Physiology of Oil Seeds. V. Germination of NC-13 Virginia-type Peanut Seeds in the Presence of Inhibitors and Ethylene.[1] [2]

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ABSTRACT

Control dormant seeds that imbibed water for 16 hr germinated 100% after 10 µl 1/1 C_2H_4 was applied for 24 hr. Dormant seeds that imbibed cycloheximide (100 µg/ml), 6-methylpurine (50 µg/ml) and 6-azauracil (50 µg/ml) for 16 hr did not germinate at either 24 or 48 hr after 10 µl 1/1 ethylene treatment. Both protein- and nucleic acid-synthesis ihhibitors prevented germination induced by ethylene in these dormant seeds.

Imbibition of 20 µM ABA by dormant seeds prevented germination, but this effect was reversed by ethylene. Tracer studies with ^{14}C- amino acids indicate that ABA does not inhibit total protein synthesis, but it does inhibit emergence in the absence of ethylene. In the presence of ABA plus ethylene, emergence occurred, but no change in total protein synthesis was detected. At 8 weeks after harvest, both germination and incorporation of 2-^{14}C-uracil into RNA were inhibited by ABA and stimulated by ethylene. By 17 weeks after harvest, only the inhibition of germination and its reversal by ethylene were notable. However, at 17 weeks after harvest, ethylene enhanced RNA synthesis when germination and protein synthesis were inhibited by cycloheximide. Development of isocitritase activity in the seeds was inhibited by ABA and the inhibition was reversed by ethylene, indicating that de novo synthesis of protein is inhibited by ABA and activated by ethylene in these seeds. The opposite effects of ABA and ethylene on germination, RNA synthesis and isocitritase activity suggest that germination is controlled at the level of RNA and/or protein synthesis in these seeds. The prevention of germination of dormant seeds in the presence of ethylene by protein- and RNA-synthesis ihhibitors supports this suggestion, but the data do not preclude an action of ABA or ethylene prior to detectable affects on RNA or protein synthesis.

Additional Index Words: Dormancy, abscisic acid, cycloheximide, 6-methylpurine, protein synthesis, RNA synthesis.

Ethylene is the major substance responsible for breaking dormancy of Virginia-type peanut seeds. The effects of exogenous ethylene, the time course of its production by the seeds, internal ethylene content of the seeds, and the effects of other plant growth regulators on ethylene production have led us to this conclusion (13-16).

One mechanism of hormone regulation of dormancy in buds and seeds appears to be control of DNA and/or RNA synthesis (29). The elucidation of this mechanism of action at the molecular level involved the use of Act D, which blocks DNA-dependent RNA synthesis, CH, which blocks protein synthesis and a variety of base analogs that inhibit RNA synthesis. Many of the plant responses to exogenous ethylene are apparently initiated at the levels of transcription or translation (1, 26). Part of the evidence for these actions of ethylene were obtained by use of nucleic acid- and protein-synthesis inhibitors.

In both dormant and non-dormant seeds, nucleic acid- and protein-synthesis inhibitors may promote (3), inhibit or have no apparent effect on radicle emergence (18, 19). However, once emergence occurs, radicle elongation is usually inhibited by these substances, particularly that component of growth stimulated by GA (3, 18, 19). In addition, the inhibitors interfere with enzyme synthesis by seeds (6, 8, 22, 27). For some enzymes such as protease, the inhibition may occur only at a particular stage of embryogenesis (11).

The natural inhibitor of plant growth, ABA, also inhibits germination, synthesis of specific enzymes and DNA and RNA synthesis in a variety of plant tissues, including seeds (2, 5, 6, 16, 21, 29). ABA inhibits germination of NC-13 Virginia-type peanut seeds and this effect correlates with its inhibition of ethylene production by the seeds (16). GA, cytokinins and ethylene, alone or in combination, have been shown to reverse the effects of ABA on emergence, radicle elongation and RNA and protein synthesis (16, 20, 21, 29). The data suggest that initial radicle emergence and subsequent elongaton depends on either new RNA synthesis or activation of existing, stable mRNA's (4, 11, 12, 24, 28). Synthesis of DNA is apparently not essential during early stages of germination (4, 10).

In barley aleurone cells, the action of ABA appears to be very specific; ABA did not affect respiration, phosphorylation, or total protein and RNA synthesis of the cells while inhibiting GA-induced α-amylase synthesis (5). Also, Villiers (28) showed that ABA inhibits the incorporation, by root tips from dormant embryos of *Fraxinus excelsior,* of uridine and thymidine into nucleic acids, but leucine incorporation into total protein was unaffected. Thus, total protein synthesis was apparently dependent on preformed, stable RNA's already stored in the embryos (4, 28). However, since these embryos remained dormant, it appears that ABA inhibited synthesis of specific types of mRNA, and thus proteins, associated with emergence of the radicle (28).

GA, cytokinins and ethylene may activate, while ABA inhibits the growth processes by their opposite effects on RNA and associated protein synthesis (2, 4, 6, 11, 12, 21, 26, 28, 29). The embryo is the major site of ethylene production in peanut seeds (13). Cytokinin was not required in the incubation medium for induction of protease activity in squash cotyledons if the embryo was present

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[2]Mention of a trademark name or a proprietary product does not constitute endorsement by the United States Departmet of Agriculture or Texas A&M University and does not imply its approval to the exclusion of other products that also may be suitable.

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[4]Abbreviations: CH:cycloheximide; 6-MP; 6-methylpurine; 6-AZU:6-azauracil; Act D:actinomycin D; ABA:-abscisic acid; GA:gibberellic acid.

(22). Thus, the embryo in seeds can function as the site for production of hormones activating growth and enzyme synthesis. A distinctive feature of mature dormant seeds may be a prolonged inhibition of existing, stable RNA species that are responsbile for synthesis of proteins necessary for growth. This contrasts with the temporary inhibition of translation of mRNA during embryogenesis of cotton seeds that are non-dormant when mature (11, 12).

In this study, some effects of ABA and inhibitors of protein- and nucleic acid-synthesis in relation to ethylene physiology of dormant and afterripened Virginia type peanut seeds are reported.

Materials and Methods

The approach used was to block the sequence DNA > RNA > protein synthesis with a DNA-, RNA-, or protein-synthesis inhibitor, treat the seeds with ethylene, and observe them for a response (germination and ethylene production where applicable). When no outward response such as germination was observed, as with CH and 6-MP, RNA and protein synthesis by the seeds was determined in the presence and absence of ethylene. This was done in an attempt to localize a biochemical site of ethylene action, even though germination was inhibited.

Germination and Gas Sampling. The procedures for determining ethylene, CO_2, and method of germination have been reported (13-16), except that the gas chromatograph used here was a Beckman GC4, and the oven was operated at 65°C. Fifty seeds were germinated in 2 liter Erlenmeyer flasks at 29° ± 1° C in the dark. For small seed samples, imbibition was in 9 x 2 cm petri dishes with Whatman No. 5 filter paper and 35 ml of solution. Imbibition was for 16 hr in water (control) or treatment solution. Ethylene treatments for germination tests were in 55 liter Plexiglas chambers with KOH traps. Ethylene treatments and incubation with radioactive precursors began immediately after imbibition. When seeds were incubated with radioactive precursors, ethylene was added directly to the incubation flasks.

Incubations with radioactive precursors. Seeds without seedcoats (See Results and Discussion) were incubated for 24 hr at room temperature (24° C) in 125 ml Erlenmeyer flasks, with 10 seeds per flask plus 7.5 ml of 0.01 M K_2HPO_4 buffer at pH 6.0. Flasks used for ethylene treatment were sealed with rubber stoppers fitted with rubber septa, and the others were covered with cheesecloth. At the end of the incubation period, the seeds were decanted from the solution, washed thoroughly with distilled water, frozen in liquid nitrogen, and stored at -20° C until extracted.

Extraction of Proteins. Soluble proteins were extracted from 10 seeds in 25 ml of 0.1 M Tris buffer at pH 7.0 by grinding in a cold mortar. Debris was removed by centrifugation at 20,000 x g for 10 min, and the supernatant was decanted. Proteins were precipitated by 100% saturation of the supernatant with $(NH_4)_2 SO_4$ followed by placement of the solution at 3° C overnight. Proteins were pelleted by centrifugation at 20,000 x g for 10 min, resuspended twice in cold saturated $(NH_4)_2 SO_4$, and dissolved in 25.0 ml of 0.1 M Tris buffer at pH 7.0. Duplicate 0.5 ml aliquots were assayed for both protein and radioactivity. Proteins were determined by the Biuret method.

Extraction of RNA. RNA was extracted from 10 seeds with 30 ml of grinding medium by the phenol-buffer procedure of Click and Hackett (7). The buffer used contained 0.1 M glycine, 0.1 M NaCl, and 0.1 M EDTA at pH 9.5. The RNA was precipitated with 2 volumes of cold 95% ethanol and left in the cold overnight. The precipitate was pelleted by centrifugation at 25,000 x g for 10 min. The pellet was redissolved in 10 ml of the pH 9.5 buffer and reprecipitated twice from the buffer using 2 volumes of ethanol each time. RNA was dissolved in 10.0 ml of buffer, and duplicate 0.5 ml aliquots were taken for determination of both RNA and radioactivity. RNA content was determined by absorbance at 260 nm.

Assay of Radioactivity. Samples were counted in a Beckman LS-200B liquid scintillation counter. The scintillation liquid was a mixture of 6 g PPO (2,5-diphenyloxazole) and 100 g napthalene (scintillation grade) per liter of dioxane. Samples were dark adapted and counted to 0.2 to 0.5% accuracy with 69.8% efficiency.

Enzyme Assay. Isocitritase (isocitric lyase, EC 4.1.3.1) was extracted and assayed by procedures similar to that of Gientka-Rychter and Cherry (8) and Smith and Benedict (27).

Results and Discussion

Effect of Inhibitors on Dormant Seeds. Preliminary germination tests with Act D and CH at 2, 10, and 20 μg/ml and 10, 25, and 50 μg/ml, respectively, did not inhibit germination of intact (with seedcoats), dormant Virginia-type peanut seeds, when the moist seeds were treated with 10 μl/1 ethylene. When seedcoats were removed, CH (50 μg/ml) inhibited germination completely at 24 hr, but some germination occurred at 48 hr of ethylene treatment (Table 1). At 100 μg/ml CH, the seeds did not germinate after 48 hr of ethylene treatment (Table 1). Ethylene was also unable to overcome the inhibition of germination by the base analogs, 6-MP and 6-AZU (Table 1). Act D, chloramphenicol, 6-azathymine (a DNA synthesis inhibitor) and 8-azaguanine at a concentration of 50 μg/ml had no inhibitory effect on germination subsequent to ethylene application.

Table 1. Effect of protein- and nucleic acid-synthesis inhibitors on germination of dormant Virginia-type NC-13 peanut seeds with subsequent application of ethylene.

Inhibitor and Concentration	Germination*			
	Apical‡		Basal	
	24 hr	48 hr	24 hr	48 hr+
	%			
Control: H_2O $-C_2H_4$	0	20	0	10
Control: H_2O $+C_2H_4$	100	-	100	-
CH: 100 μg/ml	0	0	0	0
50 μg/ml	0	10	0	48
6-MP: 50 μg/ml	0	0	0	0
6-AZU: 50 μg/ml	0	0	0	0

*Seeds were tested with seedcoats removed. The results are the average of two experiments with 3 replicate samples per experiment. Each sample contained 12-18 seeds. Tests were performed after 5 months of storage in the shell at 3 ± 2 C.

+Ethylene was applied at 10-12 μl/1 after 16-17 hr imbibition in the inhibitor. Germination was recorded at 24 and 48 hr after adding ethylene.

‡Apical and basal refers to seed position in the pod. Apical seeds are naturally less dormant than basal and are located distal to the peg attachment to the pod.

The data agree with similar hormonal effects obtained with light sensitive lettuce seeds. Light or GA could overcome the effect of Act D, but neither light nor GA could reverse the inhibition by CH (18). Ethylene overcame any effect of chloramphenicol on dormant peanut seeds, as did GA for lettuce seeds (18). But neither ethylene

nor GA reversed the effects of 6-AZU in peanuts or lettuce seeds, respectively. We have previously shown that the inhibition of germination of Virginia-type peanut seeds by ABA is reversed by ethylene (16).

The germination data suggest that ethylene action in breaking dormancy of peanut seeds results in RNA and protein synthesis, as in other dormant seeds whose dormancy is broken by other plant hormones (18, 19, 20, 23, 28, 29). This is indicated by the inhibition of germination in the presence of ethylene plus CH, and either ethylene plus 6-MP or 6-AZU, which inhibit protein and RNA synthesis, respectively. However, these substances also inhibit other processes during germination of afterripened seeds.

Effect of Inhibitors on Afterripened Seeds. Germination, growth, and ethylene and CO2 production of afterripened Virginia-type peanut seeds were inhibited by CH and 6-MP (Table 2). We have previously shown that germination and ethylene production by these seeds is inhibited by ABA (16). In the 6-MP treatment, no radicle emerged, but the hypocotyl-radicle elongated somewhat (In peanut seeds, initial growth occurs by hypocotyl elongation followed closely by radicle emergence from the hypocotyl. Measurements are for the entire structure, i.e. hypocotyl-radicle) (Table 2). We had previously indicated that two ethylene-production maxima were associated with normal germination of peanut seeds of a non-dormant Spanish-type variety (25). The first maximum was associated with emergence of the hypocotyl-radicle, and the second with radicle emergence. The concentration of 6-MP (50 μg/ml) used with the intact Virginia-type seeds apparently penetrated the seeds only to the extent that the second ethylene production maxima was inhibited, and emergence of the radicle did not occur (Table 2). The data suggest that the inhibitors function by blocking ethylene action or a result of ethylene action, whether ethylene is applied exogenously to dormant seeds or produced during germination processes. The inhibitors may function by blocking RNA or protein synthesis required in the germination processes, of which ethylene synthesis is a necessary part.

Effect of Inhibitors on Protein and RNA Synthesis in Relation to Germination. Ethylene cannot overcome the inhibitory effect of either CH or 6-MP on germination, but can reverse the inhibition induced by ABA (16). Therefore, these three inhibitors were used in an experimental series to compare their effects on germination and protein- and nucleic acid-synthesis of seeds that had imbibed the inhibitors and were subsequently treated with ethylene. Thus, any observed changes would be due to the ethylene treatment.

Cycloheximide and 6-MP completely inhibited germination (Table 3), as previously shown (Tables 1 and 2). Total protein synthesis was inhibited 80 to 85% and 40 to 50% by CH and 6-MP, respectively, at 8 or 17 weeks after harvest; ethylene was unable to overcome this inhibition of either germination or protein synthesis (Table 3). In contrast, ABA inhibited germination in the minus ethylene treatment, but had no effect on total protein synthesis (Table 3). Also, when ethylene reversed the inhibition of germination by ABA, there was still no detectable effect on total protein synthesis (Table 3). Comparison to the germinated controls emphasizes the effectiveness of ABA in retarding germination without affecting total protein synthesis. The lack of detectable stimulation of total protein synthesis by

Table 2. The effect of cycloheximide and 6-methylpurine on germination, growth, and ethylene and CO_2 production by afterripened Virginia-type NC-13 peanut seeds.

Treatment	Germination	Hypocotyl-radicle Growth	Ethylene	Carbon dioxide
	%	cm	nl/g fr wt/hr	μl/g fr wt/hr
Water	96[+]	1.83[§]	6.99[¶]	175.2
CH[*]	20	0.26	trace	91.6
6-MP	"80"[‡]	0.41	2.61	106.4

[*] CH (100 μg/ml); 6-MP (50 μg/ml).

[+] Each datum is the mean of 3 replicate samples of 50 seeds each. Germination was determined at 48 hr. Seeds were tested with seedcoats.

[‡] Not true germination, no radicle emerged. Growth consisted of elongation of the hypocotyl only.

[§] The mean of 30 representative seedlings, 10 each of three replicates. The seedlings were uniform in development. Therefore, only a representative sample was measured.

[¶] Determined at 24 hr of germination. Inhibition by CH and 6-MP remained at 48 hr. Fresh weights were measured at 48 hr.

Table 3. The effect of cycloheximide, 6-methylpurine, and abscisic acid on protein synthesis by basal Virginia-type NC-13 peanut seeds in the presence or absence of ethylene at eight and seventeen weeks after harvest.[*]

Sample	Inhibitor[+]	Germination[‡] 8	Germination[‡] 17	Specific Activity[¶] 8	Specific Activity[¶] 17	DPM Percent of Control 8	DPM Percent of Control 17
		%		DPM/mg protein		%	
		Minus Ethylene[§]					
1	Control	60	70	12,946	12,816	100	100
2	CH	0	0	1,942	2,479	15	19
3	6-MP	0	0	5,320	6,723	41	52
4	ABA	0	0	15,665	14,314	121	112
		Plus Ethylene (10 μl/1)					
5	Control	100	100	12,600	10,839	100	100
6	CH	0	0	2,107	2,731	17	25
7	6-MP	0	0	5,228	4,723	41	44
8	ABA	100	100	12,560	14,307	100	132

[*] Seeds were stored in the shell at 3 ± 2 C. Storage began about 1 month after harvest.

[+] CH (100 μg/ml); 6-MP (50 μg/ml); ABA (20 μM).

[‡] Number of weeks after harvest.

[§] All incubations were for 24 hr at room temperature (24 C) in 125 ml flasks (10 seeds/flask plus 7.5 ml 0.01 M K_2HPO_4 buffer at pH 6.0). Flasks used for ethylene treatment were sealed with rubber stoppers, and others were covered with cheesecloth. Flasks were gently shaken during the incubation period.

[¶] DPM (disintegrations per minute) were calculated by dividing CPM (counts per minute) by percent counting efficiency. Seeds received 25 μCi of a mixture of ^{14}C-amino acids, specific activity 1.1 mCi/mg.

ethylene in the presence of ABA suggests a specificity of action of the hormones, which agrees with the data of Chrispeels and Varner (5) and Villiers (28). Chrispeels and Varner (5) indicate that ABA specifically acts to inhibit GA-stimulated α-amylase synthesis in barley aleurone cells. Act D also prevents synthesis of GA-induced α-amylase if it is added to the aleurone cells at the same time as GA (6). Apparently at this time, the enzyme synthesis is dependent on transcription of new RNA's. Villiers (28) showed that in dormant *F. excelsior* embryos, ABA did not inhibit leucine incorporation into total protein. Total protein synthesis evidently occurred by existence of preformed stable mRNA already present in the dormant embryos (28). Similarly, since neither ABA nor Act D plus ethylene inhibits germination and no effect on total protein synthesis was evident when ethylene activated germination in the presence of ABA, specific protein synthesis from stable RNA's stored in dormant peanut seeds could be the means of initiation of germination by ethylene in these seeds.

Incorporation of ^{14}C-uracil into RNA and germination of the seeds at 8 weeks after harvest (minus ethylene treatment) were inhibited by CH, 6-MP and ABA compared to the germinated control (Table 4). Ethylene could not reverse the inhibitory effects of either CH or 6-MP. However, RNA synthesis was equal to the control when ethylene induced germination (plus ethylene treatment) in the presence of ABA at 8 weeks after harvest (Table 4). Thus, the opposite effects of ABA and ethylene on germination and total RNA synthesis were clear at 8 weeks after harvest. However, after an additional 9 weeks of afterripening, 17 weeks after harvest, the effect of ABA on germination was the same (100% inhibition) but the inhibition of total RNA synthesis was no longer detectable (Table 4). Similarly, ethylene stimulated germination in the presence of ABA, but an effect on total RNA synthesis was not observed (Table 4). However, in the CH treatment an enhanced RNA synthesis, induced by ethylene treatment, occurred when both germination and protein synthesis were inhibited by CH (Tables 3 and 4). These data indicate that at this stage of afterripening (17 weeks after harvest) ethylene still affects RNA synthesis by the seeds. Also, at this stage of afterripening, control of germination by ABA and ethylene apparently occurs through synthesis of RNA's that are specific for germination. This is consistent with the continued inhibition of germination and RNA synthesis by 6-MP, which was not reversed by ethylene (Table 4).

To further test the hypothesis that ABA and ethylene could conceivably control germination by acting at the level of RNA or protein synthesis in peanut seeds, isocitritase activity of the seeds was assayed. Table 5 shows that the development of isocitritase activity was inhibited 87% by ABA and restored to the control level by ethylene. Since isocitritase is an enzyme synthesized *de novo* during germination of peanut seeds (8, 9) and Act D (which blocks DNA-dependent RNA synthesis) inhibits its formation in peanut (8) and cotton seeds (27), these data demonstrate that the inhibitory effect of ABA, and its reversal by ethylene could occur at either *de novo* RNA or protein synthesis in these seeds. The data are in agreement with reports that ABA inhibits: (1) precocious germination and protease synthesis of cotton seed embryos at a particular stage of embryogenesis (11); (2) GA-induced α-amylase synthesis by barley aleurone layers (5, 6); and (3) protein synthesis by wheat embryos during early hours of germination. This inhibition was interpreted to occur at the level of translation (4). The effect of ethylene on isocitritase activity in peanut seeds appears comparable to the stimulatory effect of GA on this enzyme in havel seeds (23).

The ability of ethylene to reverse the inhibitory effects of ABA on these seeds provided a means

Table 4. The effect of cycloheximide, 6-methylpurine, and abscisic acid on nucleic acid synthesis by basal Virginia-type NC-13 peanut seeds in the presence or absence of ethylene at eight and seventeen weeks after harvest.*

Sample	Inhibitor	Germination 8	Germination 17	Specific Activity+ 8	Specific Activity+ 17	DPM Percent of Control 8	DPM Percent of Control 17
		%		DPM/μg RNA		%	
		Minus Ethylene					
1	Control	70	100	148	156	100	100
2	CH	0	0	43	86	29	55
3	6-MP	0	0	39	63	26	40
4	ABA	0	0	60	191	41	122
		Plus Ethylene (10 μl/l)					
5	Control	100	100	77	84	100	100
6	CH	0	0	35	104	45	124
7	6-MP	0	0	27	43	35	51
8	ABA	100	100	88	103	114	123

*See notes Table 3.

+Seeds received 25 μCi of 2-^{14}C-uracil specific actitivy 50.8 mCi/mmole.

‡Maximum deviation from mean value ± 5.0 DPM/μg RNA.

Table 5. The effect of ABA and ethylene on isocitritase activity of afterripened Virginia-type NC-13 peanut seeds.

Treatment	μmoles glyoxylate*/ mg protein/ 15 min X 10
Control	2.3 ± 0.3
ABA (100 μM)	0.3 ± 0.0
ABA + C_2H_4+	1.9 ± 0.3

*Enzyme activity was assayed at 112 hr from the start of imbibition for control and ABA. At this time the enzyme activity is increasing for the control and ABA + C_2H_4 treatments. Each datum is the mean ± S.D. of three experiments for control and ABA and two experiments for ABA + C_2H_4.

+100 μl/1 C_2H_4 was applied for 24 hr to the ABA treatment after 112 hr and the assay ran on the germinated seeds after an additional 112 hr from the start of C_2H_4 treatment. Enzyme activity does not begin to rise to any extent until after C_2H_4 treatment.

whereby the effects of ethylene treatment on RNA and protein synthesis could be determined during initiation of germination. The data suggest two sites of regulation by ethylene: (1) protein synthesis by stable RNA's at the level of translation or (2) protein synthesis beginning at the level of transcription. But, the data do not preclude an action of ABA or ethylene prior to detectable effects on RNA or protein synthesis (as shown by isocitritase activity).

Since ethylene induces its own production, some other protein synthesis inhibited by ABA could be enzymes for ethylene synthesis. This may explain the effectiveness of ABA, CH and 6-MP in inhibiting germination of these seeds, since high rates of ethylene production are essential during early stages of their germination (13-17).

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