their transcripts are differentially regulated (van der Straeten and van Montagu, 1991; Rodrigues-Pousada *et al.*, 1993; Gray *et al.*, 1994; Zarembinski and Theologis, 1994; Nakatsuka *et al.*, 1998). In this review, data are presented on the production of ethylene in seeds and the role played by this phytohormone in seed development and germination.

Ethylene and zygotic embryogenesis

Plant embryogenesis (for review, see Meinke, 1994) involves multiple developmental pathways coordinated by phytohormones, abscisic acid (ABA) being the most thoroughly studied (Hilhorst, 1995; Rock and Quatrano, 1995; Bewley, 1997; Marion-Poll, 1997; Thomas *et al.*, 1997; Le Page-Degivry, 1998). While there are many molecular studies on the ethylene pathway in fruits and flowers (Fluhr and Mattoo, 1996), information for vegetative tissues and embryogenesis is extremely limited. In some photosynthetic tissues, ethylene affects chlorophyll metabolism (Johnson-Flanagan and Thiagarajah, 1990; Abeles *et al.*, 1992). Because chlorophyll loss is triggered during the final stages of embryogenesis (e.g. development of desiccation tolerance), this process may be affected by ethylene. In sunflower seeds, ethylene production from ACC decreased during seed maturation, and non-dormant mature seeds were unable to synthesize ethylene until germination and growth occurred (Corbineau *et al.*, 1989). Mustard and canola seeds produce significant amounts of ethylene during embryogenesis, specifically in the early pre-desiccation stages (Johnson-Flanagan and Spencer, 1994; Child *et al.*, 1998). The thickness of the silique, an organ that does not produce ethylene, may have a key role in controlling the gas concentration during maturation.

Transitory climacteric ethylene is detectable prior to senescence in oilseed rape pods, much of which is attributable to the seeds (Meakin and Roberts, 1990). The decline in this ethylene production is accompanied by a rise in β -1,4-glucanase in the dehiscence zone of siliques (Meakin and Roberts, 1990), and polygalacturonase may also be involved (Jenkins *et al.*, 1996; Petersen *et al.*, 1996). The results concerning the pod wall of oilseed rape strongly suggest regulation of ethylene biosynthesis by ACC-oxidase rather than at the level of ACC-synthase (Child *et al.*, 1998).

ACC and MACC have been quantified during embryogenesis in very few seeds. In oilseed rape, the variation in ACC is exceedingly small during the climacteric period, implying its rapid conversion to ethylene, while the MACC level is very low (Child *et al.*, 1998). However, the MACC content of seeds increased after the production of climacteric ethylene slowed, indicating that some ACC is continually being conjugated rather than used for the production of ethylene. The presence of climacteric ethylene appears to accelerate more than initiate the abscission of the silique. Cell separation in the dehiscence zone of oilseed rape takes place only when auxin levels in that zone decline to low values, coinciding with the ethylene climacteric (Chauvaux *et al.*, 1997). Pod dehiscence involves highly regulated and controlled expression of an array of different genes at precise times and cellular location and requires a complex signal transduction network. Recently, a cDNA (SAC29) encoding a response regulator protein was isolated from *Brassica napus* pods (Whitelaw *et al.*, 1999). SAC29 was highly expressed in the dehiscence zone when ethylene and auxin reached a maximum (Meakin and Roberts, 1990; Johnson-Flanagan and Spencer, 1994). The role of SAC29 as a component of an ethylene-mediated cascade regulating dehiscence is under study.

Legume seeds are also widely studied in relation to the ethylene pathway and embryogenesis. Exogenous ethylene injected through the maturing pod wall was a powerful initiator of precocious germination in *Phaseolus vulgaris* cv. Seminole seeds (Fountain and Outred, 1990), probably owing to induction of ABA catabolism or to changes in tissue permeability to ABA caused by ethylene. A slow rate of ethylene production was found in these seeds, the testa being the source. In seeds of *Lupinus luteus*, ACC inhibited assimilate import from the seed coat to the developing embryo, and ABA played an important role in this import to the seeds (Zayakin and Nam, 1998). Recently, some aspects of embryogenesis of *Cicer arietinum* were studied in relation to ethylene biosynthesis. A full-length cDNA encoding ACC-oxidase was isolated and sequenced from embryonic axes of chick-pea seeds, which depend on ethylene

production for germination (Gómez-Jiménez *et al.*, 1998). ACC-oxidase mRNA was found both in cotyledons and the embryonic axis during early and middle embryogenic stages, but not in the cotyledons during the desiccation period (Gómez-Jiménez *et al.*, 1998). ACC content, ACC-synthase activity (Gómez-Jiménez and Matilla, unpublished data), ethylene production and *in vitro* ACC-oxidase activity reached a maximum towards the mid-stages of chick-pea embryogenesis and then declined during late embryogenesis and seed desiccation (Gómez-Jiménez *et al.*, 1998). However, the expression of the ACC-synthase gene remained practically invariable over zygotic embryogenesis in *C. arietinum* (Gómez-Jiménez and Matilla, unpublished data). These results suggest a relationship between ethylene production and normal *C. arietinum* seed development. In chick-pea seeds, the relationship between the control of embryogenesis by ABA (Colorado *et al.*, 1995) and ethylene is currently under study.

A role of ethylene in breaking dormancy

A seed is programmed to survive after being dispersed from the mother plant until the establishment of a photosynthetically competent seedling. Seed dormancy prevents germination during periods unfavourable to seedling growth. Primary dormancy develops during seed formation on the mother plant and can be relieved by dry storage or by imbibition at certain temperatures over a particular time period. The breaking of dormancy is characterized by changes in several physiological parameters that affect the subsequent germination response. There is increased sensitivity to germination-stimulating factors such as GAs, nitrate, chilling and light (Karssen *et al.*, 1989; Derkx and Karssen, 1993). Various studies suggest that membrane transitions may be involved as regulatory elements in dormancy and germination (Hilhorst *et al.*, 1996).

Since many species produce ethylene during germination and some factors that break dormancy also stimulate ethylene production, it was proposed that gas production during early imbibition may contribute to the breaking of dormancy in some species. The first observations were made in the seeds of *Trifolium subterraneum* (Esashi and Leopold, 1969), *Arachis hypogea* (Ketring and Morgan, 1971, 1972), *Spergula arvensis* (van Staden *et al.*, 1973) and *Avena fatua* (Adkins and Ross, 1981), among others. For dormant *Chenopodium album* seeds, endogenous ethylene stimulates germination (Saini *et al.*, 1985a, b) and depends on an interaction with nitrate supply from the mother plant or the *in vitro* germination medium (Saini and Spencer, 1987). As opposed to

seeds of *A. caudatus*, *L. sativa* and cocklebur (Satoh *et al.*, 1984; Kepczynski and Karssen, 1985; Abeles, 1986), non-dormant *C. album* seeds do not require ethylene for radicle protrusion, although they produce the gas. While treatment with norbornadiene (NBD) impeded the germination of dormant *C. album* seeds, inhibition was reversed by exogenous ethylene. However, aminoethoxyvinylglycine (AVG) did not inhibit seed germination when dormancy was broken by GA, nitrate and light, probably because the small quantity of gas produced was sufficient owing to the assumed great sensitivity to this phytohormone (Machabée and Saini, 1991).

Steward and Freebairn (1969) inhibited lettuce seed germination at 25°C by first heating dry seeds at 97°C for 8–16 h; ethylene (100 $\mu\text{l l}^{-1}$) released this dormancy when added during the imbibition period. Exogenous ACC was not as effective as ethylene, and the dormancy induced by the heat lowered ethylene production and ACC synthesis. Both processes increased when exogenous ethylene was applied (Fu and Yang, 1983).

Non-dormant cocklebur seeds produced much more ethylene than dormant ones (Satoh and Esashi, 1983), but exogenous ethylene was not capable of breaking dormancy (Esashi *et al.*, 1978). One cause of primary dormancy in these seeds may be the difficulty of producing ethylene in the axis (Esashi and Katoh, 1975) and the lack of high sensitivity to ethylene. Ethylene production in the axes of the non-dormant cocklebur seeds parallels ACC synthesis without the participation of MACC (Satoh and Esashi, 1983). The ACC and MACC contents in dormant and non-dormant seeds are quite similar, suggesting that dormant seeds have difficulties in oxidizing ACC. The seeds of the legume, *Stylosanthes humilis*, have a strong dormancy when freshly harvested, but this physiological dormancy is gradually relieved with time (i.e. 6 months). The authors of this work conjecture that ACC-synthase, a key enzyme in dormancy control, is absent or not operative in young seeds and that ABA appears to control ethylene production (Vieira and Barros, 1994).

One approach to the mechanism of seed dormancy is to study mutants lacking dormancy or having a weak dormancy within a species that normally has dormant seeds. Such an approach was taken in *Arabidopsis thaliana* with the *rd01* and *rd02* mutants (León-Kloosterziel *et al.*, 1994, 1996). The reduced dormancy of these mutants was caused by a single recessive mutation at two distinct loci and was embryo determined (León-Kloosterziel *et al.*, 1996). The seeds of these mutants show the same sensitivity to ABA, ethylene, auxin and cytokinin as the wild type. However, double-mutant analysis suggested an ABA and GA requirement for germination.

Responses of seeds to ethylene and compounds related or unrelated to their biosynthetic pathway

Many seeds produce ethylene during germination, but the detailed role of this phytohormone remains unclear. To provide insight into the action of ethylene, experiments were performed in which the germination medium was enriched in ethylene (ethephon), ACC, substances that alter the biosynthetic pathway, or compounds that inhibit the action mechanism (Esashi, 1991). In other studies, the experimental protocols included an examination of the effect that ethylene exerts on seed responses to an exogenous phytohormone whose action mechanism on some physiological process is relatively well understood (i.e. ABA, GAs, auxins or kinetin). In the 1970s and 1980s, experiments were conducted to confirm preliminary results from previous years. Thus, it was demonstrated that exogenous ethylene accelerates germination in cocklebur, *Amaranthus retroflexus* (Egley, 1980; Schonbeck and Egley, 1980a), aged *Striga lutea* (Egley and Dale, 1970) and aged *Brassica napus* seeds. From these data, it was suggested that ageing deteriorated the ethylene-production system (Takayanagi and Harrington, 1971). Esashi *et al.* (1976), submitting cocklebur seeds to an anaerobic pre-treatment to enhance germination, demonstrated that exogenous ethylene boosted the germination percentage even more than anaerobiosis.

In addition, in ABA-treated cotton seeds, germination was increased with ethylene, GAs and kinetin. Germination was greater when ethylene was supplemented with one of the two phytohormones. Fusicoccin, a toxin that induces cell elongation, completely reversed the effect of ABA (Halloin, 1976). Ethylene reversed ABA inhibition of the germination of *A. hypogea* (Ketring and Morgan, 1972), *C. album* (Karssen, 1976) and also reversed the polyethylene glycol (PEG) inhibition of *A. retroflexus* seeds (Schonbeck and Egley, 1981b, c). Schonbeck and Egley (1980b) demonstrated that redroot pigweed seed germination increased with exogenous ethylene concentrations under most experimental conditions, but the response was significantly altered by temperature which decreased the ethylene response thresholds. Schonbeck and Egley (1981a) hypothesized a timing sequence for redroot pigweed seeds, taking into account light (via Pfr), ethylene sensitivity, temperature, water stress and CO₂. The role of ethylene in the germination of *A. caudatus* seeds was studied by Kepczynski (1986a) by using ACC, AVG, ABA and PEG-6000. Both ABA and PEG effects were reversed by exogenous ethylene and by ACC; the effect of this ethylene precursor was correlated with increased gas production by seeds. The ABA effect was strengthened by AVG (Kepczynski, 1986a).

However, ethylene in the form of ethephon, which in many seeds stimulates germination, can also provoke inhibition. Such is the case for seeds of watergrass and rice, among others (Taylorson, 1979; Southwick *et al.*, 1986). Germination of recalcitrant *Quercus robur* seeds exposed to light was inhibited by ethephon, ACC and ABA (Finch-Savage and Clay, 1994). The germination of cocklebur seeds responded positively to ethylene at 23°C but was strongly inhibited by ethylene at high temperatures (Esashi *et al.*, 1986a). On the other hand, ethephon was the most efficient antagonist in *A. caudatus* seeds inhibited by paclobutrazol (Kepczynski *et al.*, 1988) or by jasmonates (Kepczynski and Bialecka, 1994, 1997). Ethylene reversed the inhibition induced by methyl jasmonate in the germination of cocklebur seeds. Ethylene production, ACC content and ACC oxidation were reduced by methyl jasmonate. However, MACC was not altered by methyl jasmonate (Nojavan-Asghari and Ishizawa, 1998). In some seeds the promotion of germination by ethephon depends on an interrelationship with CO₂ (i.e. cocklebur, lettuce, *Spergula arvensis* or redroot pigweed), and the sequence of the CO₂- and ethylene-sensitive phases could be changed by seed conditioning (Esashi *et al.*, 1986b, 1988). Secondary dormancy in cocklebur seeds was broken only by the simultaneous application of CO₂ and ethylene (Esashi *et al.*, 1978). The fact that NBD, an inhibitor of cocklebur seed germination, was capable of counteracting CO₂ action in some cases, but was incapable of reversing the action of ethylene, suggests that NBD acts with some side-effects besides being a competitive inhibitor of ethylene action (Ishizawa *et al.*, 1988). Exogenous ethylene was capable of alleviating the chilling injury to peas, while AVG and NBD tended to increase the chilling injury (Petruzzelli and Harren, 1997). By contrast, *Echinaceae angustifolia* seeds need a continuous-light treatment, pre-chilling and ethephon in order to reach 100% germination (Feghahati and Reese, 1994). The germination of *C. arietinum* seeds is stimulated by ACC and ethephon and depressed by aminoxyacetic acid, CoCl₂ and NBD, among others (Gallardo *et al.*, 1994a). However, the germination of these seeds is accelerated by inhibitors of the polyamine pathway (i.e. cyclohexylamine or methylglyoxal-bis-guanylhydrazone), suggesting a greater channelling of AdoMet towards ACC-synthase and, consequently, greater ethylene synthesis (Matilla, 1996). Gallardo *et al.* (1994c) showed that the presence of cyclohexylamine in the germination medium increased the activity of ACC-synthase and ACC-oxidase in addition to raising the levels of ACC and ethylene concomitantly with a strong drop in endogenous polyamines.

In addition, short-chain saturated fatty acids are

known to inhibit the germination of chick-pea seeds, and this inhibition was reversed by ACC or ethylene (Gallardo *et al.*, 1994b). The mechanism of short-chain saturated fatty acid action is unknown, but in *C. arietinum* seeds these fatty acids negatively affected all steps in the transformation from AdoMet to ethylene (Gallardo *et al.*, 1994b). Furthermore, the sensitivity of various plant tissues (e.g. peanut and cyclopia seeds) to ethylene increased as a result of short-chain saturated fatty acid incorporation into cell membranes (Whitehead and Nelson, 1992; Sutcliffe and Whitehead, 1995).

Also, when considering pollen grain germination during sexual reproduction in higher plants, it bears noting in relation to ethylene that: (1) the *in vitro* maturation of pollen grains of *Nicotiana tabacum* from the mid-binucleate stage was inhibited by aminooxyacetic acid and AVG, while ACC and ethephon were able to overcome this inhibition; and (2) ethylene production increased in isolated pollen during *in situ* maturation (Chibi and Matilla, 1994). These results, together with other previously published data (Chibi *et al.*, 1993), lead us to believe that there is competition for AdoMet by the ethylene and polyamine pathways, providing some degree of regulation of maturation and germination in *Nicotiana tabacum* pollen grains.

Finally, strigol, isolated from root exudates of cotton (Cook *et al.*, 1972), stimulates germination in witchweed by promoting ethylene biosynthesis in the seed (Babiker *et al.*, 1993a). Ethylene is a good germination promoter in witchweed (Egley and Dale, 1970), and the soil atmosphere can contain ethylene (Smith, 1976) which positively or negatively affects germination in various seeds (Taylorson, 1979). Curiously, the synthetic strigol analogue GR-24 stimulated ethylene production in *S. hermonthica* and *S. forbesii* seeds, but promoted germination only in the former (Jackson and Parker, 1991). Applying ethephon to soils can encourage germination of this devastating plant; after the plant becomes photosynthetically competent, it can be killed with herbicides (Egley, 1999). Soil experiments with seeds of witchweed or *A. retroflexus* (Schonbeck and Egley, 1981b) demonstrated that cultivation can influence germination, dormancy and deterioration of buried weed seeds. More recently, an unidentified compound extracted from a plant-derived smoke extract, at relatively high concentrations, strongly inhibited the germination of light-sensitive lettuce seeds, counteracting increased germination by ethylene (Jager *et al.*, 1996).

A role for ethylene in germination

Seed germination involves a series of hormonally regulated metabolic processes. Consequently, as

germination involves the revival of the growth of the organ that breaks the seed coat, this part of the seed may contain the true target cells for certain phytohormones (e.g. ethylene; Matilla, 1996; Kieber, 1997). Egley (1999) rightly considers germination and dormancy in this light: "dormancy (a reduced ability to germinate) results when some pre-germination events do not follow an essential sequential pattern and perhaps an orderly sequence of metabolic events is necessary to 'set the stage' for germination".

Although the great majority of seeds produce ethylene during the germination process, it is not yet clear whether this gas acts as a phytohormone in the chain of germination events (Abeles, 1986), or whether ethylene production is a result of, rather than a requirement for, germination (Fu and Yang, 1983) and consequently does not alter the pattern of events prior to or during the breaking of the seed coat. A number of papers will be reviewed to shed more light on this enigma. Various studies have demonstrated that ethylene production in certain seeds increased before radicle protrusion, and this protrusion was reduced on trapping the ethylene produced. However, in wild oat an early temporary rise in ethylene production was reported in both dormant and non-dormant seeds (Adkins and Ross, 1981), and in seeds of peanut and bean, the application of AVG effectively inhibited ethylene production without reducing germination (Hoffman *et al.*, 1983). The use of inhibitors of ethylene synthesis and action indicate the dependence of seed germination in some species on endogenous ethylene. The criteria used for correlating germination and ethylene production were: (1) parallel increase of seed ethylene production with the progress of germination; (2) inhibition of germination by AVG and CoCl_2 (inhibitors of ACC-synthase and ACC-oxidase, respectively) and NBD (inhibitor of ethylene action); and (3) the effects of all the inhibitors being overcome by exogenous ethylene alone or application of ACC.

Ethylene synthesis and action are indispensable for the germination of lettuce under favourable conditions (Abeles, 1986; Khan and Prusinski, 1989; Saini *et al.*, 1989) and *Striga hermonthica* (Logan and Stewart, 1995). In addition, the germination of *A. caudatus* and *C. arietinum* seeds requires ethylene synthesis as well as for alleviation of inhibition induced by ABA and for GA action (Kepczynski and Karssen, 1985; Kepczynski, 1986a, b; Matilla, 1996). The possibility that other compounds could replace the need for GAs in the GA-deficient mutants of *Arabidopsis* was studied by Karssen *et al.* (1989). Thus, ethylene and light together induced full germination in the *ga1* mutant in the absence of applied GA, the effect being much weaker in darkness. By contrast, in tomato, ethylene did not stimulate germination of the *gib1* mutant in light or darkness (Groot and Karssen,

1987); fusicoccin was the only compound tested that partly replaced the need for applied GA.

In a study including different seeds that produce ethylene, Lalonde and Saini (1992) demonstrated that of 10 species examined, only *Tagetes erecta* strictly required ethylene synthesis to germinate. This dependence was demonstrated for seeds of other Compositae [i.e. lettuce and cocklebur (Dunlop and Morgan 1977a; Satoh *et al.*, 1984; Abeles, 1986)]. On the other hand, the germination of dormant seeds of *C. album* depended on ethylene, although the non-dormant seeds did not seem to need the gas they produce (Machabée and Saini, 1991). By using inhibitors of ethylene synthesis and action and a laser-photoacoustic detection system to measure ethylene evolution, Petruzzelli *et al.* (1994, 1995) found that pea seeds started to release ethylene before visible germination. It is noteworthy that some seeds are highly sensitive to ethylene and thus require only a small amount for germination. Therefore, these seeds can germinate in the presence of AVG, which is not always capable of completely eliminating ethylene production (Kepczynski and Karssen, 1985; Saini *et al.*, 1989; Machabée and Saini, 1991; Gallardo *et al.*, 1994a).

Little is known about the action mechanism of ethylene in the germination of ethylene-dependent seeds. Logan and Stewart (1991, 1995) proposed that cytokinins elicited germination of *S. hermonthica* by stimulating ACC-synthase activity. However, Babiker *et al.* (1993b) proposed that cytokinins affected ethylene biosynthesis and germination of *S. asiatica* by increasing ACC-oxidase activity rather than ACC-synthase. This germination can be inhibited by AVG and NBD. It is possible that stimulation of germination by ethylene and ACC in *S. hermonthica* led to a higher rate of cell division prior to radicle protrusion and that cell division required a higher rate of aerobic respiration than elongation (Logan and Stewart, 1991, 1995).

It was suggested that alternative respiration may be involved in the normal germination process of cocklebur seeds (Esashi *et al.*, 1979). However, Esashi *et al.* (1987) later concluded that ethylene action could not be explained only in terms of regulation of the respiratory system. In a number of recent publications, the activity of β -cyanoalanine synthase (CAS), the enzyme likely to be involved in cyanide metabolism (Maruyama *et al.*, 1998), is related to regulation of the cocklebur germination. However, mitochondrial CAS, which may control cocklebur seed respiration by decreasing the cyanide level, is greatly stimulated by ethylene in all the seeds studied to date (Hasegawa *et al.*, 1995). Cytosolic CAS activity of rice was stimulated by ethylene during the pre-germination period, but this did not occur in cocklebur (Hasegawa *et al.*, 1994, 1995). Ethylene is

capable of activating electron transport through both cyanide-sensitive and -resistant pathways in cocklebur seeds (Esashi *et al.*, 1982). The stimulation of aerobic respiration by ethylene in cocklebur was associated with increased mitochondrial development during imbibition (Esashi *et al.*, 1975). Ethylene as well as cysteine and/or HCN (both substrates of CAS) increased the amino acid content in dormant and non-dormant cocklebur seeds simultaneously with increased CAS activity (Maruyama *et al.*, 1997). This ethylene-induced amino acid accumulation also occurred under anoxic conditions (Yoshiyama *et al.*, 1996b). It appears that mitochondrial CAS activated by ethylene provided asparagine and aspartate and increased the amino acid pool during the pre-germination period (Maruyama *et al.*, 1997). Ethylene action may also be related to amino acid accumulation in primed seeds (Yoshiyama *et al.*, 1996a, b).

The germination of chick-pea seeds depended on ethylene synthesis by the embryonic axis. Radicle emergence was inhibited by NBD, high temperatures, ABA, PEG, *n*-propyl gallate or CoCl_2 , and the inhibition was reversed by exogenous ACC or ethylene (Gallardo *et al.*, 1991, 1994a). Much work has been done on this species to understand the role of ethylene in the germination process (Matilla, 1996). ACC-*N*-malonyl-transferase (Martínez-Reina *et al.*, 1996) and ACC-oxidase (Muñoz De Rueda *et al.*, 1995) have been biochemically characterized; calcium may be an important cofactor for the latter activity (Gallardo *et al.*, 1999). During germination, the levels of mRNA for ACC-oxidase from chick-pea increased in the embryonic axis and reached a maximum at 24 h (maximum percentage of germination), coinciding with a maximum ACC-oxidase activity and ethylene production. Similar transcriptional activity was not detected in the cotyledons (Gómez-Jiménez *et al.*, 1998). Chick-pea germination may require activation of mRNA transcription for ACC-oxidase, which can be inhibited by ABA and osmotic stress and stimulated by IAA and polyamines (Gómez-Jiménez and Matilla, unpublished data). Exogenous polyamines (i.e. putrescine or spermine), or the presence of inhibitors of their synthesis (i.e. cyclohexylamine or methylglyoxal-bis-guanylhydrazone), activated the transformation of AdoMet to ethylene, resulting in a strong stimulation of radicle protrusion under optimal (25°C) as well as non-optimal (30–35°C) germination conditions (Gallardo *et al.*, 1992, 1994c, 1996; Matilla, 1996). Cyclohexylamine (25°C) stimulated the mitotic index in the sub-apical and apical zones of radicle apex and plumule, respectively (Gallardo *et al.*, 1994c). However, ethylene did not seem to have a significant effect on the mitotic activity of the radicle meristem, since the mitotic index was

not altered by the addition of ethephon (Matilla, unpublished data).

The ability of seeds to oxidize applied ACC was the basis of a highly sensitive vigour test (Khan, 1994). In lettuce, cabbage, tomato, snap bean and sweet corn seeds, this test detected ACC-derived ethylene before the radicle protrusion; its production was directly related to the seed vigour. Babiker *et al.* (1993b, 1994) demonstrated that germination and embryo growth of *S. asiatica* seeds was limited by a low capacity to oxidize ACC, and this activity was increased by cytokinins or auxins. Nojavan-Asghari and Ishizawa (1998) recently concluded that methyl jasmonate, an inhibitor of cocklebur germination, reduced ethylene production by inhibiting ACC-oxidase and ACC-synthase, and thereby retarded germination.

Finally, it is essential to mention osmopriming (pre-soaking of seeds in osmotic solutions that allow the seeds to imbibe water and initiate germination, but which do not permit radicle protrusion through the seed coat) and its relationship to ethylene biosynthesis. Osmopriming improves germination in certain seeds at suboptimal temperatures, and it was associated with increased respiration and gene expression (Bray, 1995), as well as ethylene production and ACC-synthase activity (Fu *et al.*, 1988). Osmopriming of sunflower with PEG-6000 enhanced the conversion of ACC to ethylene (a good indicator of seed vigour, as indicated above) and probably increased ACC-oxidase activity (Chojnowski *et al.*, 1997). The low ACC-dependent ethylene production in aged sunflower seeds could be related to a reduced *in vivo* ACC-oxidase activity as in pea and cocklebur (Gidrol *et al.*, 1991).

Ethylene and thermoinhibition in seeds

Lettuce seeds

Most studies of the alleviation of thermoinhibition by ethylene have used lettuce seeds (Abeles, 1986). Lettuce germination (i.e. radicle protrusion) is sensitive to many internal and external factors (plant-growth regulators, light, temperature and water availability) and depends on cell expansion initiated within the embryo (i.e. hypocotyl region). As the temperature of germination or imbibition is raised from optimal (25°C) to supraoptimal (30–35°C), germination is inhibited. This effect is called thermoinhibition, since the embryo itself germinates readily if the endosperm is removed (Saini *et al.*, 1989). Such thermoinhibition can be overcome by treating seeds with ethylene. Therefore, this system is highly useful in studying the action of ethylene in the germination process.

Effects and interactions among GA, kinetin and ethylene with CO₂ on the relief of thermoinhibition have been reported (Negm *et al.*, 1972; Keys *et al.*, 1975; Rao *et al.*, 1975; Khan, 1980/81). However, these reports were based on germination tests conducted in sealed systems where metabolic activities of the seeds could dramatically change the concentrations of gases such as ethylene and CO₂ (Negm *et al.*, 1972; Keys *et al.*, 1975), possibly resulting in modified effects of other treatments (Keys *et al.*, 1975; Saini *et al.*, 1986a). With the use of a flow-through gas system, no hormone treatment alone was able to overcome thermoinhibition in the dark; the action of exogenous ethylene required the presence of at least another hormone, CO₂ or light, or a combination of these (Saini *et al.*, 1986a). Ethylene synthesis was essential for the relief of thermoinhibition in the dark by applications of GA, kinetin and/or CO₂ (Saini *et al.*, 1986b). A similar requirement for ethylene under non-thermoinhibitory conditions (25°C) in the light was reported by Abeles (1986). Endogenous ethylene was also essential for the light-induced relief of thermoinhibition (Saini *et al.*, 1989). These results suggest that ethylene plays an essential role in lettuce seed germination, regardless of the conditions for germination or the means used to induce it. Finally, although lettuce seeds subjected to high temperature and other stresses produce little ethylene (Abeles, 1986; Khan and Huang, 1988), thermoinhibition itself appears not to be caused by a reduction in the ability of seeds to produce ethylene (Burdett, 1972; Dunlap and Morgan, 1977b; Abeles, 1986); high temperatures may raise the threshold concentration of ethylene needed for germination (Dunlap and Morgan, 1977b). The cytokinin or ethylene requirements for overcoming thermoinhibition and osmotic restraint (prevention of germination at non-thermoinhibitory temperatures by placing seeds in an osmoticum) can also be eliminated or diminished by removing or weakening the seed coats (Abeles, 1986). Khan and Prusinski (1989) demonstrated that thermoinhibition was synergistically alleviated by kinetin + ACC and concluded that cytokinins may play an important role in regulating ethylene biosynthesis and germination in intact seeds at high temperatures, and the seed coats may be essential for such a regulation. In addition, thermoinhibition can also be completely relieved by a combination of kinetin and 100% O₂, to a lesser extent by O₂ and ethylene, but not at all by O₂ + GA (Tables 1 and 2). A combination of O₂ + kinetin or ethylene was more effective than all three phytohormones and CO₂ in air (Small *et al.*, 1993). However, ethylene + kinetin + GA were effective in breaking and preventing skotodormancy (imposed by imbibition in continuous darkness) in *Lactuca serriola* seeds (Small and Gutterman, 1992a). Whether thermoinhibition and skotodormancy are controlled

Table 1. Effect of oxygen plus growth regulators on germination of Grand Rapids lettuce seeds at 25°C (control) and 38°C (thermoinhibited). Concentrations used: GA₃, 100 mg l⁻¹; kinetin, 10 mg l⁻¹; ethylene, 200 µl l⁻¹; CO₂, 10% (v/v); O₂, 100%. Numbers are germination percentages recorded after 48 h (means ± SE, n = 3). Adapted from Small *et al.* (1993)

Treatment	25°C		38°C	
	Light	Dark	Light	Dark
Air control	92 ± 5	47 ± 4	0	0
Air + kinetin	98 ± 1	57 ± 13	10 ± 2	1 ± 1
Air + GA ₃	95 ± 4	89 ± 4	1 ± 1	0
Air + ethylene	93 ± 2	88 ± 4	0	0
Air + kinetin + GA ₃ + CO ₂ + ethylene	99 ± 1	93 ± 2	72 ± 4	68 ± 1
O ₂	94 ± 3	90 ± 5	23 ± 5	22 ± 1
O ₂ + kinetin	99 ± 1	97 ± 6	93 ± 2	89 ± 5
O ₂ + GA ₃	97 ± 4	99 ± 2	23 ± 4	26 ± 4
O ₂ + ethylene	96 ± 2	97 ± 5	89 ± 3	48 ± 2

Table 2. Effect of aminoethoxyvinylglycine (AVG), norbornadiene (NBD) and Hg(ClO₄)₂ on germination percentage in the light at 25°C (control) and 38°C (thermoinhibition) of Grand Rapids lettuce seeds. Concentrations used: NBD, 2 ml l⁻¹; Hg(ClO₄)₂, 250 mM; kinetin, 10 mg l⁻¹; O₂, 100%; ethylene, 200 µl l⁻¹; AVG, 2 mM. Numbers are recorded after 48 h (means ± SE). Adapted from Small *et al.* (1993)

Treatment	Germination	
	25°C	38°C
Air control	92 ± 5	0
Air + AVG	56 ± 4	0
Air + NBD	18 ± 6	0
Air + Hg(ClO ₄) ₂	92 ± 4	0
Air + AVG + NBD	1 ± 1	0
Air + ethylene	93 ± 3	0
Air + AVG + ethylene	96 ± 4	0
Air + NBD + ethylene	95 ± 5	0
Air + AVG + NBD + ethylene	96 ± 0	0
Air + kinetin	93 ± 4	9 ± 3
Air + kinetin + AVG	52 ± 4	0
Air + kinetin + NBD	29 ± 4	0
Air + kinetin + AVG + NBD	12 ± 2	0
Air + kinetin + Hg(ClO ₄) ₂	92 ± 3	5 ± 2
Air + kinetin + AVG + NBD + ethylene	92 ± 4	5 ± 1
O ₂	95 ± 0	21 ± 3
O ₂ + kinetin	96 ± 2	93 ± 3
O ₂ + kinetin + AVG	93 ± 3	84 ± 1
O ₂ + kinetin + NBD	92 ± 4	84 ± 5
O ₂ + kinetin + AVG + NBD	78 ± 6	76 ± 1
O ₂ + kinetin + Hg(ClO ₄) ₂	96 ± 1	92 ± 1
O ₂ + kinetin + ethylene	98 ± 3	96 ± 2
O ₂ + kinetin + AVG + ethylene	96 ± 2	94 ± 6
O ₂ + kinetin + AVG + NBD	96 ± 2	92 ± 2
O ₂ + kinetin + AVG + NBD + ethylene	96 ± 2	92 ± 2

by similar mechanisms has not been clarified; but the efficacy of hormones in their alleviation appears to be different (Small and Gutterman, 1992a, b). Ethylene participates in the germination process of thermoinhibited *Lactuca sativa* seeds or those germinated at the optimal temperature (Abeles, 1986), as well as in seeds that were matricconditioned with the moist solid carrier Micro-Cel E (a synthetic calcium silicate with matric properties and a high water-holding capacity) at 15°C for 20 h (this treatment allowed the seeds to germinate at 35°C) (Khan, 1996). Lastly, priming is an effective method to overcome thermoinhibition in lettuce seeds (Cantliffe, 1981), but the mechanism by which seed priming bypasses thermoinhibition is not understood. Cantliffe *et al.* (1984) concluded that seed priming appeared to lead to the irreversible initiation of cell elongation, thus overcoming thermoinhibition. This cell elongation could be caused by an increased accumulation of free amino acids in the radicle tip (Takeba, 1980). The relationship between ethylene and increased osmotic potential is not well understood.

Chick-pea seeds

Germination (i.e. emergence of embryonic axis) of *Cicer arietinum* seeds under optimal conditions (25°C) depends on ethylene production by the embryonic axis (Gallardo *et al.*, 1994a). Germination was inhibited at supraoptimal temperatures (30–35°C), and thermoinhibition was reversed by the application of ethylene or ACC or when channelling of AdoMet towards ACC and ethylene was increased (Matilla, 1996) (Table 3). In chick-pea seeds the main results were as follows: (i) maximum ethylene production in the embryonic axis occurred when growth was entirely due to cell elongation before mitotic

Table 3. Characteristics of the thermoinhibition in chick-pea (*Cicer arietinum*) seeds

Physiological process involved	Reference
Inhibition of protrusion at 30–35°C compared with a 25°C control	Gallardo <i>et al.</i> (1991)
Alleviation with inhibitors of polyamine synthesis	Muñoz De Rueda <i>et al.</i> (1995)
Alleviation with spermine or putrescine	Gallardo <i>et al.</i> (1992, 1996)
Accumulation of free and bound polyamines	Muñoz De Rueda <i>et al.</i> (1994a)
Rise of ACC-synthase and decrease of ACC-oxidase	Gallardo <i>et al.</i> (1991)
Inhibition of ethylene production and increase in MACC	Gallardo <i>et al.</i> (1991)
Channelling of AdoMet towards polyamine pathway	Muñoz De Rueda <i>et al.</i> (1994a)
Rise of ACC-synthase and ACC-oxidase with polyamine synthesis inhibitors	Gallardo <i>et al.</i> (1995)

activity began (Sánchez-Calle *et al.*, 1989). (ii) Thermoinhibition lowered ethylene production and ACC levels; however, ACC-synthase activity and the MACC content increased concomitantly at supraoptimal temperatures (30°C and 35°C) (Gallardo *et al.*, 1991). (iii) Polyamine biosynthesis inhibitors alleviated thermoinhibition (Muñoz De Rueda *et al.*, 1994b), induced ACC-synthase and ACC-oxidase, and increased ethylene production (Gallardo *et al.*, 1995). (iv) Alleviation of thermoinhibition by spermine (Gallardo *et al.*, 1992) or putrescine (Gallardo *et al.*, 1996) involved the ethylene pathway, suggesting that polyamines can play a major part in controlling germination in *Cicer arietinum* seeds (Matilla, 1996). Therefore, one of the causes of thermoinhibition in chick-pea seeds is a fall in the production of ethylene, apparently due to the greater activity of ACC-malonyl transferase, a key enzyme in this seed (Martínez-Reina *et al.*, 1996), and greater channelling of AdoMet towards the polyamine pathway. ACC-malonyl transferase activity and AdoMet channelling bring about low levels of ACC and lower endogenous ethylene in the embryonic axis.

Other seeds

The seeds of *Amaranthus retroflexus* germinate in the absence of light at 35–40°C. Lower temperatures reduced germination, an effect that can be reversed by ACC or ethrel (Kepczynski *et al.*, 1996). The reason for this may be that the sensitivity of seeds to exogenous ethylene decreased at lower temperatures, to reduced conversion of ACC to ethylene (Kepczynski and Kepczynska, 1997), or to lower levels of endogenous ACC. High-temperature treatments of *Amaranthus lividus* seeds during imbibition decreased ethylene production in germinating seeds probably due to membrane damage caused by the lipid-peroxidation process (Bhattacharjee and Mukherjee, 1998). In sunflower seeds the induction of thermodormancy appeared to be associated with loss of the ability to convert ACC to ethylene (Corbineau *et al.*, 1988). An

additive or synergistic effect of ethylene and GA₃ in breaking seed dormancy has also been demonstrated in *Rumex crispus* (Samimy and Khan, 1983), *Xanthium strumarium* (Esashi *et al.*, 1975) and *Lactuca sativa* (Keys *et al.*, 1975). The results of these studies appear to confirm that all factors related to the breaking of any type of thermodormancy somehow stimulate ethylene production in the seed. However, there are exceptions, such as the seeds of *Manihot glaziovii* and *Avena sativa*, in which thermoinhibition must be due to factors other than reduced ethylene production (Poljakoff-Mayber *et al.*, 1990; Drennan and van Staden, 1992).

Conclusions

Although zygotic embryogenesis of seeds is the least characterized in relation to the role of ethylene, existing data point towards the involvement of this phytohormone in cell growth, seed maturation and, possibly, with the acquisition of primary dormancy. It is highly probable that the ethylene biosynthetic pathway is active in all the seed organs throughout the initial and middle stages of embryogenesis, but that it tends to be located exclusively in the embryonic axis in the final stages, and some of its components remain stored in this organ during the period of seed desiccation, to be used at the onset of the imbibition period of viable seeds.

The mechanism by which endogenous ethylene participates in seed germination is not currently known. However, some advances have been made in this respect. Thus, in seeds of *Xanthium pennsylvanicum*, it has been proposed that ethylene regulates germination by acting on mitochondrial-CAS activity concomitantly with the increase in the amino acid pool and sulphhydryl bases (Yoshiyama *et al.*, 1996a, b; Maruyama *et al.*, 1998). In chick-pea seeds, the competition for AdoMet by ethylene and the polyamine pathways provides some degree of regulation at the onset and in the course of germination (Matilla, 1996). Thus, when ethylene

production is stimulated by the presence of polyamine biosynthesis inhibitors, an acceleration of the transformation of AdoMet into ethylene and stimulation of germination occurs (Gallardo *et al.*, 1994c). This and other results in *Cicer arietinum* strongly suggest that ethylene synthesis may be one of the triggers of germination rather than being a consequence of it (Matilla, 1996).

Endogenous ethylene production may be essential for alleviation of thermoinhibition in some seeds. Ethylene synthesis or sensitivity to ethylene action often decreases at high temperatures. However, the mechanism by which ethylene promotes germination at thermoinhibitory temperatures is not clearly understood. Ethylene may not act by lowering the mechanical resistance of the endosperm to embryonic growth (Prusinski and Khan, 1990). According to Abeles (1986), Dunlap *et al.* (1990) and Dutta and Bradford (1994), ethylene-dependent seeds require the gas to enhance radial cell expansion in the embryonic hypocotyl, rather than on the tissues enveloping it. However, Logan and Stewart (1995) proposed that stimulation of germination by ethylene leads to a higher rate of cell division prior to radicle emergence and that cell division requires a higher rate of aerobic respiration than elongation. By using the hydrotime model in *Lactuca sativa*, Dutta and Bradford (1994) concluded that ethylene extended the high-temperature limit for germination by acting in the embryo to maintain a lower water potential threshold for the initiation of growth as temperatures

increase. On the other hand, when *Cicer arietinum* seeds (which depend on ethylene synthesis to germinate) are subjected to thermoinhibition (30–35°C), ethylene production falls markedly in the embryonic axis (Fig. 1) as a consequence of the inhibition of ACC synthesis mediated by a greater conjugation to MACC as well as inhibition in ACC-oxidase activity and a greater channelling of AdoMet towards the polyamine pathway than towards ethylene (Gallardo *et al.*, 1991; Matilla, 1996).

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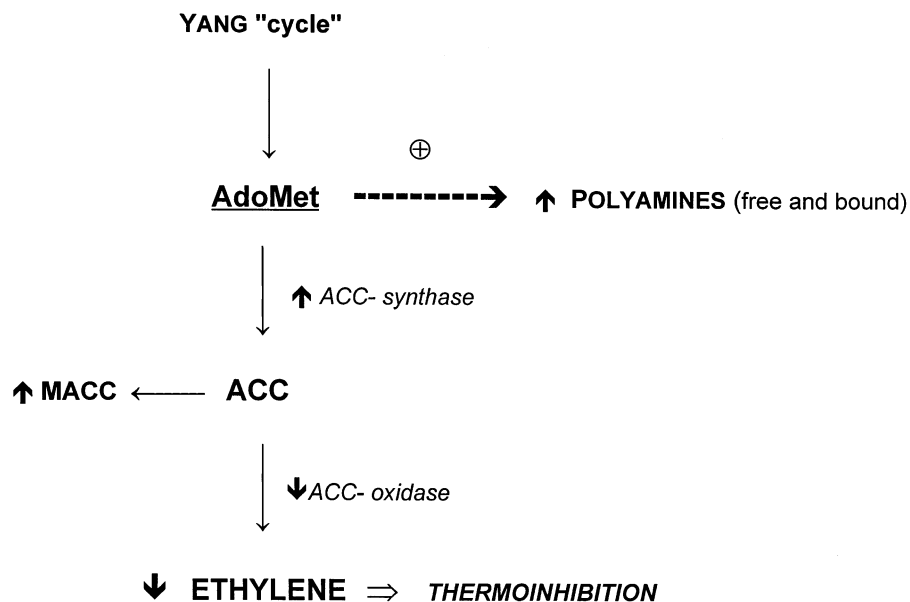


Figure 1. Events that occur in the embryonic axis of *Cicer arietinum* seeds that might be involved in the induction of the thermoinhibitory process. (⊕) Alleviation of thermoinhibition with cyclohexylamine, methylglyoxal-bis-guanylhydrazone, putrescine or spermine. Increase (↑), decrease (↓) and channelling of AdoMet towards polyamine pathway (---→).

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