Sensitivity to ethylene as a major component in the germination of seeds of *Stylosanthes humilis*

Dimas Mendes Ribeiro and Raimundo Santos Barros*

Departamento de Biologia Vegetal, Universidade Federal de Viçosa, 36571-000 Viçosa, MG, Brazil

Abstract

Physiological dormancy of scarified seeds of Townsville stylo (*Stylosanthes humilis* H.B.K.) is broken by ethylene. When the biosynthesis of this gas was impaired by 2-aminoethoxyvinylglycine (AVG) plus Co^{2+}, the response to ethylene at very low concentrations was appreciable in non-dormant seeds and nil in the dormant ones. Complete inhibition of germination of non-dormant seeds occurred only when they were treated with AVG plus Co^{2+} under an ethylene-free atmosphere, a condition in which no trace of the gas in the atmosphere of Erlenmeyer flasks could be detected. Injection of ethylene into that system triggered germination of both dormant and non-dormant seeds, demonstrating a requirement for the gas. Non-dormant seeds were at least 50-fold more sensitive to ethylene than the dormant ones. Perception of ethylene occurred within a very short time (at most 15 min), since exposure of both dormant and non-dormant seeds to the gas, at a steeply declining concentration, sufficed to cause substantial germination.

Keywords: dormancy, ethylene, germination, hormone sensitivity, inhibition, *Stylosanthes humilis*

Introduction

The concept of tissue sensitivity to growth regulators was mostly advanced in the 1980s, when it became firmly established (Trewavas, 1981, 1982). Thus, the response of a tissue to a hormone depends not only on the amount present, but also on its sensitivity to the regulator (Bradford and Trewavas, 1994). Because sensitivity is a highly dynamic variable, in certain situations it becomes the limiting factor in the response to the regulator.

For cells or organs (e.g. seeds), sensitivity of a population to a hormone is easily determined by constructing a dose–response curve; from this the spectrum of frequency distribution of response against concentration is obtained to show variation in sensitivity among the individuals of that population. The most sensitive individuals respond to the regulator at very low concentrations, while the least sensitive exhibit a response only under high concentrations (Trewavas, 1982; Bradford and Trewavas, 1994; Klee, 2004). This spectrum of response can be displayed both in space and in time. Hence, temporal changes in sensitivity can also be examined by applying the regulator at several concentrations along a time course, and registering the response of a tissue. Variations in sensitivity to a regulator may be due to changes in the number of receptor molecules to that particular regulator in a cell or tissue (Trewavas, 1981, 1982). However, in tomato a decrease in the number of functional receptor molecules was suggested to increase fruit sensitivity to ethylene (Klee, 2004), due to the negative control of the ethylene signalling chain leading to a response (Hua and Meyerowitz, 1998; Klee, 2004; Stepanova and Alonso, 2005). Several other factors might also underlie the basic mechanisms of changes in sensitivity (Firn, 1986).

Changes in sensitivity to a hormone are affected by both endogenous and environmental cues, including the developmental stage of a tissue or organ and the time course of response. For instance, Trewavas (1982) suggested that dehydration of some cereal seeds during maturation leads to an increase in the responsiveness of their aleurone layers to gibberellins. Likewise, short photoperiods appear to prepare lateral buds of willow (*Salix viminalis*) to respond to abscisic acid (Barros and Neill, 1986). Preincubation at relatively high temperatures and burial in the soil increase the ethylene sensitivity of imbibed seeds of *Amaranthus retroflexus* (Schonbeck and Egley, 1981). It is likely that increases in sensitivity to growth regulators constitute part of the after-ripening phenomenon shown by some seeds.

*Correspondence
Fax: +55 31 3899 2580
Email: rsbarros@ufv.br

Seeds of Townsville stylo (*Stylosanthes humilis* H.B.K.), a tropical annual forage legume, possess a relatively hard cover and, when freshly harvested, also display physiological dormancy that is gradually overcome with seed ageing (Vieira and Barros, 1994). Germination of scarified, dormant seeds is promoted by the ethylene-producing compounds, 2-chloroethylphosphonic acid (CEPA, an ethylene-releasing compound) and 1-aminocyclopropane-1-carboxylic acid (ACC) (Vieira and Barros, 1994), or by ethylene itself (Ribeiro and Barros, 2004a). Furthermore, imbibed non-dormant seeds produce as much as 10- to 12-fold more ethylene than dormant seeds (Pelacani *et al.*, 2005). This means that a greater capacity to synthesize ethylene is associated with germination of non-dormant seeds. The response of seeds to CEPA also increases along with seed post-harvest age, suggesting an increase in sensitivity of the seeds to ethylene. In this work, by employing a new technique whereby ethylene production by the seeds is inhibited (Ribeiro and Barros, 2004b), an evaluation was made of the contribution of the ethylene sensitivity factor in the germination of Townsville stylo seeds.

Materials and methods

Plant material and germination conditions

Plants of *Stylosanthes humilis* H.B.K. were raised in 3-litre plastic pots in a greenhouse in Viçosa (20°45′S, 42°15′W), Minas Gerais State, Brazil, and the seeds produced were harvested periodically and kept in the laboratory under dry conditions (21–28°C). In this way, seeds of several postharvest ages (thereafter referred to as days (d)-old), i.e. with different degrees of dormancy, were available for experiments.

Seeds were freed from their husks, scarified with fine sandpaper, sterilized with 0.5% (w/v) NaOCl for 10 min and thoroughly washed with distilled water. Afterwards, they were infiltrated by partial vacuum with the test solutions at pH 7.0, also containing 0.05% (v/v) Tween 80. Fifty seeds were then transferred to 50-ml Erlenmeyer flasks with two layers of Whatman number 1 filter paper and imbibed with 3 ml test solution. Flasks were sealed with rubber serum caps and used for germination tests in the dark, at 30°C in a day/night growth chamber (Forma Scientific Inc., Ohio, USA). Seeds with a protruded radicle of length *c*. 3 mm were considered as germinated. Daily germination counts were carried out for 4 or 5 d.

Inhibition of ethylene biosynthesis and accumulation

Inhibition of ethylene biosynthesis was attempted using 2-aminoethoxyvinylglycine (AVG, 0.1 mM) and $Co(NO_3)_2$ (1 mM), inhibitors of the activities of ACC synthase and ACC oxidase, respectively; both compounds were supplied simultaneously to the seeds.

An ethylene-free atmosphere inside the Erlenmeyer flasks was created by placing a polypropylene cup (26 mm diameter, 6 mm high) on the paper layers in the bottom of the flasks. The cups were also lined with three filter paper layers and wetted with a 0.25 M mercuric perchlorate solution (0.3 ml). No trace of ethylene was detected in the atmosphere of sealed flasks. Ethylene was released from the perchlorate solution by pouring into it an equal volume of 4 M NaCl solution (Abeles, 1973).

To inhibit ethylene biosynthesis in an ethylene-free environment, seeds were placed around the cup and treated with AVG plus Co^{2+} solution; 24 h later, the perchlorate solution was poured into the cup and the flasks sealed with rubber caps (Ribeiro and Barros, 2004b).

Production and quantitation of ethylene

Ethylene was produced from CEPA, as described by Abeles *et al.* (1992). Known amounts of the gas (as measured by gas chromatography, see below) were injected into the flasks through the rubber caps with a syringe.

Ethylene was quantified as described by Saltveit and Yang (1987). A 1 ml air sample was taken from the Erlenmeyer flasks with a gas-tight syringe and injected into a gas chromatograph (Hewlett-Packard 5890, series II), equipped with a flame ionization detector and a stainless-steel column (1 m long, 6 mm wide) packed with Porapak-N, 80–100 mesh. Nitrogen carrier gas and hydrogen flow rates were $30 \, ml \, min^{-1}$ and that of air $320 \, ml \, min^{-1}$. Column, injector and detector temperatures were 60°C, 110°C and 150°C, respectively. Ethylene peaks were registered by a HP 3395 A integrator coupled to the chromatograph, and quantified by comparison with authentic ethylene standards.

Statistical design

The statistical design of the assays was based on a completely randomized distribution with five replicates (Erlenmeyer flasks with 50 seeds). Germination percentages (%G) were transformed into arcsin $(\%G/100)^{1/2}$ values to follow a normal distribution. Differences among means were tested according to Scott and Knott (1974) in the case of germination assays, or by the Tukey test in the case of ethylene quantitation. Comparisons were performed at 5% significance.

Results and discussion

Generally, as dormancy is overcome with time, a requirement for an exogenous hormone to promote germination decreases gradually, until it is no longer required. Thus, a capacity to synthesize the regulator is acquired or increased, and/or seed sensitivity to the regulator is also increased. In common lambsquarters (*Chenopodium album*), for instance, inhibitors of ethylene biosynthesis inhibit germination and ethylene production in stimulated dormant seeds. Under identical conditions germination of non-dormant seeds is not greatly affected (Machabée and Saini, 1991), implying that the small amount of ethylene produced by inhibited non-dormant seeds is enough to trigger germination. In other words, non-dormant seeds became more sensitive to the regulator. Although the capacity for synthesizing the regulators responsible for dormancy breakage and their amounts have been examined in seeds of several species, changes in sensitivity accompanying seed ageing have been poorly investigated.

Ethylene has been implicated in dormancy breakage and germination of seeds of several species (Kępczyńsky and Kępczyńska, 1997), although Trewavas (1987) argues that only under appropriate conditions does sensory adaptation occur to make ethylene a meaningful stimulant. Ribeiro and Barros (2004a, b), however, showed that this gaseous hormone is required for dormancy breakage and germination of Townsville stylo seeds (and see below).

By employing seeds of different postharvest ages (2, 41, 164 and 343 d), dose–response curves were constructed when ethylene biosynthesis by Townsville stylo seeds was impaired or largely depressed by AVG plus Co^{2+}; hence, the assay dealt mainly with changes in sensitivity. Figure 1 shows that the greater the postharvest age, the greater the seed response to ethylene. By taking into account the concentration required to elicit a 50% response as the criterion to evaluate sensitivity (Firn, 1986), this corresponded to 1.68 nM for completely non-dormant seeds (343 d-old), to 17.4 nM for seeds 164 d-old, to 63.3 nM for seeds 41 d-old and to 87.3 nM for seeds that were 2 d-old. Hence, the first group of seeds was about 52-fold more sensitive to ethylene than the most dormant ones. The same trend was observed when considering the maximum rates of response (germination per unit ethylene concentration), i.e. the peaks of the first derivative for each curve occurred at 1.2, 13, 35 and 64 nM, for seeds in decreasing order of age. And since the ratio of sensitivity to control (the most dormant seeds) decreased with increasing concentrations of the regulator (Trewavas, 1987), the concentration range for the projected saturation response was much narrower: 12, 60, 76 and 77 μM, for the seeds in the same descending order of age. The behaviour of the
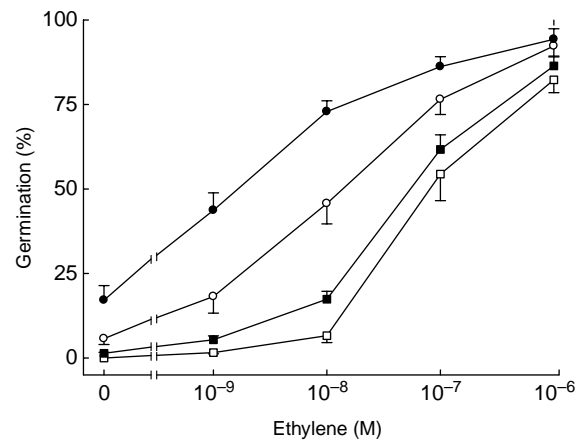
Figure 1. Response to ethylene of Townsville stylo seeds with different dormancies treated with 2-aminoethoxyvinylglycine (AVG) plus Co^{2+} at 30°C. In the control (pure water, pH 7.0), seeds exhibited the following germination, according to their postharvest ages: (□) 2 d: 0%, (■) 41 d: 10 ± 2%, (○) 164 d: 54 ± 2%, and (●) 343 d: 95 ± 1%. Means of 5 replicates ± standard errors.

164 d-old seeds started departing from that of the two other younger lots (Fig. 1), in accordance with the observations of Vieira and Barros (1994), who showed that dormancy loss is accelerated by 6 months after harvest of seeds of Townsville stylo, culminating with complete germination by 12–15 months.

Some germination (*c*. 20%) was observed in non-dormant seeds treated with ethylene biosynthesis inhibitors and without the addition of that gas (Fig. 1), which might cast some doubt on the role of the regulator in seed germination. However, because the unavailability of endogenous free ACC seems to constitute an impairment for germination of dormant seeds (Pinheiro, 2004) and because they also respond to applied ACC and CEPA (Vieira and Barros, 1994), and more promptly to ethylene itself (Ribeiro and Barros, 2004a), ethylene is likely to be involved in germination. Three approaches were adopted to ascertain this. In the first, seeds were provided with AVG plus Co^{2+}, and germination and ethylene accumulation in the flask atmosphere were monitored. Table 1 shows that germination and ethylene production were impaired completely by the inhibitors only in dormant seeds; in water some ethylene was produced and, as consequence of its accumulation, some germination occurred (Ribeiro and Barros, 2004a). The inhibitors depressed ethylene production by non-dormant seeds to a very large extent, but germination was still appreciable, above 20% (Table 1), under the same conditions without added ethylene (Fig. 1). Comparable amounts of ethylene were accumulated in flasks containing inhibited non-dormant or non-inhibited dormant

Table 1. Effects of 0.1 mM aminoethoxyvinylglycine (AVG) plus 1 mM $Co(NO_3)_2$ on germination of, and on ethylene production by, dormant (32 d-old) and non-dormant (421 d-old) seeds of Townsville stylo at 30°C. Means followed by same letter in a column do not differ significantly at the 5% level

Treatment: time (h) of measurement	Dormant		Non-dormant	
	Germination (%)	Ethylene (nM)	Germination (%)	Ethylene (nM)
Water (control)				
12	0 c	0 c	20.0 ± 2.3 c	7.0 ± 1.4 c
24	0.8 ± 0.49 b	1.83 ± 0.51 b	66.8 ± 5.6 b	70.2 ± 17.6 b
48	1.6 ± 0.75 a	4.21 ± 1.34 a	79.2 ± 1.4 a	85.3 ± 11.1 b
72	2.0 ± 0.63 a	5.04 ± 0.75 a	85.4 ± 1.0 a	123.3 ± 13.6 a
96	2.4 ± 0.40 a	4.83 ± 0.43 a	86.7 ± 2.7 a	122.9 ± 13.5 a
AVG + Co^{2+}				
12	0	0	0 d	0 d
24	0	0	1.2 ± 0.5 c	0.08 ± 0.04 d
48	0	0	2.0 ± 0.6 c	0.93 ± 0.13 c
72	0	0	10.8 ± 1.7 b	2.98 ± 0.33 b
96	0	0	21.6 ± 3.6 a	3.93 ± 0.55 a

seeds; germination of dormant seeds, however, was negligible (Table 1), indicating that other factors besides the amount of gas were modulating differently the response to ethylene of the two seed lots. A differential response to inhibitors has also been observed in dormant and non-dormant seeds of lambsquarters (Machabée and Saini, 1991) and in stimulated and non-stimulated thermo-inhibited seeds of lettuce (Saini *et al.*, 1989).

The second approach to diminishing the ethylene present involved capturing the gas from the flask atmosphere in a mercuric perchlorate solution. Germination was greatly reduced in dormant seeds and only slightly affected in non-dormant ones, although in both cases ethylene was not present in the flask atmospheres at 12 h from start of incubation or later (Table 2). Upon treatment of the perchlorate with a NaCl solution on the third day (by injection into the cup solution inside the flask with a syringe needle perforating the rubber seal), ethylene was desorbed [treatment $Hg(OCl)_2 \rightarrow NaCl$], showing that it was being produced by the seeds and immediately fixed in the perchlorate. At the end of experiment, ethylene was also desorbed from the perchlorate in treatment $Hg(OCl)_2 \rightarrow Hg(OCl)_2$, confirming that the seeds had produced the gas. On both occasions the ethylene amounts desorbed from perchlorate were much greater in flasks containing non-dormant seeds. Table 2 also shows that as a consequence of ethylene release in the middle of the experiment, germination of, and ethylene production by, dormant and non-dormant seeds increased.

Using the techniques described above, ethylene biosynthesis was not completely inhibited in non-dormant seeds (Tables 1 and 2), making it difficult to ascertain the role of this regulator on the germination of Townsville stylo seeds. The third approach

consisted of combining the two techniques. Seeds were imbibed for a day with the inhibitors AVG plus Co^{2+}, and afterwards a perchlorate solution was introduced to remove ethylene from the flask atmosphere (Ribeiro and Barros, 2004b). Only with this treatment was neither ethylene produced nor germination permitted in either dormant or non-dormant seeds (Table 3, upper part). This was confirmed by the fact that no trace of ethylene was found upon treatment of perchlorate with NaCl.

Germination did not occur in the absence of ethylene accumulation in the flask atmospheres, as shown in Tables 1, 2 and 3 (upper part). This, however, might simply mean that ethylene was a product of germination instead of its cause (Kępczyńsky and Karssen, 1985). If ethylene was allowed to accumulate in the flasks in the last set of assays, instead of being retained in the perchlorate solution, the results would have been similar to those in Table 1. This could be achieved by stopping the action of perchlorate in the middle of the experiment (see Table 3, lower part). As expected, no germination occurred and no ethylene was produced by both dormant and non-dormant seeds when the biosynthesis of the gas was impaired under an ethylene-free atmosphere throughout the experiment [treatment $AVG + Co^{2+}/Hg(OCl)_2 \rightarrow Hg(OCl)_2$]. By neutralizing the action of perchlorate on the third day, and thus allowing ethylene biosynthesis to occur, the gas was accumulated in the atmosphere of flasks containing non-dormant seeds [treatment $AVG + Co^{2+}/Hg(OCl)_2 \rightarrow NaCl$], mimicking the situation observed in Table 1. To summarize, Tables 1 and 3 (lower part) show that inhibited dormant seeds neither produced ethylene nor germinated, and ethylene even in very low amounts was required for germination of non-dormant seeds.

Table 2. Effects of the presence of 0.25 M Hg(OCl)$_2$ solution in the flasks on seed germination of Townsville stylo (%, G in each treatment) and on ethylene accumulation in the flask atmosphere (nM, E in each treatment) at 30°C. On the third day from the start of incubation, the Hg(ocl)$_2$ amount in cups was completed with an identical volume (0.3 ml) of water, fresh Hg(OCl)$_2$ or NaCl (to desorb ethylene) (indicated by treatments following the arrows). Desorbed ethylene amounts are shown in parentheses. Means followed by the same capital letter (ethylene), or followed by the same small letter (germination) in a column do not differ significantly at the 5% level

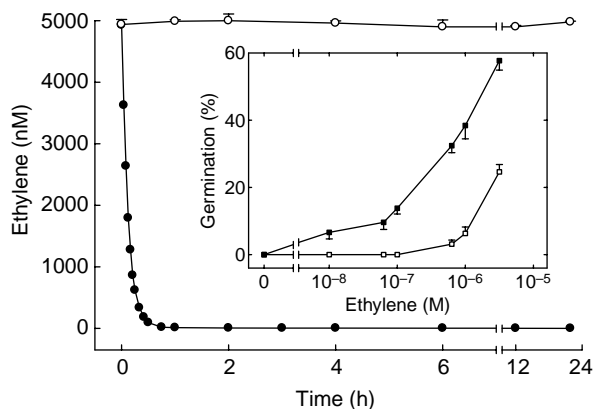| Contents in cup within flask		Time of measurement (h)						
		12	24	48	72	84	96	120
Dormant (19 d-old)								
H$_2$O → H$_2$O	G	1.2 ± 0.8	3.6 ± 0.04a	10.0 ± 1.4a	13.2 ± 1.6a	14.0 ± 0.9a	14.8 ± 0.5a	16.4 ± 1.5a
	E	1.0 ± 0.2	2.2 ± 0.4	15.3 ± 2.2	29.4 ± 2.4A	34.8 ± 1.1A	40.8 ± 2.8A	42.0 ± 2.5A
Hg(OCl)$_2$ → Hg(OCl)$_2$	G	0	0.8 ± 0.5b	3.2 ± 0.5b	4.0 ± 0.6b	5.2 ± 0.5b	5.8 ± 0.5b	5.8 ± 0.5b
	E	0	0	0	0C	0B	0B	0.0 (18.0 ± 1.5 B)
Hg(OCl)$_2$ → NaCl	G	0	1.2 ± 0.5b	4.0 ± 0.6b	4.6 ± 0.5b	15.2 ± 1.0a	16.4 ± 0.8a	18.0 ± 0.9a
	E	0	0	0	0 (19.5 ± 0.5B)	29.5 ± 1.6A	41.9 ± 1.6A	42.3 ± 1.6A
Non-dormant (263 d-old)								
H$_2$O → H$_2$O	G	62.4 ± 5.2a	68.8 ± 4.1a	71.2 ± 3.6a	74.4 ± 2.8a	75.6 ± 4.3a	76.4 ± 1.9a	77.6 ± 1.7a
	E	15.4 ± 1.6	60.4 ± 2.9	112.0 ± 2.8	132.6 ± 3.9A	142.4 ± 2.8A	152.5 ± 3.8A	154.3 ± 6.1A
Hg(OCl)$_2$ → Hg(OCl)$_2$	G	46.0 ± 4.0b	52.8 ± 2.4b	54.4 ± 1.7b	55.6 ± 2.0b	56.0 ± 3.6b	56.8 ± 1.0b	57.2 ± 1.5b
	E	0	0	0	0C	0B	0B	0.0 (76.6 ± 5.0B)
Hg(OCl)$_2$ → NaCl	G	41.6 ± 1.6b	50.8 ± 2.4b	52.8 ± 1.9b	53.2 ± 1.8b	62.0 ± 5.7a	77.6 ± 1.2a	78.8 ± 1.7a
	E	0	0	0	0.0 (101.9 ± 0.5B)	142.6 ± 7.2A	153.7 ± 9.8A	154.6 ± 7.0A

Table 3. Effects of ethylene biosynthesis inhibitors in the incubation medium in an ethylene-free atmosphere [adsorption of ethylene achieved using 0.25 M $Hg(OCl)_2$] on Townsville stylo seed germination and on ethylene concentration in the flask atmosphere at 30°C. In the second set of experiments (lower part), the $Hg(col)_2$ in the cups was completed with an identical volume (0.3 ml) of either fresh $Hg(OCl)_2$ or NaCl, added on the third day of incubation, as indicated by arrows. In parentheses are shown ethylene amounts quantitated after desorption with NaCl. Means followed by same letter in a column do not differ significantly at the 5% level

Incubation medium/contents in cup within flask	Germination (%) at h of incubation				Ethylene (nM) at h of incubation			
	12, 24, 48, 72	84	96	120 h	12, 24, 48, 72	84	96	120 h
Dormant (18-d-old)								
Water/Water	–	–	–	$9.6 \pm 1.0a$	–	–	–	$13.5 \pm 1.9a$
Water/$Hg(OCl)_2$	–	–	–	$5.2 \pm 1.5b$	–	–	–	$0\ (7.5 \pm 2.0b)$
AVG + Co^{2+}/Water	–	–	–	0 c	–	–	–	$0c$
AVG + Co^{2+}/$Hg(OCl)_2$	–	–	–	0 c	–	–	–	$0\ (0c)$
Non-dormant (303-d-old)								
Water/Water	–	–	–	$91.2 \pm 3.0a$	–	–	–	$153.2 \pm 6.1a$
Water/$Hg(OCl)_2$	–	–	–	$76.0 \pm 2.1b$	–	–	–	$0\ (87.0 \pm 8.3b)$
AVG + Co^{2+}/Water	–	–	–	$32.0 \pm 3.0c$	–	–	–	$4.7 \pm 1.7c$
AVG + Co^{2+}/$Hg(OCl)_2$	–	–	–	0 d	–	–	–	$0\ (0$ d$)$
Dormant (31-d-old)								
AVG + Co^{2+}/$Hg(OCl)_2 \rightarrow Hg(OCl)_2$	0	0	0	0	0	0	0	$0\ (0)$
AVG + Co^{2+}/$Hg(OCl)_2 \rightarrow$ NaCl	0	0	0	0	0	0	0	0
Non-dormant (347-d-old)								
AVG + Co^{2+}/$Hg(OCl)_2 \rightarrow Hg(OCl)_2$	0	0 b	0 b	0 b	0	0 b	0 b	$0\ (0$ b$)$
AVG + Co^{2+}/$Hg(OCl)_2 \rightarrow$ NaCl	0	$16.4 \pm 2.9a$	$24.4 \pm 3.3a$	$43.6 \pm 2.6a$	0	$4.3 \pm 0.4a$	$5.7 \pm 0.6a$	$7.7 \pm 1.2a$

A more direct way of investigating the role of ethylene in germination is by adding the gas into the flasks described above; this, however, is problematical due to the fast adsorption of the regulator by the perchlorate solution (Tables 2 and 3, lower part). By injecting ethylene into the flasks containing either dormant seeds, non-dormant seeds or no seeds at all, exactly the same response was obtained (not shown); therefore, the flasks with no seeds were chosen for studying the adsorption of ethylene by perchlorate. Injection of ethylene into the flask atmosphere showed that the gas was trapped very quickly in the perchlorate solution in a negatively exponential way (Fig. 2). At 15 min following injection, 88% of the ethylene had already been fixed; at 30 min 98%, and at 60 min 99.9% had been adsorbed. Despite the very rapid removal of ethylene, there were significant effects on germination, which varied with its concentrations (Fig. 2, insert). In an environment in which ethylene biosynthesis apparently did not occur (see Table 3), there was no germination of either dormant or non-dormant seeds when ethylene was not added (zero concentration), demonstrating the need for this hormone. The

Figure 2. Fixation of ethylene (5000 nM) injected into closed Erlenmeyer flasks containing two layers of filter paper with an aminoethoxyvinylglycine (AVG, 0.1 mM) plus Co^{2+} (1 mM) solution and a polypropylene cup with 0.3 ml 0.25 M $Hg(OCl)_2$ solution. Open symbols represent flasks without $Hg(OCl)_2$ solution in the cup. Adsorption of ethylene occurred according to $y = 0.22 + 1142.57e^{-19401196t} + 12.49e^{-0.52t} + 3844.71e^{-7.05t}$ ($R^2 = 0.99$). Insert shows the effects of ethylene added to the flasks, above, containing dormant (28-d-old, open symbols) or non-dormant (361-d-old, closed symbols) Townsville stylo seeds. Seeds were remained imbibed in AVG plus Co^{2+} for 24 h; afterwards, the cup at the bottom of the flask received 0.3 ml $Hg(OCl)_2$ solution. The flasks were then sealed, and ethylene at several concentrations was injected. Germination was counted on the fifth day at 30°C. Means of 5 replicates ± standard errors.

dose–response curves in Fig. 2 (insert) show that non-dormant seeds were much more sensitive to the gas than the dormant ones. A clear measurable response, one of the several criteria to evaluate sensitivity (Firn, 1986), was exhibited by non-dormant seeds (7% germination, first response point) at 18.3 nM, a concentration about 55-fold lower than that necessary to cause similar germination of dormant seeds. These results explain why germination occurred in non-dormant seeds at very low concentrations of ethylene, and did not do so in dormant seeds at similar concentrations (Tables 1 and 3, lower part). Both response curves (insert, Fig. 2) are similar [dormant: $y = -0.13 + 32.98(1 - e^{-314745x})^{1.24}$, $R^2 = 0.99$; non-dormant: $y = 0.31 + 58.95(1 - e^{-578340x})^{0.49}$, $R^2 = 0.98$], one being only displaced in relation to the other along the concentration axis, indicating that, at least in Townsville stylo seeds, changes in sensitivity to ethylene are a quantitative trait.

Ethylene is involved in dormancy breakage (Fig. 1) and germination (Fig. 2, insert) of Townsville stylo seeds, even at very low concentrations in the case of non-dormant seeds. Tables 2 and 3 (upper part) and Fig. 2 (insert) show that the gaseous regulator acts even when it is being rapidly depleted from the flask atmosphere, i.e. perception of the gas by seeds occurs in a short time. This was tested by providing ethylene to dormant and non-dormant seeds inhibited by AVG plus Co^{2+} in an ethylene-free atmosphere. The Erlenmeyer flasks were then periodically opened, and their atmospheres exhausted with a fan in a fume hood; following this procedure, no ethylene remained in the flask atmosphere. The shortest exposure time tested, 15 min, appeared to have already saturated the response process, since longer exposures of seeds did not cause further increases in germination (Table 4; and see experiment of Fig. 2, insert, when the flasks were kept closed for 5 d). After 15 min of exposure, ethylene content in the flask was depleted by about 88% (Fig. 2 and Table 4); it is possible that the minimum exposure time required was even shorter than 15 min. By taking into account seed ages and the average magnitude of response shown in Table 4 (dormant: 24% and non-dormant: 55% germination), and interpreting these data according to curves of Fig. 1, it can be inferred that non-dormant seeds perceived the quickly declining ethylene concentration, from 5000 to 600 nM, within 15 min in the flask atmosphere, and then to zero upon flask opening, as if it were 2.6 nM. On the other hand, dormant seeds perceived the declining ethylene concentration as if it were 36 nM, i.e. a figure 14-fold greater. Again, from interpolation of the curves in Fig. 1, it is evident that to cause a germination of 24% in non-dormant seeds, an ethylene concentration about 1900-fold lower than that causing identical germination of dormant seeds

Table 4. Effect of addition of ethylene (5000 nM) into closed Erlenmeyer flasks on germination of dormant (37-d-old) and non-dormant (412-d-old) Townsville stylo seeds inhibited by 0.1 mM aminoethoxyvinyl-glycine (AVG) plus 1 mM Co(NO$_3$)$_2$; 24 h after the start of imbibition, the flasks received 0.3 ml of 0.25 M Hg(OCl)$_2$ solution, then were sealed and ethylene injected. Flasks were then opened at each time shown, and their atmospheres exhausted with a fan in a fume hood. Ethylene remaining in the flask atmosphere after each period of fixation by perchlorate is shown. Germination was recorded on the fifth day

		Germination (%)	
Exposure to ethylene (h)	Remaining ethylene (nM)	Dormant	Non-dormant
0	4999.97 ± 87.0	0	0
0.25	610.10 ± 28.0	22.5 ± 2.8	52.3 ± 2.1
0.5	100.25 ± 5.6	22.8 ± 4.1	52.6 ± 1.2
1	9.74 ± 0.38	23.3 ± 1.4	53.2 ± 3.9
2	4.61 ± 0.92	23.9 ± 2.2	54.4 ± 4.0
3	2.82 ± 0.25	24.7 ± 2.2	54.6 ± 3.6
4	1.76 ± 0.14	24.9 ± 2.5	55.7 ± 1.0
6	0.77 ± 0.15	25.2 ± 3.2	56.6 ± 3.5
12	0.25 ± 0.07	26.1 ± 1.5	57.5 ± 2.5
24	0	26.4 ± 3.9	58.1 ± 3.3

would suffice. Further evidence of differences in sensitivity was observed in the time for initiation of germination in the latter experiment, 3 h for non-dormant seeds, and 6 h for dormant ones (not shown). Perception and information processing to trigger the ethylene response in seeds of Townsville stylo thus proceeds in a very short time.

Conclusions

Not only the integumentary barrier, but also physiological dormancy, ensures seed dispersal, germination and seedling establishment under favourable conditions. Physiological dormancy of Townsville stylo seeds is not well documented, likely due to the response of the seeds to several environmental cues that break dormancy, and/or to the millions of seeds shed in the soil where the dormancy is gradually lost (Vieira and Barros, 1994). Dormancy of freshly harvested seeds is broken only under high ethylene concentrations, i.e. their sensitivity to the regulator is very low (Fig. 1). As the seeds age, sensitivity to ethylene increases, so much so that non-dormant seeds are capable of responding to the gas at extremely low concentrations (Figs 1 and 2, insert; Tables 1 and 3, lower part). Figs 1 and 2 (insert) show that sensitivity is about 50-fold (or more) greater in non-dormant than in dormant seeds. If this difference is multiplied by the low ethylene concentrations eliciting germination of non-dormant seeds inhibited by AVG plus Co^{2+} (Tables 1 and 3, lower part) (as recommended by Bradford and Trewavas, 1994), a product is obtained that is similar to the concentrations required for germination of dormant seeds (Fig. 1). Perception of ethylene by both dormant and non-dormant seeds

occurs in a short time, and therefore seems not to constitute a bottleneck for germination. Capacity for ethylene biosynthesis of freshly harvested seeds of Townsville stylo is low due to their very low contents of free ACC (Pinheiro, 2004). Upon ageing, that capacity is increased, as well as sensitivity to ethylene. Then, seeds are able to respond to ethylene resulting from microbial activity in the soil or other sources (Egley, 1980; Schonbeck and Egley, 1981; Munné-Bosch *et al.*, 2004). In such situations, sensitivity assumes a particular importance for the survival of the species, and this very high sensitivity also explains why ethylene does not appear to be associated with germination of non-dormant seeds of some species, likely because of it being below the limits of detection, as suggested by Saini *et al.* (1989) and Machabée and Saini (1991).

References

Abeles, F.B. (1973) *Ethylene in plant biology*. New York, Academic Press.
Abeles, F.B., **Morgan, P.W. and Saltveit, M.E. Jr** (1992) *Ethylene in plant biology*. 2nd edition. New York, Academic Press.

Barros, R.S. and Neill, S.J. (1986) Periodicity of response to abscisic acid in lateral buds of willow (*Salix viminalis* L.). *Planta* **168**, 530–535.

Bradford, K.J. and Trewavas, A.J. (1994) Sensitivity thresholds and variable time scales in plant hormone action. *Plant Physiology* **105**, 1029–1036.

Egley, G.H. (1980) Stimulation of common cocklebur (*Xanthium pensylvanicum*) and redroot pigweed (*Amaranthus retroflexus*) seed germination by injections of ethylene into soil. *Weed Science* **28**, 510–514.

Firn, R.D. (1986) Growth substance sensitivity: The need for clearer ideas, precise terms and purposeful experiments. *Physiologia Plantarum* **67**, 267–272.

Hua, J. and Meyerowitz, E.M. (1998) Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. *Cell* **94**, 261–271.

Kępczyński, J. and Karssen, C.M. (1985) Requirement for the action of endogenous ethylene during germination of non-dormant seeds of *Amaranthus caudatus*. *Physiologia Plantarum* **63**, 49–52.

Kępczyński, J. and Kępczyńska, E. (1997) Ethylene in seed dormancy and germination. *Physiologia Plantarum* **101**, 720–726.

Klee, H.J. (2004) Ethylene signal transduction. Moving beyond Arabidopsis. *Plant Physiology* **135**, 660–667.

Machabée, S. and Saini, H.S. (1991) Differences in the requirement for endogenous ethylene during germination of dormant and non-dormant seeds of *Chenopodium album* L. *Journal of Plant Physiology* **138**, 97–101.

Munné-Bosch, S., **Peñuelas, J.**, **Asensio, D. and Llusià, J.** (2004) Airborne ethylene may alter antioxidant protection and reduce tolerance of holm oak to heat and drought stress. *Plant Physiology* **136**, 2937–2947.

Pelacani, C.R., **Barros, R.S.**, **Ribeiro, D.M. and Frigeri, R.B.C.** (2005) Breaking dormancy of *Stylosanthes humilis* seeds with low pH solutions. *Acta Physiologia Plantarum* **27**, 387–393.

Pinheiro, F.J.A. (2004) Quebra da dormência de sementes de *Stylosanthes humilis* H.B.K. por compostos selênicos, DSc Thesis, Universidade Federal de Viçosa, Brazil.

Ribeiro, D.M. and Barros, R.S. (2004a) Germination of dormant seeds of *Stylosanthes humilis* as promoted by ethylene accumulation in closed environments. *Brazilian Journal of Plant Physiology* **16**, 83–88.

Ribeiro, D.M. and Barros, R.S. (2004b) A technique devised for detecting the ethylene requirement for germination as illustrated with *Stylosanthes humilis* seeds. *Seed Science and Technology* **32**, 863–866.

Saini, H.S., **Consolación, E.D.**, **Bassi, P.K. and Spencer, M.S.** (1989b) Control processes in the induction and relief of thermoinhibition of lettuce seed germination. Actions of phytochrome and endogenous ethylene. *Plant Physiology* **90**, 311–315.

Saltveit, M.E. and Yang, S.F. (1987) Ethylene. pp. 367–396 *in* Rivier, L.; Crozier, A. (Eds) *Principles and practice of plant hormone analysis*. London, Academic Press.

Schonbeck, M.W. and Egley, G.M. (1981) Changes in sensitivity of *Amaranthus retroflexus* L. seeds to ethylene during preincubation II. Effects of alternating temperature and burial in soil. *Plant, Cell and Environment* **4**, 237–242.

Scott, A.J. and Knott, M. (1974) A cluster analysis method for grouping means in the analysis of variance. *Biometrics* **30**, 507–519.

Stepanova, N.A. and Alonso, J.N. (2005) Ethylene signalling and response pathway: a unique signalling cascade with a multiple of inputs and outputs. *Physiologia Plantarum* **123**, 195–206.

Trewavas, A. (1981) How do plant growth substances work? *Plant, Cell and Environment* **4**, 203–228.

Trewavas, A.J. (1982) Growth substance sensitivity: The limiting factor in plant development. *Physiologia Plantarum* **55**, 60–72.

Trewavas, A.J. (1987) Sensitivity and sensory adaptation in growth substance response. pp. 19–38 *in* Hoad, G.V.; Lenton, J.R.; Jackson, M.B.; Atkin, R.K. (Eds) *Hormone action in plant development: A critical appraisal*. London, Butterworths.

Vieira, H.D. and Barros, R.S. (1994) Responses of seed of *Stylosanthes humilis* to germination regulators. *Physiologia Plantarum* **92**, 17–20.